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**XXVI CICLO**

**FOLATE RECEPTOR TARGETING THROUGH A PTEROYL-  
NONAPEPTIDE CONJUGATE ON OVARIAN CANCER CELL  
LINES**

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The present thesis is divided in three parts:

#### Section 1.

Is composed by a general introduction on the problem of ovarian cancer and drug resistance. An overview of the most promising novel strategies in the management of platinum-resistant ovarian cancer is also reported. In particular, the “AIRC-DROC targeting drug resistance in ovarian cancer” project, as well as my contribution to this project, have been discussed.

#### Section 2.

Collects the results obtained by studying some peptides that inhibit TS at its dimeric interface are reported in different articles.

#### Section 3.

Reports the results obtained from the evaluation of the efficiency of a FR $\alpha$ -mediated strategy to target ovarian cancer cell lines with peptide inhibitors of TS dimeric interface, recently described in a manuscript in preparation.

## **SECTION 1.**

### **INTRODUCTION**

#### **1.1. Ovarian Cancer**

Ovarian cancer (OC) is the sixth most common type of cancer in women and the seventh most common cause of cancer death in women [1]. The estimated number of new ovarian cancer cases in Europe in 2012 was 65538 with 42704 deaths [2], although the incidence rates are at least twice as high in Europe and North America than in Asia and Africa [3]. Most cases of OC occur in women older than 50 years, but the diagnosis may be made at any age, including infancy [4]. The exact cause of ovarian cancer remains unknown, but many associated risk factors have been identified [5]. In most cases, OC is diagnosed already in advanced stage [6], because of the lack of specific symptoms that accompany the early disease and drug resistance to the standard systemic therapies.

Five-year survival rate range is from 20% to 30%, however, for women who are diagnosed when the disease is confined to the ovary, cure rates are approximately 70% to 90% [7]. Although advances in conventional therapies have improved the median survival in advanced stage ovarian cancer over the past few decades [8, 9], the overall cure rate remains poor, and often patients with ovarian cancer could relapse and develop resistance to chemotherapy [10].

##### **1.1.1. Ovarian cancer classification**

Ovarian cancers are usually classified according to histological, genotypic and phenotypic features.

According to the cell type, ovarian cancers are classified in epithelial (about 90%), stromal (5%), or germ cell (less than 5%) [11].

Stromal and germ cell tumours are less common. Stromal cell tumours may occur in women of any age and they could induce precocious sexual development in pre-pubertal girls. Moreover, they may be associated with endometrial hyperplasia, cystic disease of the breast, and endometrial carcinoma in adults, ascites in 40 percent of fibroma-thecoma tumours, may be associated with ascites and hydrothorax. Germ cell tumours, instead, occur in patients younger than one year and in patients 15 to 19 years old. They are highly malignant and usually unilateral [12].

Epithelial ovarian cancer (EOC) usually occurs in women older than 50 years, but a 15 percent of them are borderline or have a low malignant potential, with a 10-years survival rate

up to 99 percent for stage 1. The World Health Organisation histological typing of epithelial ovarian tumours recognises the following distinct subtypes: serous, endometrioid, clear cell, mucinous, Brenner (transitional cell), mixed epithelial tumours, undifferentiated and unclassified [13] .

Among these invasive serous carcinomas are the most common histological type accounting for up to 80 per cent of advanced ovarian cancers [14].

In recent years, ovarian cancer has been recognised as a heterogeneous disease and a new dualistic model for the pathogenesis divides epithelial tumours into type 1 and type 2 ovarian carcinomas [15,16]. Type 1 cancers are generally low-grade and indolent tumours. They also include low-grade serous, endometrioid, mucinous, clear-cell and malignant Brenner tumours. Several gene mutations occur early in the evolution of type 1 cancers, in particular KRAS, BRAF, ERBB2, PTEN, PIK3CA, ARID1A. A stepwise sequence of tumour development is now well recognised from benign precursor lesions to malignant lesions in type 1 cancers.

High-grade serous, high-grade endometrioid, malignant mixed mesodermal and undifferentiated tumours are high-grade and aggressive type 2 cancers. They are very frequently associated with TP53, BRCA1/2 and a combination of germline and somatic mutations [17]. However, there is no clear precursor lesion for type 2 cancers, which metastasise to the ovaries and the peritoneal cavity [1]. Moreover, tumours with mild to moderate cytologic atypia and low mitotic rates are classified as low-grade, whereas patients with severe cytologic atypia and high mitotic rates are considered high-grade serous tumours. Clinically, women with low-grade serous tumours, which account for about 10 percent of serous cancers, tend to present at a younger age and have a longer survival compared with women with high-grade tumours [18]. There is an increasing realisation that low-grade serous tumours do not respond to traditional chemotherapy regimens [19] and that alternative approaches are required particularly for the treatment of recurrent tumours.

Endometrioid epithelial cell tumour is a less common cancer, bilateral and the majority of patients with this disease are early stage and low grade. The prevalence of endometrioid ovarian cancers has decreased in recent years, likely due to better pathological diagnosis, and currently they account for about 10 percent of ovarian cancers. Endometriosis, and in particular endometriotic cysts, have been implicated as putative precursor lesions to endometrioid ovarian cancer. ARID1A mutations have been detected in endometriotic cysts and in endometrioid ovarian cancer, suggesting a causative role [20]. Clear-cell cancers account for about 5% of ovarian cancers, although the incidence varies worldwide. The prognosis for stage 1 clear-cell cancers is relatively good, while advanced stage clear-cell

cancers have a worse prognosis than serous ovarian cancers, probably because of resistance to the standard chemotherapeutic agents used in ovarian cancer. Clear-cell cancers are also strongly associated with endometriosis and a significant proportion carry ARID1A mutations [20]. Even mucinous epithelial cell tumours, which account for about 25 percent of all ovarian cancer, may occur in association with endometriosis. Its origin is unclear but it is associated with pseudomyxoma peritonei. Primary ovarian transitional carcinomas are rare but carcinomas with transitional features are quite common. Mixed carcinomas are rare and are likely to represent one end of the high-grade serous spectrum [15]. Borderline tumours comprise about 10–15 percent of ovarian tumours and they are the most common type and form part of the spectrum of low-grade serous cancers. These tumours are managed primarily by surgery and respond poorly to chemotherapy [15].

### **1.1.2. Stages and prognosis in OC**

The prognostic importance of subtype [21] has been demonstrated by clinical trials. The grading system (FIGO staging [22]) derived from reviewing the following tumour characteristics: architectural features, mitotic counts and nuclear atypia and a grade of 1–3 is most commonly assigned [23,24]. The carcinogenesis in ovarian cancer arises from the epithelium covering the ovarian surface, as shown in Figure 1.1.2.1. In the stage I cancer has invaded one or both ovaries, in particular it can be found in only one ovary (stage IA), or both ovaries (stage IB). After then, cancer cells are localized on the outer surface of one or both ovaries, or the tumour capsule was torn, or cancer cells are present in the fluid of the peritoneal cavity (stage IC). In the Stage I the five-years survival rate is about 90 percent, while in stage 2 it decreases from 60 to 80 percent. In the stage II, cancer has spread into the pelvis. In particular it can spread to the uterus and- or fallopian tubes (stage IIA), to other tissues of the pelvis (stage IIB) and then neoplastic cells can reach the peritoneal cavity (stage IIC). In Stage III, cancer has spread to other abdominal organs and structures. In this stage, the five-years survival rate decrease until 20 percent. In particular, cancer cells can spread over the surface (stage IIIA) and then into the peritoneum (stage IIIB), where cancer can be larger than 2 cm and-or abdominal lymph nodes are compromised. The Stage IV is characterized by distant metastases and the five-years survival rate decreases until 10 percent. They are rare, but may occur in the liver, lungs, pleura, adrenal glands and spleen [4,6].

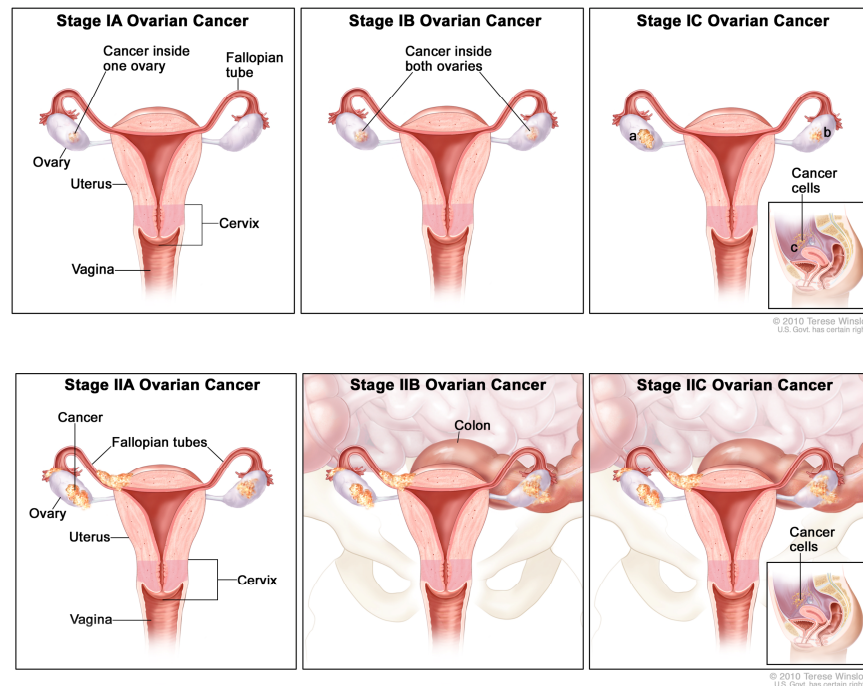


**Figure 1.1.2.1.** Characteristic of Stages I and II of Ovarian cancer [25].

### 1.1.3. Risk Factor

The carcinogenesis of ovarian cancer is still unknown, [26] but genetic and environmental factors are associated with EOC.

*Genetic risk.* Most women diagnosed with ovarian cancer have the sporadic variety, but family history and associated genetic syndromes is the greatest risk factor. Overall, hereditary predisposition accounts for at least 10 percent of all epithelial ovarian cancers. Hereditary breast and ovarian cancer syndrome, which account for approximately 90% of these cases, and hereditary nonpolyposis colorectal cancer syndrome (Lynch syndrome HNPCC) which account for the remaining 10 percent of these cases [4,27,]. Hereditary breast and ovarian cancer syndrome occurs in one in every 500 women and it is an autosomal dominant mutation in the BRCA1 or BRCA2 gene, and is associated with a 23-54 percent lifetime risk of ovarian cancer [4, 28-30]. Overall, hereditary accounts for only 12 percent of ovarian cancer occurrences [31] but increase breast, pancreatic, prostate cancer risk [4, 28-30].

Women with hereditary ovarian cancer tend to develop the disease more than 10 years earlier than women with non-hereditary ovarian cancer. The importance of identifying BRCA mutations has increased and, in addition to risk-reducing surgery and surveillance for breast cancer in the patient and in family members, there are new treatments emerging specifically for BRCA-related cancers [1]. Hereditary nonpolyposis colorectal cancer syndrome is an autosomal dominant mutation that increases the risk of nonpolyposis colorectal, endometrial,

breast, and ovarian cancers. The lifetime risk of ovarian cancer in carriers of this syndrome is 12 percent [32].

*Environmental risk.* Several modifiable risk factors (e.g. obesity, smoking, a high-starch or high-fat diet, sedentary lifestyle) could be associated with an increased risk of ovarian cancer. Increased daily fibre intake, the use of carotene, vitamin C, vitamin E, and unsaturated fatty acids, as well as increased physical activity, are moderately associated with a decreased risk of ovarian cancer [33]. Risk factors for the development of sporadic ovarian cancer are age, obesity, and polycystic ovary syndrome. Several studies indicate that height and body weight are associated with risk, in particular among non-users of hormone replacement therapy. However, the effect of hormone replacement therapy (HRT) on the risk of ovarian cancer remains controversial [34].

According to the IARC, there is sufficient evidence that epithelial ovarian cancer is caused by tobacco smoking, and limited evidence regarding perineal use of talc-based body powder and exposure to X-radiation and gamma ( $\gamma$ )-radiation (for medical purposes) [35]. On the other hand, there are a few factors known to be associated with a decreased risk of ovarian cancer, such as multiparity, use of oral contraceptives as progesterone, late menarche and early menopause, possibly breastfeeding, incomplete pregnancies, hysterectomy and tubal ligation. They act as protective agent against repeated ovulatory rupture and repair that creates opportunities for malignant gene mutations [36-38].

#### **1.1.4. Diagnosis**

Because there is a close correlation between stage at presentation and survival, early detection of ovarian cancer represents the best hope for mortality reduction and long-term disease control [39]. However, diagnosis is often difficult and the combination of symptoms analysis, physical examination, serum marker analysis, imaging investigation and other approach seems to be more promising.

In early ovarian cancer, symptoms are often nonspecific and patients present it for more than six months before diagnosis. The most common presenting symptom in children and adolescents is abdominal pain, although precocious puberty, irregular menses, or hirsutism may also be present [39]. Recognised symptoms of all stages include abdominal or pelvic pain, constipation, newly occurred meteorism, changes in bowel habits, diarrhoea, urinary frequency, vaginal bleeding, abdominal distension and fatigue. Although patients with ovarian cancer may present with inguinal lymphadenopathy, it is more common for the cancer to spread through retroperitoneal lymph nodes. In advanced ovarian cancer ascites and

abdominal masses lead to increased abdominal girth, weight loss to massive abdominal swelling bloating, nausea, vomiting, anorexia, dyspepsia and early satiety. Moreover the extension of disease across the diaphragm to the pleural cavities can produce pleural effusions and the development of respiratory symptoms. Patients may become aware of an abdominal or nodal mass either in the inguinal region, axillae or the supraclavicular fossa [40-43]. Thus, pelvic examination should be performed in order to maximize the opportunity to detect a mass when a patient presents with any of symptoms described above. For example, rectovaginal examination can be useful to detect a significantly enlarged ovary (greater than 10 cm), ovarian irregularity, a nodular or fixed pelvic mass, bilateral lesions, and nodules in the cul-de-sac [41].

Since the history and examination are often nonspecific for ovarian cancer, further testing should be performed in women older than 40 years who have persistent unexplained gynaecologic or gastrointestinal symptoms in order to allow early detection and suitable screening. Laboratory tests in these patients should include a complete blood count, comprehensive metabolic panel, and measurement of serum CA 125 levels is routinely used to aid diagnosis [44]. However, the measurement of CA 125 levels is non-specific for ovarian cancer, in fact other conditions, benign diseases (e.g. endometriosis, pelvic inflammatory disease) and functional ovarian cysts, physiologic states (menstruation, and pregnancy) may be associated with an increased CA 125 level [42]. Disease processes associated with ascites and other malignancies (e.g. breast, lung, colon and pancreatic cancer) may also alter CA 125 levels [6, 42- 46] and the interpretation of the results may be difficult.

Serum carcinoembryonic antigen (CEA) and CA 19-9 levels are sometimes measured in situations where it is unclear whether an ovarian mass is of gastrointestinal origin, or a primary mucinous ovarian tumour. Similarly, in these situations, colonoscopy and/or gastroscopy are sometimes considered [44]. Increased levels of beta subunit of human chorionic gonadotropin, serum alpha-fetoprotein, neuron-specific enolase, and lactate dehydrogenase occur in patients with germ cell tumours, and are primarily used to evaluate adnexal masses found during adolescence or young adulthood. Other laboratory biomarkers are available but not commonly used by primary care physicians [6].

For all these reasons, imaging investigation of the abdomen and pelvis is recommended for women in whom ovarian cancer is suspected. Transvaginal ultrasonography has improved the visualisation of ovarian structures, thus improving the differentiation of malignant versus benign conditions [44]. The presence of a large lesion, multi-locular cysts, solid papillary projections, extramural fluid, echogenicity, wall thickening irregular internal septations and

ascites are highly suggestive of ovarian cancer. Moreover, when ultrasonography reveals suspicious properties, gadolinium-enhanced magnetic resonance imaging may be useful for further evaluation [46]. A 'risk of malignancy' index can be calculated from clinical factors, ultrasound and CA 125 and can be used to refer patients to a specialist gynaecological oncology team. Computed tomography (CT) scans are routinely used to determine the extent of disease and to aid in surgical planning, while magnetic resonance imaging (MRI) scans do not form part of routine investigations [47]. However, there is currently no apparatus-based diagnostic procedure that can replace surgical staging of ovarian cancer and reliably assess the feasibility of surgery [48].

#### **1.1.5. Treatment and prognosis in OC**

FIGO staging remains the most powerful indicator of prognosis and influence the choice of approach and treatment [1]. Up to 70 percent of patients with epithelial ovarian cancer present at stage III or IV [49]. Tumour stage, age, general health, and post-operative residual tumour are independent, significant parameters in the survival prognosis of patients with ovarian cancer. Primary surgery remains the most common and preferred approach [1]. In particularly the standard initial management of EOC consists of aggressive surgical cytoreduction, including total abdominal hysterectomy and bilateral salpingo-oophorectomy (BSO), and platinum/taxane combination chemotherapy, such as carboplatin (formerly Paraplatin) and paclitaxel (Taxol), respectively.

Extensive and largely retrospective experience has shown that optimal surgical debulking with no residual tumour or residual tumour deposits that are less than 1 cm in size is associated with improved patient outcomes [4, 50]. Surgical staging even involves the removal of pelvic and para-aortic lymph nodes and omentum, as well as other supplemental procedures such as appendectomy [24]. Appendectomy is a typical supplemental procedure for mucinous ovarian cancer because of the possibility of metastasis.

Taking advanced of adequate staging survival of patients with low risk, in earlier stage disease there is a good chance of long-term cure and the 10-year survival rate is 95 to 99 percent without adjuvant chemotherapy [51]. This favourable prognosis allows for consideration of conservative fertility-sparing measures, such as unilateral salpingo-oophorectomy, and adjuvant chemotherapy is reserved for patients with postoperative residual disease or identifiable invasive implants [24]. Longitudinal laparotomy is the standard procedure for exploring the abdominal cavity, and this will both provide prognostic information and define whether chemotherapy is needed [52]. Moreover surgical treatment

completion with total abdominal hysterectomy and unilateral salpingo-oophorectomy should be considered after the reproductive years because of the possibility of late recurrence. Radiation is most often used for palliative purposes or for localized persistent disease after chemotherapy [1].

In advanced epithelial ovarian cancer (FIGO Stages IIB to IV), the aim is complete cytoreduction of all macroscopic visible disease [53-55]. In order to achieve this, a maximal surgical effort is required, including intestinal resection (in approximately 30% to 50% of cases of advanced OC) peritoneal stripping (on the upper abdomen such as partial resection of the liver or pancreas) cholecystectomy, diaphragmatic resection, splenectomy and removal of bulky para-aortic lymph nodes. However the value of systematic pelvic and para-aortic lymphadenectomy in advanced disease remains controversial [56-59]. In advance stage disease, and particularly in those patients with higher risk of recurrence (stage 1B/C grade 2/3, any grade 3 or clear-cell histology), several studies showed that adjuvant chemotherapy improves overall and disease-free survival [60,61].

The optimal duration of treatment remains controversial [62]. The standard treatment for advanced ovarian cancer is six cycles of platinum- and taxane-based chemotherapy (paclitaxel 175 mg/m<sup>2</sup> and carboplatin AUC 6-5, both administered intravenously every 3 weeks) [63]. Carboplatin and analogues of cisplatin, where cyclobutanedicarboxylate moiety was substituted with the two chloride ligands of cisplatin, are much less toxic than cisplatin whilst retaining significant chemotherapeutic activity [64]. Paclitaxel is a taxane that binds tubulin polymers and stabilizes the microtubule against depolymerization to inhibit cell division [65]. The efficiency of this combination is superior to platinum monotherapy [66], and to cisplatin and cyclophosphamide combination (GOG 111), which is equally effective but is more toxic and less convenient to administer [67]. Moreover, in the last decade several clinical trials, which have added a third drug as topotecan, gemcitabine or pegylated liposomal doxorubicin (PLD) (ICON-5/GOG 182 trial), did not show promising results in progression free survival (PFS) or overall survival (OS) [68,69].

Docetaxel (Taxotere, Sanofi-Aventis), an analogue of paclitaxel [70], showed a little different toxicity profile with respect to paclitaxel [71]. Thus, the combination of docetaxel-carboplatin or pegylated liposomal doxorubicin (PLD)-carboplatin can be considered an alternative for those patients who develop an allergy to or do not tolerate paclitaxel [71,72]. Actually, it has not yet been possible to demonstrate an advantage either for the addition of further cytostatics as part of a triplet or as sequential or maintenance therapy, or for treatment prolongation or

dose escalation over conventional combined treatment with six cycles of platinum and taxane [6].

Intraperitoneal (IP) chemotherapy consists on the administration of part of the chemotherapy, usually the platinum agent, directly into the peritoneal cavity through a catheter [73,74]. It seems to be a useful alternative to intravenous, systemic chemotherapy [6] in EOC. In fact ovarian cancer is relatively chemo-sensitive and the cancer remains confined within the peritoneal cavity for much of its natural history. Even if the IP chemotherapy benefit has been reported in several study studies, it has not been adopted as a standard of care. In particular, it should be considered in patients with small volume (<1 cm) or no residual disease after surgery, because of greater toxicity and complications associated with catheters delivering difficulty [75,76].

## 1.2. Cisplatin

The platinum analogue cisplatin is currently the most active chemotherapy agent in ovarian cancer. The antitumor activity of cisplatin (cis-diamminedichloroplatinum (II)) was discovered serendipitously in 1961 by Rosenberg and colleagues [77]. Since its approval by the Food and Drug administration in 1978, cisplatin continues to be one of the most effective anticancer drugs used in the treatment of solid tumours. It has been used as a first-line therapy for several cancers, including testicular, ovarian, cervical, head, and neck and small-cell lung cancers either alone or in combination with other anticancer agents. It is also used as an adjuvant therapy following surgery or radiation. In addition to cisplatin, its analogues, such as carboplatin and oxaliplatin, are also currently being used in the clinic [78,79].

Cisplatin or *cis*-diamminedichloroplatinum(II) is a neutral, square-planar, coordination complex of divalent Pt (Figure 1.2.1.) [81].

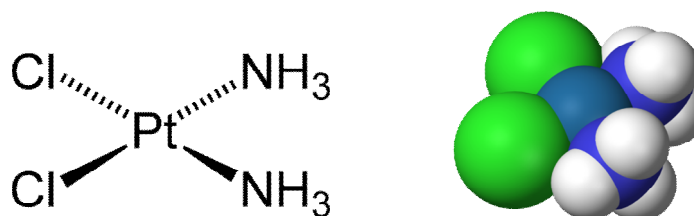


**Figure 1.2.1.** Chemical structure and three-dimensional representation of cisplatin.

Importantly, the *cis* configuration is required for its antitumor activity [80]. It has two labile chloride groups and two relatively inert amine ligands [81]. Thus, it has to be activated

through a series of spontaneous aquation reactions, in the intracellular space, which replace the cis-chloro ligands of cisplatin with water molecules. Following administration, one of the chloride ligands is slowly displaced by water. The aqua ligand in the resulting  $[\text{PtCl}(\text{H}_2\text{O})(\text{NH}_3)_2]$  is itself easily displaced, allowing the platinum atom to coordinate to a basic site in DNA. Subsequently, crosslinking of two DNA bases occurs via displacement of the other chloride ligand [82]. The chloride ions concentration is an important factor in determining the hydrolysis or aquation of cisplatin. Upon entering the cell, the chloride concentration drops down to 4 mM, which facilitates the aquation process [81] and influences the reactivity of cisplatin. Activated cisplatin (aquated form) is a potent electrophile that will react with a variety of endogenous nucleophile including glutathione (GSH), methionine, metallothionein, the sulfhydryl groups on proteins and nucleophilic groups on nucleic acids. Thus, when cisplatin enters the cells, it is potentially vulnerable to cytoplasmic inactivation by these and other intracellular components. However, DNA is the primary biological target of cisplatin [81-83] and as opposed to classic alkylating agents, the platinum atom of cisplatin forms covalent bonds with nucleophilic N7- of purine bases in DNA which results in the formation of DNA adducts, as DNA-protein cross-links, DNA monoadducts, interstrand (a lower percentage) and intrastrand DNA cross-links [84], that effectively preventing normal DNA function (Figure 1.2.2.). In particular, 1,2-intrastrand d(GpG) adducts form nearly 90% of the adducts, while 1,2-intrastrand d(ApG) adducts are less common. Moreover 1,3-intrastrand d(GpXpG) adducts occur but are readily excised by the nucleotide excision repair (NER) [85,86]. Other adducts include inter-strand crosslinks and non-functional adducts that have been postulated to contribute to cisplatin activity. Formation of cisplatin- DNA adducts cause a severe local distortion that disrupts the DNA structure and interferes with DNA replication and transcription, which results in cell cycle arrest in the G2-phase [ 85,87].

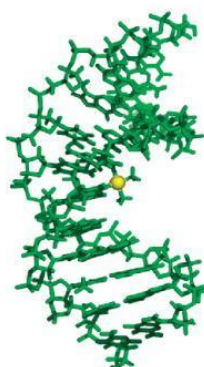


**Figure 1.2.2.** Representation of DNA adducts.

Despite a correlation between gross levels of platinum bound to DNA and the extent of cytotoxicity has been found [85], the inhibition of DNA synthesis and cytotoxicity appears to be completely uncorrelated to the amount of bound cisplatin [90].

In fact, the efficacy of this chemotherapeutic drug depends on their ability to induce DNA damage, and also on the cell's ability to detect and respond to DNA damage.

Cisplatin primarily induces cell death by apoptosis, therefore a defect in apoptotic signaling could also confer cisplatin resistance [89,90].

The cytotoxic process likely initiates with the recognition of DNA damage by over 20 individual candidate proteins, which recognize and bind the DNA on the physical distortions induced by the intra-strand platinum adducts [91]. These proteins include component of the mismatch repair (MMR) complex, the non histone chromosomal high mobility group 1 and 2 (HMG1 and HMG2) proteins, the human RNA polymerase I transcription upstream binding factor' (hUBF), and the transcriptional factor TATA binding protein (TBP) [92,93]. Whether one or more of these proteins are involved in sensing the damage is not certain [94].

The DNA damage recognition proteins, apart from the activation of downstream effectors after having bind the DNA, carry on many other functions, such as promoting cytotoxicity by first interacting with the DNA adduct and then shielding it from repair (HMG1 protein) [95], or initiating the transcription by RNA polymerase (hUBF and TBP) [96]. It appears reasonable to suggest that each of the recognition proteins may initiate one or more specific events, so that DNA damage results in several seemingly unrelated biological effects. This is utterly confirmed by the known ability of cisplatin adducts to disrupt replication and transcriptional processes, effects that do not necessarily correlate directly with cell death [97].

It is fundamental to keep in mind that cisplatin activates both pro-survival and pro-apoptotic signals, and the final fate of the cell depends upon the ratio of intensity and of duration of them. Cisplatin is known to activate cell cycle checkpoints, which temporally induce a transient S-phase arrest, followed by inhibition of the Cdc2-cyclin A or B kinase to affect a durable G2/ M arrest [95]; Cells typically remain blocked in G2/M phase, thus is rare to observe a sensible cisplatin accumulation in G1phase. The inhibitory effect of DNA adducts of cisplatin on the G1-phase cyclin-dependent kinases (CDKs), mediated by the Cdk4 inhibitor p16INK4A, contributes to this checkpoint block. Cell cycle arrest, generally as a consequence of DNA damage, is necessary to enable the nucleotide excision repair (NER) complex to remove the adducts and promote cell survival, and the apoptosis is triggered only if the damage is too consistent to be repaired. Thus, repair, checkpoint activation and apoptosis are tightly linked; interesting, the tumour-suppressor p53 protein is present in all

three processes [96,97]. Although the mediation of p53 in the cellular toxic effects of cisplatin is a direct consequence of DNA damage, a number of events must first occur to induce and activate the p53 protein molecule by its phosphorylation. In particular a kinase, involved in checkpoint activation, ATR (ATM- and Rad3-related protein), is preferentially activated by cisplatin [98,99]. ATR kinase phosphorylates p53 [100] and more recently, ATR has been linked to the activation of specific pathways of the mitogen-activated protein kinase (MAPK) cascade [101,102], which phosphorylates p53. The major MAPK subfamily members, which include the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNKs, also referred to as stress-activated protein kinase (SAPK), and the p38 kinases, participates in integrating extracellular signals to regulate cell proliferation, differentiation, cell survival, and apoptosis [103], and its inhibition leads to cisplatin resistance [104]. Therefore p53 induced and/or activate transactive several genes [105] associated with cell cycle arrest, DNA repair, and apoptosis. They include CDK inhibitor p21Waf1/Cip1, growth arrest and DNA damage-inducible gadd45a gene, and the pro-apoptotic bax gene [106,107].

The p53 protein can also transactivate mdm2, which is a negative feedback regulator of p53 activity [108].

However, when DNA damage exceeds a critical threshold, and presumably overwhelms cellular repair capacity, the net biological effect favours activation of apoptosis. This form of cell death is complex, and begins with the translocation of the cisplatin-induced Bax from the cytosol to the mitochondria, where a cascade of events, involving the release of apoptogenic factors (such as cytochrome c) activates the caspase 9–caspase 3 pathway, and results in apoptosis [109,110]. In particular, cisplatin induced cell death acts by interfering with Bax:Bcl-2 ratio, which regulates the apoptotic process. Cisplatin can increase this ratio inducing both BAX and cleavage of Bcl-2 that results cell death [111]. It also occurs through the Fas/FasL activated caspase 8–caspase 3 pathway, which is facilitated by p53 function, but does not necessarily involve the mitochondria [112,113]. However, this pathway is not well understood as caspase 8 or apoptosis can be activated by cisplatin independent of Fas/FasL in some systems [114,115].

Currently, p53 role in cisplatin-induced apoptosis this pathway is unclear. In fact, several reports have demonstrated that disruption of p53 function sensitizes tumour cells to the platinum drug, and do not make them resistant, as would be expected. It is useful to note that this counterintuitive finding is associated with tumour cells that appear to have an apoptotic dysfunction [116]. Probably it could be the result of loss in the contributory role of

p21Waf1/Cip1 [117] in G2/M arrest, resulting in premature entry into mitosis, with cell death being the final outcome [118].

Indeed, it has been demonstrated that another protein product of a p53-related gene, p73, can also be induced by cisplatin to mediate apoptosis. This finding is a demonstration of the ability of cisplatin to induce cytotoxicity through a mechanism not involving p53. Induction of p73-dependent apoptosis by cisplatin requires both drug-activated c-Abl tyrosine kinase and cellular proficiency of the MMR complex, which, as with HMG1, links damage recognition to apoptotic signaling. c-Abl activated by cisplatin can also upregulate the MEKK–MKK–JNK pathway [119], but the implied association between this specific MAPK pathway and p73 has been uncertain previously. However, the case for this association has been strengthened by recent evidence, which shows that activation of p73 by c-Abl also requires the activity of p38 as a representative of the MAPK subfamily member [120].

### **1.2.1. Platinum Resistance**

The success of cisplatin therapy is compromised due to severe multiorgan toxicities that may include failure of the kidneys and bone marrow, intractable vomiting, peripheral neuropathy, deafness, seizures, and blindness [121], as well as resistance by tumour cells to cisplatin. It has been observed that many solid tumours are intrinsically resistant, instead among initially sensitive tumours, acquired resistance commonly develops during the treatment. Acquired resistance is a particular problem, since tumours may become resistant also to other drugs with different mechanisms of action [122,123]. Platinum resistance mechanisms can be divided in two main groups: factors that limit the formation of cytotoxic platinum-DNA adducts [123] and factor that prevent cell death after platinum-DNA adduct formation [86].

*Factors that limit the formation of cytotoxic platinum-DNA adducts.* Classical resistance mechanisms involve factors that influence cisplatin uptake. In particular, reduction in cisplatin accumulation has been found in many resistant cell lines [125].

The blood flow, which regulates the delivery of chemotherapy and oxygen, causes cisplatin uptake alterations. Human tumour cisplatin concentrations do vary with pulse and blood pressure, with metastatic site, and with tumour type [126]. Moreover, blood viscosity could influence tumour blood flow and drug delivery [127,128].

Furthermore, another factor that influences cisplatin uptake is pH. In particular tumour extracellular low pH markedly increased cisplatin uptake [129]. In addition, it has been observed that cisplatin efficacy increase at low intracellular pH [130]. Thus, in cisplatin

resistant cells that present a significantly increased pH, cisplatin DNA binding is reduced [131]. Other factors that decrease cisplatin uptake and cytotoxicity in vitro are mannitol, NaCl, [132] KCl, while CaCl<sub>2</sub> and CuCl<sub>2</sub> may increase net cisplatin accumulation and cytotoxicity in vitro [133].

Moreover, another contribution to resistance comes from platinum binding to proteins either in plasma or at interstitium, which reduce cisplatin cytotoxic [134], and its uptake into cells [135] and tissues [136].

Since cisplatin uptake is linear, non saturable, it could be enter into cells by passive diffusion [137,138] or by facilitated transporters [139], either with low affinity or in great abundancy [140]. Several carrier-mediated transporters, including the Na<sup>+</sup>, K<sup>+</sup>-ATPase [141] and members of solute carrier (SLC) transporters [142] are involved in cisplatin uptake.

The plasma membrane copper transporter-1 (CTR1) contributes to platinum cellular uptake [143,144]. Interestingly, both copper and cisplatin cause rapid down-regulation of CTR1 in ovarian cancer cells by the proteasome-mediated pathway [145]. In particular, copper transporters undergo rapid cytoplasmic internalization after cisplatin exposure, reducing transporter surface expression and limiting further cisplatin uptake. A recent study demonstrated that copper transporter-2 or CTR2 limits accumulation of cisplatin and the level of CTR2 correlates with the sensitivity of ovarian carcinoma cells to cisplatin [146].

Furthermore, microtubule alterations with defective formation of the endocytic recycling compartment may lead to cisplatin resistance and reduced uptake of cisplatin and other molecules [147].

Since multiple transporters may be involved in cisplatin uptake, depending on the cellular context, it is difficult to correlate cisplatin sensitivity/resistance with a particular transporter. Many cell lines with acquired resistance to cisplatin often exhibit reduced drug accumulation, which is probably due to a decreased uptake of cisplatin rather than an increase in drug efflux [148, 149]. Cisplatin cellular uptake is also altered by metabolic inhibitors, which do not affect efflux [150,151]. A reduction in uptake of folates, epidermal growth factor (EGF), iron, glucose and others, may be involved in reduced cisplatin uptake, in conjunction with down-regulation of various transporters, defective endocytosis, and down-regulation of the small GTPases rab5, rac1, and rhoA which regulate endocytosis [152]. Some recent studies show that sphingolipid pathway modulation [153] and cholesterol depletion [154] increase cellular platinum uptake and efficacy.

Thus, cisplatin efflux from cells or from nucleus to cytoplasm is a parameter that might be associated with resistance [155, 156]. The copper transporters ATP7A and ATP7B have been

implicated in cisplatin export [157]. Other pumps that may also play a role in cisplatin resistance include the MRP2 (multidrugresistance-associated-protein-2), cMOAT (canalicular Multispecific Organic Anion Transporter), glutathione-X conjugate pump [158-160], MRP1, p-glycoprotein [161], and major vault [162,163]. In ovarian cancer (OC) cells, lung resistance-related protein (LRP) down-regulation reversed resistance, increased cellular cisplatin accumulation, increased cisplatin in isolated nuclei, and decreased cisplatin efflux from nuclei [164]. Again, clinical importance of each of these different efflux mechanisms remains uncertain.

Multidrug resistance protein (MRP) is a member of a family of transport proteins that facilitates the extrusion of a variety of glutathione (GSH)-coupled and unmodified drugs out of cells, but over-expression of MRP alone does not confer resistance [165]. The formation of conjugates between GSH and platinum drugs is a slow process and it may be an important step for their inactivation and elimination from the cell [87]. There is a strong association between increased platinum drug sensitivity and lower GSH levels. Thus, glutathione and metallothioneins are the abundant intracellular thiols involved in the drug resistance [166,167]. Glutathione (GSH  $\gamma$ -glutamylcysteinylglycine), the most abundant tripeptide thiol in the cells, is a potent nucleophile that reacts with alkylating agents as cisplatin in a 2:1 molar and the glutathione-Pt complex is effluxed from the cell in an ATP-dependent manner by the glutathione transporter family, termed the GS-X pumps [168]. Thus, GSH may protect cells both by intercepting reactive platinum complexes before they can react with DNA and by supporting DNA repair, probably by stabilizing repair enzymes such as DNA polymerase [169]. Since the higher levels of GSH do not correlate with decreased sensitivity to cisplatin [170], probably high GSH levels sensitize cells to cisplatin by up-regulation of the copper transporter hCtr1 [171]. Moreover, resistance might be augmented by GSH-related enzymes activity, as glutathione S-transferase P1-1 (GSTP1-1) [172] and other enzymes in the glutathione transferase family (GST $\mu$  or the GSTO1) [173]. However, the effect of GSH overexpression in cisplatin resistance is still under debate [165].

Inactivation may also occur by binding of the platinum drugs to metallothionein (MT) proteins. MTs are a family of sulfhydryl-rich, small molecular weight proteins. The four isoforms of MTs (MT1-MT4) are ubiquitously expressed in humans and are inducible by a variety of drugs, including cisplatin [174]. They are involved in zinc and copper homeostasis, heavy metal detoxification, protection from apoptosis [175], decreased cisplatin efficacy [176]. Recent reports suggest that the increased expression of metallothionein correlates with cellular resistance against cisplatin [177,178]. Modulation of MT levels can alter cisplatin

sensitivity but the contribution of MT to clinical platinum drug resistance is unclear [165]. The isoforms, MT-1 and MT-2, have been shown to react faster with cisplatin [179] than glutathione [180]. The basal levels of MT-1 and MT-2 are often significantly increased in cancer cells [181], resulting in even stronger scavenging of divalent platinum, and contributing to acquired resistance against cisplatin [182]. Recent reports, suggest that MT-3 is overexpressed in hypoxic conditions, and the reaction between MT-3 and Pt(II) is kinetically preferred. The authors further proposed that the Zn(II) released from this reaction can result in the up-regulation of the MT-1 and MT-2 isoforms [178].

*Factors that prevent cell death after platinum-DNA adduct formation.* Since once platinum-DNA adducts are formed, cells must either repair or tolerate the damage in order to survive, thus, the capacity to repair DNA damage rapidly and efficiently plays a role in determining a tumour the cellular sensitivity to platinum drugs. Several studies reported that cisplatin-resistant cell lines show increased levels of DNA repair [183,184].

This mechanism occurs predominantly by nucleotide excision repair (NER) system (particularly ERCC1 and ERCC1/XPF) and the related genes XPA and BRCA1 [185]. It has been observed that this pathway is defective in the inherited disease xeroderma pigmentosum (XP) and cells derived from XP patients are exquisitely sensitive to cisplatin [186].

A number of studies correlated the overexpression of ERCC1 or XPA proteins with cisplatin resistance [187,188], also in ovarian cancer [189]. In addition, epigenetic changes, such as hyper-methylation of ERCC1, have been suggested as a mechanism for enhanced cisplatin sensitivity [190]. A recent study demonstrated that XPA binding domain of ERCC1 was required for the repair of cisplatin-damaged DNA [191]. NER is also linked to the cellular signalling pathways and it may prevent cisplatin-induced apoptosis [192]. Functional NER was also required for cisplatin-induced transcription of Bcl-xL via nuclear factor-kappa B (NF- $\kappa$ B) [193]. Moreover cisplatin can also induce transcription-coupled repair (TCR) [194], while p53 protects against cisplatin-induced apoptosis in a TCR-dependent manner [195].

In addition, the homology-directed DNA repair (HR) that allows error-free repair of the double-strand breaks caused by the excision of cisplatin-DNA adducts has been implicated in the repair of cisplatin-induced DNA damage [196] and in cisplatin resistance. The base excision repair enzyme DNA polymerase B is over-expressed in several cisplatin-resistant cell lines [197] and it frequently causes incorporation of incorrect bases during platinum adduct repair [198].

Cisplatin resistance has also been associated with over-expression of the DNA damage recognition protein HMG1 [199] and of damaged-DNA-binding-protein-2 (DDB2) (which is involved in UV damage repair) [200].

Thus, platinum-DNA damage tolerance is a phenotype that may result from alterations in a variety of cellular pathways. Among these, the loss of function of the DNA post-replicative mismatch repair (MMR) system has been observed in platinum-resistant cells.

The main function of this system is to scan newly synthesized DNA and to remove mismatches that result from nucleotide incorporation errors made by the DNA polymerases. The repair function of MMR proteins has been reported to be uncoupled from their function in mediating cisplatin-induced cell death [201,202]. An increase in platinum sensitivity and in apoptosis follows the DNA post-replication mismatch repair (MMR)-mediated processing of platinum adducts. Thus, cells deficient in MMR [203] have increased cisplatin resistance and reduced apoptosis [204]. Since for MMR to enhance apoptosis requires an intact c-Abl and p73 system, a lack of p73 expression may lead to an increase of cisplatin resistance [205]. Concurrent p53 loss also enhances resistance development [206].

Since p53 is involved in the effect of cisplatin, defective apoptosis may contribute to resistance to both p53-dependent and -independent cisplatin cytotoxicity [207]. Cells with p53 deletions or mutations [208] are often resistant to cisplatin as results of global down regulation of caspase and Bax expression, but increased Bcl-2 expression [209]. Moreover cisplatin-resistant cells present abnormal mitochondrial membrane potential, intracellular distribution, or structure, and with up-regulation of cytochrome-c in the mitochondria in response to cisplatin rather than release into the cytoplasm [210]. The over-expression of Xiap (X-linked inhibitor of apoptosis protein) and PI3-K/Akt pathway induction could inhibit apoptosis [211]. Furthermore, cisplatin generation of reactive oxygen species causes dephosphorylation and degradation of Bcl-2, while nitric oxide (NO) induces its S-nitrosylation, inhibiting its ubiquitination and upregulating Bcl-2 expression. Some resistant ovarian cancer cell lines had increased expression of Fas-associated death domain-like interleukin-1 $\beta$ -converting enzyme-like inhibitory protein (FLIP). Cisplatin decreased FLIP and induced caspase-8 and caspase-3 cleavage and apoptosis in cisplatin-sensitive but not in resistant cells. FLIP downregulation in chemoresistant cells increased cisplatin-induced apoptosis [212].

### **1.2.2. Multidrug resistance (MDR)**

One of the leading causes of chemotherapy failure in ovarian cancer is the development of MDR, a phenomenon by which cancer cells develop the ability to survive in the presence of structurally and functionally different anticancer agents [213].

Possible reasons for the onset of MDR include cancer cell genetics and phenotype, tumour microenvironment and inadequate drug exposure. Several factors described previously (*see section 1.2.1.*) have been documented as key contributors to MDR, which result in limited and drug penetration, heterogeneous distribution of drug and regions with slowly proliferating cells that are less chemosensitive. As well, inadequate drug exposure at tumour sites can lead to MDR. This may result from an insufficient therapeutic dose reaching the tumour, short drug residence time and/or drug metabolism and excretion [214].

### **1.3. Strategies in the management of platinum-resistant OC**

Despite initial chemosensitivity to platinum/taxane combinations, most patients with advanced EOC relapse after first-line therapy [215]. In particular a categorisation, recently updated and confirmed by the GCIG 4th Ovarian Cancer Consensus Meeting, defines ‘platinum-refractory’ as patients progressing during therapy or within 4 weeks after the last dose; ‘platinum-resistant’ patients progressing within 6 months of platinum-based therapy; ‘partially platinum-sensitive, patients progressing between 6 and 12 months; and ‘platinum-sensitive’ patients progressing with an interval of more than 12 months (GCIG Consensus) [216]. Furthermore, the category of ‘platinum-resistant/ refractory’ comprises patients whose disease recurs after one or several lines of treatment. Its biological behaviour may be very variable, with differing growth rates and distribution of symptoms requiring different approaches to treatment, which should be focused on quality of life and control of symptoms. Traditionally, this is a poor prognosis population with a short expected OS, usually less than 12 months. Recurrent epithelial ovarian cancers appears to be associated with the value of surgical cytoreduction, and the prognosis and probability of response to second-line therapy and subsequent lines depends in great part on the progression-free interval after the last dose of the preceding line of chemotherapy [217,218, 228]. Various attempts to find out drugs for better survival outcomes in ovarian cancer can be largely divided into three ways. The first includes standard chemotherapies with modification of dose and schedule or different combinations of chemotherapeutic agents, while the second involve molecular targeted agents with or without chemotherapy [219]. Moreover a third way consists in exploring novel drug delivery systems (DDS) for chemotherapies [220]. Developing and evaluating innovative

clinical trials with biologically relevant molecular targeted agents was among these priorities [221].

### **1.3.1. Standard chemotherapies**

There are currently four standard chemotherapy regimens used for patients who become resistant to platinum: paclitaxel, topotecan, pegylated liposomal doxorubicin (PLD), and gemcitabine [219]. Occasionally, platinum drugs continue to be used in the ‘platinum-resistant’ population with, for example, a dose-dense regimen [222,223].

Paclitaxel is a taxane derivative that can stabilize microtubules to inhibit cell division. It was approved for treatment of recurrent and resistant OC. Paclitaxel showed promising response rates in ovarian cancer [224-226]. Alternative weekly dosing schedule for paclitaxel had a better safety profile than the 3-weekly schedule in recurrent OC patients [227,228].

Topotecan, pegylated liposomal doxorubicin (PLD), and gemcitabine are agents that induce irreversible DNA damage. Topotecan acts by topoisomerase 1 inhibition, which leads to both single and double stranded DNA breaks that eventually promote apoptosis. Phase III trial showed that it to be at least as effective as paclitaxel. Thus, it was approved for treatment of OC after failure of initial or subsequent chemotherapy [229]. Moreover, multiple clinical phase II trials have demonstrated that alternative dosing schedules improve the tolerability of topotecan [230,231].

Pegylated liposomal doxorubicin (PLD) (Doxil in the USA, Caelyx in Canada and Europe) is a new treatment for advanced ovarian cancer, which delivers doxorubicin encapsulated in long-circulating. Stealth liposomes, result in a prolonged circulation and enhanced tumour targeting of the drug, together with a markedly different safety profile compared with native doxorubicin.

The active component of doxorubicin (PLD) is an anthracycline that intercalates DNA, leading to inhibition of replication and, subsequently, inhibition of proper cell division. Efficacy of PLD in platinum-resistant OC has been confirmed in several Phase II trials [232]. Additionally, the CALYPSO trial demonstrated that the combination of carboplatin–PLD was not inferior to paclitaxel–carboplatin in terms of PFS, but it was better tolerated [233]. Phase III study are evaluating the effectiveness in ovarian cancer (CR100654, DOXILOVC3001, NCT01840943) [234].

Trabectedin is a novel marine antineoplastic alkaloid that targets DNA. It showed a survival benefit in combination with PLD (OVA-301 trial), probably by the restoration of platinum-sensitivity and artificially prolonging the platinum-free interval [235,236].

Gemcitabine is a nucleoside analogous that can incorporate into the DNA, leading to induction of apoptosis. The combination with carboplatin had already been approved for treatment of recurrent platinum-sensitive OC. Several Phase III trials showed that the efficacy of gemcitabine was comparable to PLD compared in platinum-resistant OC patients as well [237,238].

### **1.3.2. Novel chemotherapies**

Novel strategies for platinum-resistant ovarian cancer include agents that target DNA or microtubules polymers.

SGI-110 is a novel DNA-hypomethylating agent that restores silenced tumour suppressor gene expression in cancer cells by reversal of DNA methylation [239]. A Phase II study is currently evaluating the effect of SGI-110 in combination with carboplatin in platinum-resistant recurrent ovarian cancer patients (NCT01696032) [234, 240].

Another agent that targets DNA is SJG-136 (NSC 694501). This synthetic pyrrolobenzodiazepine dimer exerts antitumor activity through the formation of sequence-specific cross-links in the minor groove of DNA. SJG-136 caused significant growth delays in ovarian carcinoma even if significant toxicities were observed. In particular, it showed cytotoxic effects in cell lines resistant to other DNA-interactive agents, including human ovarian cancer cell lines resistant to cisplatin [241]. Phase II trials of SJG-136 are being planned in platinum resistant or refractory ovarian carcinomas (NCI-2011-02519, VICC-GYN-1003, N01CM62208, CDR0000682791, MCC-VICC-GYN-1003, 8489, NCT01200797) [234].

Canfosfamide is a glutathione analogous prodrug that is activated by glutathione S-transferase P1-1, which is overexpressed in OC, and leads to the induction of apoptosis [242,243]. Phase III trials showed that it was well tolerated as single agent, while in combination with PLD the median PFS was improved in platinum-resistant OC patients [244].

Other DNA target agents are the histone deacetylase (HDAC) inhibitors. HDACi derepress genes that results in growth inhibition, differentiation and apoptosis of cancer cells.

In particular, vorinostat (SAHA) have confirmed therapeutic benefits in cutaneous T-cell lymphoma (CTCL) without combination therapy and have also demonstrated some therapeutic benefit in other malignancies. Epigenetic agents in combination regimens for cancer therapy are being actively studied [245]. Phase I, II studies are evaluating vorinostat, carboplatin and gemcitabine combination in ovarian cancer (09-026, NCT00910000) [234].

Other chemotherapies target microtubules, dynamic filamentous cytoskeletal proteins that are responsible for cellular integrity and architecture, mitosis, intracellular transport, cell signalling and gene expression. Tubulin exists in the cell as dimers of  $\alpha$  and  $\beta$  subunits, which complex with a variety of regulatory proteins. There is a dynamic equilibrium between free and polymerized tubulin causing a state called "dynamic instability," which is a target of anticancer drugs, which inhibit tubulin through polymerisation (taxanes, epothilones) or depolymerisation (vinca alkaloids).

The taxol analogues, Cabazitaxel, demonstrated activity in multidrug- and docetaxel-resistant cancer cell lines [246]. A phase II study are currently on going in platinum refractory ovarian cancer (TaxOvar, NCT01747239) [234].

Patupilone (Epothilone B, EPO906) and its semisynthetic derivative ixabepilone (BMS-247550) are microtubule-stabilizing agents. Patupilone showed a similar response rate (RR) than PLD in a Phase III trial in platinum-refractory or -resistant epithelial ovarian [247].

Ixabepilone showed promising results in Phase II trial in patients with recurrent or persistent platinum- and taxane-resistant ovarian cancer [248]. Phase II trials of ixabepilone plus liposomal doxorubicin are being planned in advanced ovarian epithelial cancer (CDR0000441122, AECM-0504007857, NCI-7229, NCT00182767) [234].

Vinorelbine, is the first 5'NOR semi-synthetic vinca alkaloid. Phase II trials of vinorelbine and gemcitabine combination chemotherapy are being planned in platinum-resistant epithelial ovarian/ cancer patients treated (KCSG GY10-10, NCT01196559) [234, 249].

Also BAL 101553, a highly water soluble prodrug of the synthetic novel small molecule BAL27862, arrested tumour cell proliferation and induces cell death in cancer cells through disruption of the microtubule network. BAL101553 showed activity against a broad range of tumour types [250]. BAL101553 is currently under evaluation in phase I/II study in adult patients with advanced solid tumours who have failed standard therapy or for whom no effective standard therapy is available (CDI-CS-001, NCT01397929) [234].

### **1.3.3. Targeted therapies**

Targeted therapies may represent the future of platinum-resistant OC treatment. However, genetic profiling of tumours before and after a treatment escape will provide insight into the drug resistance mechanisms. Based on the outcome of these studies, it may be possible to predict which combinations of targeted therapies (or chemotherapies) can prevent treatment escape. Current approaches to targeted therapy for OC can be classified into different groups, with each therapy having its own mechanism of action as well as specific advantages. For

example, some of these therapies target several pathways as folate, angiogenesis pathway and signal transduction pathway [219]. Immunotherapy also represents an alternative and rational approach for the treatment of ovarian cancer [251,252]. Others target therapies are directed against DNA repair mechanisms [219].

#### **1.3.4. Advanced drug delivery strategies**

Since inadequate drug exposure at tumour sites can lead to MDR, different approaches to overcome MDR have been pursued, including the use of drug efflux transporter inhibitors, synthesis of more active drug analogs and development of prodrugs. Furthermore, drug delivery systems (DDS) have also been developed to bypass systemic and peritoneal transport barriers in order to achieve higher therapeutic drug concentrations at diseased sites. DDS include nano-sized DDS, microspheres, implants and injectable depots. These DDS have the potential to provide sustained drug release over extended periods in the peritoneal cavity. Sustained and localized delivery may also improve intratumoral drug distribution and penetration and allow for circumvention of MDR [213].

#### **1.4. Angiogenesis pathway targeting**

Angiogenesis plays a fundamental role in normal ovarian physiology, as well as in the pathogenesis of ovarian cancer, promoting tumour growth-progression through ascites formation and metastatic spread [253, 254]. This process involves the recruitment of mature vasculature and circulating endothelial cells [255], proangiogenic soluble mediators and tyrosine kinase receptors [256] resulting in cellular proliferation, differentiation, metastasis, angiogenesis [257], endothelial survival, enhancement of vascular permeability, and effects on hemodynamic status [258, ] by activation of a number of downstream intracellular signalling cascades such as the PI3/Akt protein kinase B pathway, MAPK and Raf/MEK/Erk pathway [257,259].

*Vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) inhibitors.* VEGF and VEGFR are expressed in ovarian cancer and have been associated with poor survival [260,261]. The VEGF family consists of seven ligands, VEGFA–VEGFE [262]. Strategies to target the VEGF pathway include monoclonal antibodies directed against VEGF or its receptors and small molecule tyrosine kinase inhibitors that act intracellularly to prevent autophosphorylation and signalling of downstream pathways.

Bevacizumab (Avastin®, Genentech; South San Francisco, CA, USA) is an anti-VEGF humanized monoclonal antibody, which binds onto VEGF with high affinity neutralizing its activity. Phase III trials [263, 264] showed that bevacizumab was efficient as first-line therapy in combination with chemotherapy (paclitaxel-carboplatin) and as maintenance therapy also in combination with carboplatin and gemcitabine chemotherapy (NCT00434642 [OCEANS] [265]. Moreover phase III trial with the bevacizumab and chemotherapy (pegylated liposomal doxorubicin [PLD], topotecan, or paclitaxel) combination in platinum-resistant EOC, showed promising results [266]. GOG 213 phase III trial is currently recruiting patients to evaluate adjuvant combination chemotherapy (paclitaxel or docetaxel, plus carboplatin) with bevacizumab in platinum-sensitive, recurrent EOC (NCT00565851), following cytoreductive surgery [267]. AGO-OVAR17 phase III trial is currently recruiting patients to evaluate the optimal treatment duration of bevacizumab maintenance following front-line therapy (bevacizumab plus carboplatin/paclitaxel) in patients with primary EOC (NCT01462890; BOOST) [268].

Another anti-angiogenic agent is Aflibercept, or VEGF-Trap, which is a fusion protein containing the VEGF binding regions of both VEGFR-1 and 2 linked through the Fc region of a human IgG1. Aflibercept binds VEGF-A and neutralizes all VEGF-A isoforms plus placental growth factor (PDGF) [269]. This agent is currently being explored in platinum resistant ovarian cancer and pre-clinical data showed inhibition of the angiogenic effect, including narrowing of vessels, endothelial cell apoptosis, stop of blood flow and reduction of tumour burden and ascite formation [270,271]. VEGF trap also prolonged survival when combined with paclitaxel [272]. Preliminary results from a phase I/phase II trial of aflibercept in combination with docetaxel in patients with recurrent ovarian cancer are promising [273].

Tivozanib hydrochloride (formerly AV-951, KRN-951) is a potent and selective VEGFR tyrosine kinase inhibitor, which was well tolerated with a recognizable, manageable pattern of side effects, reproducible biologic and clinical activity [274]. A Phase II study [STU00073756, NCT01853644] is evaluating tivozanib effects in recurrent ovarian cancer [234].

*Platelet derived growth factor receptor (PDGFR) and fibroblast growth factor receptor (FGFR) inhibitors.* PDGF and FGF are other factors involved in angiogenesis and associated with ovarian carcinomas and poor survival [275,276]. Moreover, both PDGF and FGF signalling pathways [277,278] appear to be involved in VEGF resistance described across various solid tumours, suggesting that combined inhibition of VEGF and PDGF and/or FGF may more completely block angiogenesis than VEGF inhibition alone [278,279]. Several

agents that target the PDGFR have been studied. These include imatinib mesylate selective Abl, c-Kit, and PDGFR inhibitor, which failed to demonstrate clinical benefit in patients with ovarian cancer [280]. Dasatinib [281], another PDGFR selective inhibitor, is currently studying in the Treatment of Persistent or Recurrent Epithelial Ovarian Cancer (GOG 170 M Phase II study).

Moreover, several small molecule multitargeted tyrosine kinase inhibitors are being studied in ovarian cancer [267].

BIBF 1120 (Boehringer Ingelheim; Germany, or Intedanib, Nintedanib, vargatef) targets VEGFR, PDGFR, FGFR, members of the v-src sarcoma viral oncogene homolog (Src) family, fms-like tyrosine kinase 3 (Flt-3). Results of a phase II study are promising in patients with recurrent EOC [282]. BIBF 1120 has been used as a single agent, but also in combination with carboplatin /paclitaxel in epithelial ovarian cancer patients [283]. An ongoing phase III trial is investigating the combination of nintedanib with chemotherapy standard and as maintenance therapy in EOC (NCT01015118, AGO OVAR12/LUME-Ovar1) [234, 268].

Pazopanib (or GW 786034, GlaxoSmithKline; London, UK) inhibits the activity of VEGFR, PDGFR, FGFR, and c-kit. Results of a phase II study of pazopanib in pretreated recurrent EOC are promising [284]. The evaluation of the antitumor activity and acceptable tolerability for pazopanib in recurrent EOC are ongoing in a phase III trial (NCT00866697) [AGO-OVAR16] [268]. Phase I-II studies are evaluating pazopanib and chemotherapy combination (I3107000 NCT01238770; CDR0000703686 EORTC-55092, EU-21119, EUDRACT-2010-024077-39, NCT01402271) [234].

Cediranib (or AZD2171, Recentin<sup>TM</sup>, AstraZeneca; Wilmington, DE, USA) neutralizes the effect of molecules such as VEGFR, FGFR, and PDGFR. Phase II studies in patients with either platinum-resistant or -sensitive recurrent EOC showed a clinical benefit [285,286]. A Phase III trial is currently assessing the safety and efficacy of platinum-based chemotherapy with cediranib in platinum sensitive relapsed EOC [268].

Sorafenib neutralizes the effect of VEGFR and PDGFR, stem cell factor receptor (c-kit), (Raf). Phase II trials with sorafenib in combination with chemotherapy in advanced and recurrent OC EOC, failed [287]. Instead, preliminary results from an ongoing phase II study of intermittent sorafenib and bevacizumab administration in patients with recurrent EOC showed prolonged PR and clinical benefit [288]. Moreover, a randomized phase II study is evaluating sorafenib as maintenance therapy in advance EOC (NCT00791778 [Study 12007]).

Sunitinib inhibits VEGFR and PDGFR among other molecules. Although, pre-clinical data have shown that it can inhibit tumour growth and reduce microvessel density count [289], some studies exhibited modest efficacy results [290]. However, other studies are still ongoing. AMG 706 is an investigational inhibitor of VEGFR , PDGFR receptor, and stem-cell factor receptor. A Phase II Evaluation of AMG706 (NCT00574951) in the Treatment of Persistent or Recurrent Epithelial Ovarian Fallopian Tube or Primary Peritoneal Cancer is currently active [290, 291].

Finally, AMG-386 (Amgen; Thousand Oaks, CA, USA) is a peptide-Fc fusion protein that inhibits the binding of both Angiopoietina and Tie receptors [292]. A Phase II study with AMG 386 and paclitaxel in recurrent ovarian cancer showed that the median PFS was prolonged with a significant dose response effect and safety profile was tolerable [293]. Thus, has been evaluated in EOC, in several phase III clinical trials and in combination with paclitaxel (TRINOVA-1[NCT01204749]), pegylated liposomal doxorubicin (TRINOVA-2 [NCT01281254], and as first-line in association with carboplatin/paclitaxel and then as maintenance therapy for EOC (TRINOVA-3 [NCT01493505]. Now, weekly AMG 386 is being investigated in phase III studies in primary (TRINOVA-3; ClinicalTrials.gov, NCT01493505) and recurrent settings (TRINOVA-1; ClinicalTrials. gov, NCT01204749) [286].

A number of studies are currently on going to evaluate the effect of anti-angiogenic drugs, mainly via the usage of potential biomarkers. In particularly VEGF could be used as a biomarker to predict the effect of bevacizumab [293], PDGF may act as a biomarker for predicting the effect of anti-angiogenic drugs [294].

*Epidermal growth factor receptor (EGFR) inhibitors.* EGFR over-expression has also been observed in ovarian cancer, but the relationship between over-expression and prognosis in ovarian cancer remains controversial [295,296]. The EGFR family consists of four related transmembrane tyrosine kinase receptors: ErbB1 (HER 1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) [257]. Strategies that target the EGFR in gynaecologic diseases include monoclonal antibodies [297-300] (trastuzumab, pertuzumab, EMD7200) and tyrosine kinases inhibitors [301-303–314] (gefitinib, erlotinib, lapatinib and CI-1033).

A Phase II trial on ovarian cancer patients treated with trastuzumab reported a moderate response rate [297]. Several studies examined pertuzumab, a humanized recombinant monoclonal antibody that inhibits the dimerization of HER2 with EGFR, HER 3, and HER4, in patients with ovarian cancer [303], but as single agent there were only modest responses

[304]. However, several studies with pertuzumab are ongoing. The EORTC have recently completed a trial investigating erlotinib as maintenance therapy following first-line chemotherapy in patients with ovarian cancer (NCT00263822), while results of a phase II trial of erlotinib and bevacizumab in patients with advanced ovarian cancer (NCT00696670) are uncertain [305]. However, it has been observed that EGFR tyrosine kinase mutations in patients with platinum resistant disease were correlated with a response to EGFR inhibitors [303,306]. Since EGFR activation has been reported to promote VEGF [307], the combination of EGFR inhibitors (erlotinib) and VEGF inhibitors (bevacizumab) are exploring in recurrent ovarian. Phase II trial of Bevacizumab or Bevacizumab and Erlotinib as First Line Consolidation Chemotherapy after Carboplatin, Paclitaxel, and Bevacizumab (CTA) (NCT00520013) are currently under investigation [308].

MM-121 is a monoclonal ErbB3-directed antibody that significantly inhibits tumour growth in vivo [309]. Phase I/II clinical trials are currently ongoing with MM-121 and paclitaxel in epithelial ovarian cancer (MM-121-04-02-08 ARD11586, NCT01447706).

Vandetanib is an example of drug with dual action, inhibiting both VEGFR and EGFR tyrosine kinase activity thereby preventing angiogenesis, cell migration and proliferation. It is currently being tested in combination with liposomal doxorubicin in recurrent ovarian cancer in a phase I/II trial (NCT00862836). Moreover, a phase II study is evaluating docetaxel alone or in combination with vandetanib (NCT00872989) [310].

*Steroid receptor coactivator (Src) inhibitors.* Src is a downstream non-receptor protein tyrosine kinase that plays a multitude of roles in cell signalling and carcinogenesis, and is associated with poor prognosis in ovarian carcinogenesis [311]. It promoted chemotherapy resistance and tumour survival in ovarian cancer cell lines [312]. Src inhibition has been shown to augment the activity of paclitaxel and cisplatin in ovarian cancer cell lines and to reverse chemo-resistance [313]. Src inhibition also has been found to have a potent anti-angiogenic effect evidenced by down-regulation of VEGF and IL-8 [314]. Thus, agents that target Src have been studied. These include Dasatinib, multitargeted tyrosine kinase inhibitor that inhibits SRC-family kinases, as well as PDGF $\beta$  and others, which has been tested alone and in combination with chemotherapy. The triplet combination could be safely administered and it demonstrated substantial clinical activity [315].

Saracatinib (AZD0530) is a highly selective inhibitor of Src and is being evaluated in phase 2 studies and in randomized trials [267].

*Activin receptor-like kinase-1 (ALK1) inhibitors.* Multiple lines of evidence implicate the BMP9/BMP10/ALK1 pathway as a key regulator of angiogenesis. In particular ALK1 expression is elevated in neovascular endothelium during tumour growth. ALK1 is a type I receptor in the transforming growth factor (TGF) superfamily with high affinity for BMP9 and BMP10 proteins, which are functional activators of ALK1 in endothelial cells [316].

Dalantercept, or ACE-041, is a soluble receptor fusion protein consisting of the extracellular domain of human ALK1 linked to the Fc portion of human IgG1. It binds with high affinity BMP9 and BMP10, thereby inhibiting activation of endogenous ALK1. It was generally well tolerated and demonstrated encouraging preliminary anti-cancer activity. It exhibited a safety profile distinct from that of VEGF pathway inhibitors and displayed multiple signs of antitumor activity, including a partial response and long-term stable disease [316]. The effectiveness of ACE-041 will be evaluated in phase II study combination (GOG-0170R NCI-2012-01936, NCT01720173) [234].

*Other antiangiogenic agents.* VB-111, a novel viral therapy agent for systemic administration, targets the endothelial cells in the tumour vasculature, driving them to apoptosis. VB-111 comprises a non-replicating adenovector (Ad-5, E1-deleted), which contains a proprietary modified murine pre-pro endothelin promoter (PPE-1-3X) and a Fas-chimera transgene (Fas and human TNF receptor 1). The Fas pathway effectively mediates cell death in normal and in malignant cells. Its activation by Fas ligand, which is highly expressed in normal tissues, is highly toxic. Hepatotoxicity is circumvented by the specificity of the PPE-1-3X promoter, which restricts activation of the Fas pathway to angiogenic endothelial cells. This specificity is further enhanced, as the Fas component of the chimeric transgene is activated via binding of the chimeric TNFR-1 receptor with TNF- $\alpha$ , which is less prevalent in normal tissues. The transcriptional rate of endothelin-1 is also augmented in the presence of cytokines like TNF- $\alpha$ , which also contributes to tumour specificity. This drug showed tumour activity, favourable safety and tolerability profile [317]. It is currently under evaluation in combination with Paclitaxel (VB-111-157 11-418, NCT01711970) [234].

BNC105P is a novel vascular disrupting agent (VDA). It is the disodium phosphate ester prodrug of BNC105, a tubulin polymerization inhibitor (TPI) that exhibited a high selectivity for tumour endothelial cells [318]. The combination of BNC 105P with carboplatin and gemcitabine is currently under evaluation in various clinical trials (Phase I/ II, GYN12-154 / ANZGOG-1103, NCT01624493) [234].

### 1.5. Signal transduction pathway targeting

Signal transduction pathway comprises a number of downstream intracellular signalling cascades, which are involved in regulation of multiple cellular processes that stimulate cell proliferation, invasion and angiogenesis. Since they are often deregulated in many human cancers, they represent targets for a novel therapeutic approach.

#### *Mesenchymal-epithelial transition factor (c-MET) and hepatocyte growth factor (HGF c-MET) signalling pathway inhibitors*

Abnormalities in the receptor tyrosine kinase c-MET and its ligand HGF c-MET signalling pathway have been reported to correlate with poor clinical outcomes and drug resistance in patients with cancer [319].

Cabozantinib (XL-184) is a novel, small-molecule, multi-targeted receptor tyrosine kinase inhibitor with particular activity against HGF (tyrosine-protein kinase Met), VEGFR and proto-oncogene tyrosine-protein kinase receptor Ret [320]. It is currently under evaluation in clinical trials in Persistent or Recurrent Epithelial Ovarian cancer (NCI-2012-02058, GOG-0186K, U10CA027469, NCT01716715) [234].

Rilotumumab (AMG102) is an anti-HGF monoclonal antibody that interferes with c-MET activation by HGF. It is currently evaluated in clinical phase I/II studies alone or in combination with the EGFR-blocking antibody panitumumab. Previous studies have shown that rilotumumab decreases c-MET phosphorylation and can stabilize the progression of certain solid tumours [319]. A phase II study with AMG102 Patients With Persistent or Recurrent Ovarian Epithelial Cancer is currently in going (NCI-2012-02058 GOG-0186K, U10CA027469, NCT01716715) [244].

*The mitogen-activated protein kinase (MAPK) signalling pathway inhibitors.* MAPK signalling pathways involve four distinct MAPK cascades, extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), p38 and ERK5. Each of these cascades comprised of three sequentially acting kinases, activating one after the other (MAPKKK/MAP3K, MAPKK/MAP2K, and MAPK). Mitogen-activated protein kinase or MAP2K or MAPKK are commonly known as MEK, which selectively phosphorylate serine/threonine and tyrosine residues within the activation loop of their specific MAP kinase substrates. The Ras/Raf/MEK/ERK signalling is activated in human cancers via several different mechanisms [321].

A number of MEK inhibitors have progressed into clinical trials.

Trametinib (GSK1120212, JTP 74057), a selective inhibitor of MEK 1 and 2, has emerged as the first MEK inhibitor to show favourable clinical efficacy in a phase III trial. Several phase II/III clinical trials of trametinib alone or in combination with other agents are in going [NCT01553851, NCT01682083, NCT01362296, NCT01619774, NCT01245062, details are available on [clinicaltrials.gov](http://clinicaltrials.gov)].

MEK162 (ARRY 438162) is a second generation inhibitor that targets MEK 1/2 [321]. Phase III clinical trial are in going with MEK162 in Low-grade Serous Ovarian Cancer (ARRAY-162-311 2013-000277-72, NCT01849874) [234].

LY2228820 dimesylate is a highly selective small molecule inhibitor of p38 $\alpha$  Mitogen-Activated Protein Kinase, and p38 $\beta$  mitogen activated protein kinases (MAPKs) that is currently under clinical investigation for human malignancies [322] also in phase II/I study with gemcitabine and carboplatin combination (12517, I1D-MC-JIAE; NCT01663857) [234].

*PI3K/AKT/mTOR pathway inhibitors.* PTEN is a lipid phosphatase that is associated with cell cycle G1-phase arrest and apoptosis through the PI3K/AKT/mTOR pathway [323]. The loss of functional PTEN often occurs in gynaecological malignancies [324] and leads to the constitutive activation of PI3K effectors in the absence of exogenous stimuli.

Potential therapies targeting the mTOR pathway include Temsirolimus (CCI-779), everolimus (RAD001), and deforolimus (AP23573). In ovarian cancer, AKT (Ser/Thr-specific protein kinase, also known as protein kinase B) activity is frequently elevated and is closely associated with the up-regulation of mTOR signalling. High levels of AKT activity in vitro resulted in hypersensitivity to mTOR inhibitors [325].

In vivo study with RAD001 inhibited tumour growth, angiogenesis, and production of ascites in xenografts of SKOV-3 cells [326]. GOG trial 170I has recently closed a Phase II Evaluation of Temsirolimus in the treatment of Persistent or Recurrent Epithelial Ovarian. Currently recruiting studies include (NCT00926107), mTOR inhibitor Temsirolimus (CCI-779) to treat ovarian cancer with Ca125 relapse only, a Phase I study of DOXIL and Temsirolimus in Resistant Solid Malignancies (NCT00703170), and a Phase I study of Docetaxel and Temsirolimus in resistant solid malignancies (NCT00703625) [234].

Moreover, the nucleoside analogue triciribine phosphate monohydrate (TCN-P) is a selective inhibitor of Akt activation. In Phase I and II studies demonstrated clinical activity against multiple tumour types[326] and the combination TCN-P with carboplatin combination will be evaluated Phase I/II study (MCC-17035, NCT01690468) [244].

*Insulin-like growth factor 1 receptor (IGF1) inhibitors.* IGF1 is another receptor involved in malignant transformation of cells by inhibition of apoptosis, tumour progression and metastases [327]. AMG 479 is a human monoclonal antibody directed against the IGF1 receptor. It is a potent inhibitor of the PI3–AKT pathway in xenograft models of pancreatic cancer and in addition enhances the anti-tumour effects of anti-EGFR targeted therapies (panitumumab, erlotinib and gefitinib) [328]. A phase II study of AMG 479 or placebo in combination with chemotherapy in the first-line treatment of advanced ovarian cancer is being planned (NCT00718523) [234].

Fenretinide, *N*-4-hydroxyphenyl retinamide (4-HPR) is a synthetic amide of retinoic acid, which inhibited cell growth through the induction of apoptosis, in ovarian carcinoma cell lines, probably by the depolarization of the mitochondrial membrane and activation of caspase pathway. Additional mechanisms are under investigations, and IGF1 has been recently proposed as surrogate biomarker of fenretinide efficacy [329]. Phase I/II, Phase (SPOC-2011-001 NCT01535157) are currently in going with fenretinide (4-HPR) and ketoconazole combination in recurrent ovarian cancer [234].

*Wee- 1 inhibitors.* Tyrosine kinase Wee1 acts as a gatekeeper of the G2/M checkpoint of progression through the cell cycle. Thus, its inhibition could result in cell growth arrest and inability to perform proper cell division [339].

The pyrazolo-pyrimidine derivative MK-1775 is the most potent and highly selective inhibitor of Wee1 [339]. MK-1775 plus carboplatin is currently evaluated in phase II study in treat p53 mutated refractory and resistant Ovarian Cancer (M10MKO / MK1775-009 NCT01164995) and in Combination With Paclitaxel and Carboplatin in Platinum-Sensitive Ovarian tumours With the P53 Gene Mutation (MK-1775-004) NCT01357161 [234].

*Aurora Kinase Inhibitors.* Aurora kinases are protein kinases that act as mitotic regulators. Thus, its activity is cell cycle dependent and active during the G2M phase of the cell cycle [331]. Aurora kinase A is over expressed in epithelial ovarian carcinomas and associate with tumour grade and survival [332].

MK-0457 is a pan-aurora kinase inhibitor small molecule, which significantly reduced tumour burden in both chemosensitive and chemoresistant human ovarian cancer cell lines [333]. It has been reported a significantly improved reduction in tumour growth after combination treatment with docetaxel and a synergic effect with paclitaxel, which results in apoptosis in ovarian cancer cells [334].

MLN8054, an orally active small molecule inhibitor of aurora kinase [335] that inhibited the tumour growth in human tumour xenografts in nude mice, through the induction of mitotic accumulation and apoptosis. MLN8054 is currently being explored in the management of patients with platinum-refractory or resistant epithelial, (NCT00853307) [234].

MLN8237, or alisertib, significantly reduced ovarian carcinoma cell migration and adhesion and the activation-associated phosphorylation of the cytoskeletal regulatory protein SRC. In vivo tumour growth and dissemination were inhibited by alisertib treatment as a single agent. Moreover, combination of alisertib with paclitaxel resulted in more potent inhibition of tumour growth and dissemination compared with either drug alone [336].

*Cyclin Dependent Kinases (CDK) inhibitors.* CDK family of serine/threonine kinases regulate progression through each stage of the cell division cycle and as such are major targets for deregulation in cancer. Several checkpoints are involved: particularly, G1 and G2 checkpoints are involved in response to DNA damage.

In particular PD0332991 is highly selective CDK4/6 (Cyclin Dependent Kinases 4 and 6) inhibitor, which demonstrated efficacy in Colo-205 colon tumour and SF-295 glioblastoma xenografts [337]. A Phase 2 Study is in going with PD0332991 in epithelial ovarian cancer (11-003234, NCT01536743) [244].

## **1.6. DNA repair mechanisms targeting**

Other therapeutic strategies target DNA repair mechanisms, such as Poly (ADP-ribose) polymerase (PARP) proteins. PARP is a family of key nuclear enzymes involved in the repair of DNA single-strand breaks (SSBs) using the base excision repair pathway [338], keeping low-fidelity non homologous-end-joining DNA repair machinery in check [339]. PARP-1 and PARP-2 are the only members of the PARP family that are known to be activated by DNA damage and they are essential for the maintenance of genomic stability [340]. Patients with EOC who harbour germline mutations in BRCA1/2 genes (sporadic ovarian cancers), approximately 10% of cases of EOC, exhibit defects in homologous recombination DNA repair [341]. A recent study suggests that over 50% of high-grade serous ovarian cancer had loss of BRCA function, either by genetic or epigenetic events [342], and is susceptible to PARP inhibitors [340].

Olaparib (AZD2281, KU-0059436; AstraZeneca) a potent oral PARP inhibitor, induces synthetic lethality in BRCA-deficient tumour cells [343]. The potential of olaparib has been

tested in multiple clinical trials in both platinum-sensitive and -resistant OC patients with or without *BRCA* mutations and a correlation between olaparib effects and platinum sensitivity has been observed [344]. The antitumor activity of olaparib was demonstrated in patients with platinum-sensitive recurrent high-grade serous ovarian cancer (HGS-OC) irrespective of *BRCA*1/2 germ-line mutations status. Also, olaparib maintenance therapy significantly improved PFS with tolerable toxicity profiles [345]. Indeed, a Phase III trial testing olaparib as a first line treatment will be performed in *BRCA*-mutated OC patients (NCT01844986). Pre-clinical data support the relationship between PARP inhibition and the VEGF pathway [346]. For example, a phase I trial of olaparib in combination with the VEGFR inhibitor cediranib is also open to recurrent ovarian or TNBC patients (NCT01116648) [347] and preliminary results are encouraging.

Niraparib (MK4827), an oral PARP inhibitor, showed selective lethality in HR repair deficient tumours with *BRCA* loss or non-*BRCA* HR defects [348]. Currently, phase III studies are evaluating Niraparib in a Maintenance Study With Niraparib Versus Placebo in Patients With Platinum Sensitive Ovarian Cancer (NCT01847274) and in Her2 Negative, Germline *BRCA* Mutation-positive Breast Cancer (BRAVO)(NCT01905592) [324].

In another phase II study, rucaparib (AG-014699/PF-0136738) is being tested as monotherapy in ovarian cancer patients with known *BRCA* deficiencies [349]. Preliminary results are promising [345].

The PARP inhibitor Veliparib (or ABT-888, Abbott) has been found to enhance the activity of multiple DNA-damaging agents (temozolomide, cisplatin, carboplatin, cyclophosphamide and irinotecan, as well as radiation). It is an orally active drug that has been shown to cross the blood-brain barrier in preclinical studies. It is also being studied as a single agent in *BRCA*-mutated solid tumour patients, as well as a sensitizer to therapeutic ionizing radiation [350]. The phase I study of ABT-888 in combination with carboplatin, paclitaxel and bevacizumab as first-line treatment for stage II-IV ovarian cancer is actively enrolling patients (NCT00989651) [234]. Moreover, phase I and II studies have also been planned to evaluate Veliparib and Topotecan Hydrochloride combination in patients with solid tumours, relapsed or refractory Ovarian cancer, or primary peritoneal cancer (NCI-2011-00312 MC0861, U01CA069912, CDR0000657976, MAYO-MC0861, 8329, NCT01012817).

Further investigations include the development of predictive markers that will predict not only the drug's pharmacodynamic effects, but also clinical benefit from PARP inhibition, as well as the identification of resistance mechanisms to PARP inhibitor therapy [351].

Birinapant is another agent that targets PARP among other molecules. It is a mitochondria-derived activator of caspases (Smac) and a potent antagonist of IAP (inhibitor of apoptosis protein). Birinapant causes rapid cIAP1 degradation, caspase activation, PARP cleavage, and NF- $\kappa$ B activation, which result in cell death in an IAP-dependent manner [352]. Currently, it is under evaluation in advanced ovarian cancer (Phase II study 120191, 12-C-0191, NCT01681368) [234].

### **1.7. Immunotherapy**

Immunotherapy represents an alternative and rational approach for the treatment of ovarian cancer, since the immune system is protective against this disease.

Lenalidomide (Revlimid®), is a highly potent thalidomide derivative that presents anti-angiogenic activity through inhibition of FGF, VEGF, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) induced endothelial cell migration, due at least in part to inhibition of the Akt phosphorylation. Moreover, lenalidomide induces T-cell proliferation via increased IL-2 and IFN- $\gamma$  production, which are correlated with tumoral T-cell infiltration and recurrence/survival. Single-agent lenalidomide therapy is generally well tolerated and preliminary analysis suggested that it might have some clinical activity in patients with recurrent ovarian cancer. This agent has the convenience of oral administration and the potential for enhanced quality of life compared to chemotherapy treatments [353]. Lenalidomide is currently evaluated in combination with carboplatin and pegylated liposomal doxorubicin, phase I REV (GINECO-OV214), phase II NCT01111903 clinical studies [234].

The NGR-hTNF (asparagine–glycine–arginine–human TNF) is prepared by fusing the N terminus of TNF with the C terminus of the peptide NGR, which is able to selectively recognise a CD13 isoform overexpressed on tumour endothelium, involved in the development of new vessels from pre-existing vessels. In preclinical models NGR-hTNF showed tumour regressions and its immunotherapeutic properties thought the targeted delivery of TNF to tumour. It induced apoptosis of tumour endothelial cells and increased endothelial permeability, drug penetration in tumour by a rapid disassembly of vascular endothelial-cadherin-dependent adherence junctions and formation of intercellular gaps that are followed by vascular damage at later time points. NGR-hTNF exerted synergistic effects *in vivo* when combined with various chemotherapeutic drugs, especially doxorubicin, without increasing the *in vitro* cytotoxicity of chemotherapy, likely because of indirect effects on host

cells rather than direct effects on tumour cells. Interestingly, the highest doxorubicin sensitivity *in vivo* is observed in tumour microenvironments with increased vascular permeability [354]. The effects of NGR-hTNF plus carboplatin and doxorubicin are currently under evaluation in phase II (NGR018 2010-023613-61), and Phase I ( NCT01358071) [234]. VTX-2337 is a small molecule agonist of Toll-like Receptor 8 (TLR8) that activates multiple components of the innate immune system. In particular, it activates NK cells, leading to increased IFN $\gamma$  production and increased cytolytic activity. This novel TLR8 agonist induced an immune response to tumours and improved clinical responses to clinically approved monoclonal antibody therapies, especially in individuals who showed reduced ADCC activity [355]. The study GOG-3003NCT01666444 are currently on going with VTX-2337 and pegylated liposomal doxorubicin (PLD) combination.

Ipilimumab (MDX-CTLA-4, Yervoy) is a fully human IgG1 monoclonal antibody that binds and blocks the activity of CTLA, which is constitutively expressed on regulatory T cells and acts as an immune-checkpoint receptor. Thus, the effect of Ipilimumab is mediated by immune modulation. In most cases it showed clinical benefit and improvement of OS [356]. The study CA184-201, NCT01611558 on Recurrent Platinum Sensitive Ovarian Cancer Patients is currently recruiting participants.

Furthermore, a novel IDO-1 (indoleamine dioxygenase-1) inhibitor, INCB024360, represents an attractive cancer therapeutic strategy via up-regulation of cellular immunity. IDO1 mediates oxidative cleavage of tryptophan, an amino acid essential for cell proliferation and survival. *In vitro* study INCB024360 showed significantly inhibition of tumour growth in a lymphocyte-dependent manner on tumour-bearing mice. Moreover, in patients with cancer the IDO pathway was activated in multiple tumour types [357]. A Phase 2 study is in going with INCB 24360-210 (NCT01685255) for Subjects With Biochemical-recurrent-only EOC, Following Complete Remission With First-line Chemotherapy.

### **1.8. Folate pathway targeting**

Since cancer cells have an excessive growth rate, they are also highly dependent on folate metabolism for replication of DNA: for this reason, folate pathway enzymes and its membrane-bound receptor are targets for biologic therapy [358].

In addition, ovarian cancer cells often express folate receptors (FRs) that are not overexpressed on somatic cells, making them attractive targets for anticancer therapy [359].

Thus efforts to disrupt folate metabolism were the basis for development of some of the first rationally designed chemotherapeutic drugs. The first drugs developed that interfere with folate metabolism are the inhibitors of thymidylate synthase [360,361].

### 1.8.1. Thymidylate Synthase (TS) and Folate cycle

Thymidylate Synthase (TS) is considered a key target for hyperproliferative diseases, infectious diseases, and in particular for anticancer chemotherapy because is involved in a key step in DNA biosynthesis. The reaction catalyzed by TS is the only *de novo* pathway to dTMP synthesis [362]. TS is present in almost all living organisms including bacteria, DNA viruses, and protozoa, and is one of the most conserved enzyme known in the evolutionary scale [363]. TS is involved in a complex pathway called TS cycle or also Folate cycle, and in feedback regulation of own expression.

The folate cycle is a three-enzyme cycle that involves TS, dihydrofolate reductase (DHFR) and serine-hydroxymethyl transferase (SHMT) (Figure 1.8.2.1.).

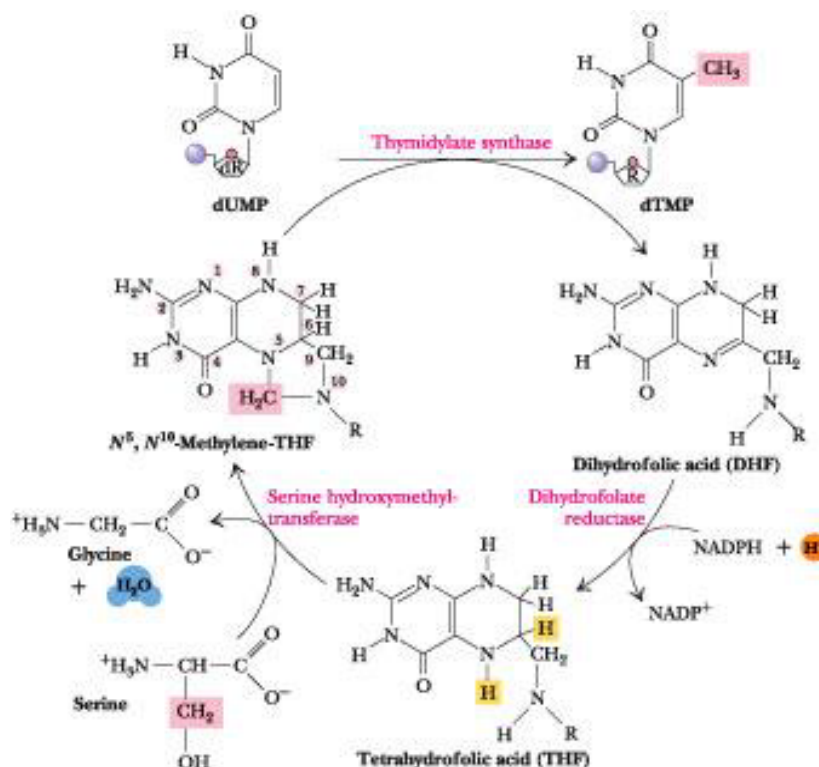


Figure 1.8.1.2. Folate Cycle.

It is devoted to the regeneration of the reduced and active folate form in particular N<sup>5</sup> N<sup>10</sup>-methylenetetrahydrofolate (mTHF), which acts as a donator of one-carbon units to the methionine cycle, for use in methylation reactions, and the synthesis of purines and

pyrimidines is catalyzed by TS. The functional relationship among these is maintained by an obligate sequence of chemical reactions. In fact, the final product of the reaction is only released when the acceptor molecule is correctly bound to the enzyme. DHFR catalyzes the reduction of dihydrofolate to tetrahydrofolate (THF) by NADPH- dependent reaction. SHMT transfers the methylene group from L-serine to THF, between the N5 and N 10 positions, and additional carbon atom is transferred to dUMP. This is a  $\alpha$ -cleavage reaction that used pyridoxal- 5'phosphate (PLP) cofactor [364].

#### **1.8.1.1. Catalytic function of Thymidylate Synthase**

TS is a folate-dependant macromolecular protein that catalyzes the reductive methylation of 2'-deoxyuridine -5'-monophosphate (dUMP) to thymidine-5'-monophosphate (dTMP), which is subsequently phosphorylated to thymidine-5'-diphosphate (dTDP) and thymidine-5'-triphosphate (dTTP), a substrate of DNA polymerase, to be used for DNA replication and repair. The reaction involves the cofactor mTHF that acts as a donor of one-carbon units to the methionine cycle, for use in methylation reactions. The synthesis of purines and pyrimidines is catalyzed by TS [364].

The catalytic reaction begins with the TS and dUMP reaction, which leads to the formation of the non-covalent binary complex. The ternary complex (TS-dUMP-cofactor) is rapidly formed by the addition of the cofactor. Then, TS is protonated in position 10, thus converting the inactive form to the active one. The ternary complex becomes the covalent ternary complex. When the catalytic thiol (Cys-198) of TS gives a nucleophilic attack on C6 of the uracil ring of dUMP, a Michael-type adduct is formed and the carbon unit of the cofactor gives an electrophilic attack on C5 of dUMP trans with respect to Cys-198. The hydrogen at C5 of the uracil ring is abstracted by an unidentified base on the receptor site. The methylene group is reduced to a methyl group through hydride transfer from C6 of the cofactor, which is oxidized at the N5-C6 bond; at the same time, the thiol group undergoes  $\beta$ -elimination to reinstate the double bond and afford the reaction product, dTMP. It is subsequently phosphorylated to dTTP by cellular kinases; dTTP is a substrate of DNA polymerase and is incorporated in the DNA. The *de novo* cellular thymidylate nucleotides, fundamental elements for DNA synthesis and repair, are strictly connected with TS activity and with cell survival.

In fact, TS inhibition leads to a drastic reduction in cell survival, due to the toxic thymineless state. This cytotoxicity is probably effected by DNA fragmentation resulting from dTTP depletion, which increases misincorporation of 2'-deoxyuridine-5'-triphosphate (dUTP). Cells containing the salvage enzyme thymidine kinase, which uses circulating thymidine, can

circumvent this cytotoxicity. However, in normal tissue and in some tumour cells, the circulating thymidine concentration may not be sufficient to achieve a significant effect [365].

#### **1.8.1.2. Feedback regulation of TS expression**

Chu E. and co-workers proposed a model to explain the autoregulatory function of TS [364]. The protein in its inactive unbound form interacts with those mRNA fragments that promote its own expression [366], thus acting as a translational inhibitor. This model explains the resistance against TS inhibitors that often develops in anticancer chemotherapy. In fact, the inhibitor bound complex induces an over-expression of TS protein both as a late response (thus lowering the efficacy of the drug) and by a reduced degradation of TS inhibitor complex [367].

Moreover, since in vitro studies showed that TS can bind a number of other mRNAs, as mRNA of cancer-related genes as c-myc, bcl-2 and p53 (*see section 1.2.*), this enzyme could participate to the regulation of synthesis of other proteins and oncogenes involved in cell cycling [368]. Probably the over-expression of hTS is correlated with the loss of RNA regulatory capacity when the protein is bound to its inhibitors [369]. Nevertheless, this role is not well understood and it is still being studied. In tumour tissues, p53 and bcl-2 genes are present in high concentration and TS could block the p53 gene specifically promoting tumour generation. Drug-resistant cells also present high levels of TS, which can affect the ultimate response of tumour cytotoxic drugs. In fact, the drug binding may play a critical role in retarding cell progression through the S-phase and initiating apoptosis after drug exposure. Moreover, TS binding activity to other mRNAs could be reduced by the presence of mutation in the coding sequence of the proteins cited above [370].

#### **1.8.1.3. Thymidylate Synthase structure**

In PDB, more than 100 TS structures from 9 different organisms have been reported. The structure of about 30 different kinds of TS have been studied, and it appears to be one of the most highly conserved enzymes [371]. The TS mammalian structure has been resolved even for TS-substrates/ligands complex [372]. It is an obligate homodimer of 2 subunits of 35,000 daltons and the whole protein consists of about 316 aminoacids (Figure 1.8.1.3.1.). The hTS dimer has two active sites, each formed by residues from both monomers. Therefore the dimeric assembly is necessary for the catalytic activity and monomers can adopt active and inactive forms. The active and inactive forms differ in the conformation of the loop from residue 181 to residue 197, which contains the catalytic cysteine, residue 195. This residue

points into the active site in the active form and toward the dimer interface in the inactive [373]. The hTS dimer-to-monomer dissociation constant ( $K_d$ ) was recently reported to be 200 nM [374].

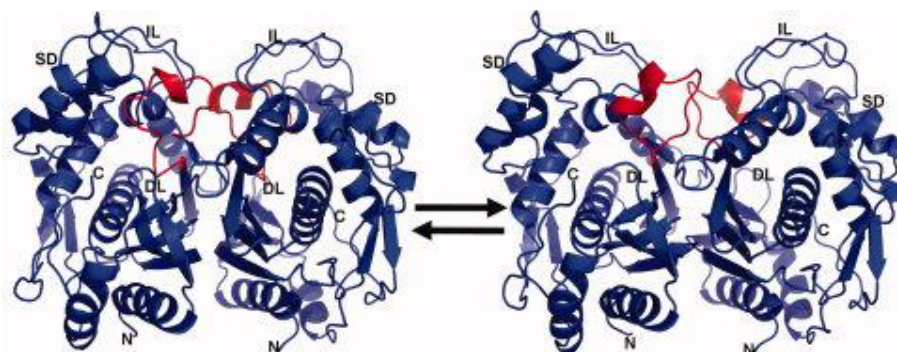


**Figure 1.8.1.3.1.** Representation of the transition between active and inactive conformation of thymidylate synthase.

#### 1.8.1.4. Folate cycle Inhibitors

The most known thymidylate synthase classical inhibitor is 5-fluorouracil (5FU) and it has been shown to be effective in salvage therapy for refractory ovarian cancer patients with low burden disease [375]. This structural analogue of thymidine and uracil is a pro-drug that quickly enters the cells by a facilitated trans-membrane carrier system, where it is deoxyribosylated and then phosphorylated: uridine phosphorylase and orotate phosphoribosyltransferase convert 5-FU into the ribonucleoside or ribonucleotide, respectively; thymidine phosphorylase can also salvage 5-FU into the deoxynucleoside, FdUrd. Kinases convert FdUrd to FUMP and to FUDP, which provides a branch point in metabolite fate. FUDP is phosphorylated to FUTP, which is a substrate for RNA polymerases. FUDP can also be converted to the deoxynucleotide (FdUDP) by ribonucleotide reductase (RNR). FdUDP is further phosphorylated into FdUTP, which is a substrate for DNA polymerases. The enzyme dUTP pyrophosphatase (dUTPase) performs an absolutely essential function to prevent genomic uracil incorporation by catalyzing the hydrolysis of dUTP into dUMP; *E. coli* and *S. cerevisiae* lacking dUTPase are inviable [376] dUTPase also breaks down FdUTP to FdUMP, which is a noteworthy aspect of 5-FU metabolism. While breaking down FdUTP prevents 5-FU from being incorporated into DNA, it creates FdUMP in the process. FdUMP forms an irreversible ternary complex with TS, leading to the inactivation of the enzyme [377]. Suicide inhibition of TS by FdUMP also causes a drop in TTP, which entails an accumulation of dUMP (so, dUMP and FdUMP at higher concentration may overwhelm dUTPase and be incorporated into the genome by DNA polymerases), moreover

and TTP feedback inhibits TK and allosterically regulates RNR (TTP normally increases dGDP formation and decreases dUDP formation); loss of this feedback inhibition presumably increases the conversion of FdUrd to FdUMP by TK and the conversion of FUDP to FdUDP by RNR. Thus, there are several interrelated events that can lead to the introduction of 5-FU into DNA. In the case of 5FU, a covalent hTS inhibitor, high hTS protein levels were attributed to both mRNA regulation and a decrease in enzyme degradation efficiency caused by inhibitor binding [378].

Inhibitors such as aminopterin [379] and methotrexate [380] were the first antifolate drugs to demonstrate significant antitumour activity in ovarian cancer cells. Methotrexate is a folate analogue that was originally designed to target DHFR. Inhibition of DHFR decreases THF levels, which results in attenuated DNA/protein/lipid methylation, inhibition of thymidylate synthase (TS). Thus, interference with DNA synthesis inhibits 5-aminoimidazole-4-carboxamideribonucleotide (AICAR) transformylase and blocks de novo purine synthesis. Moreover, MTX treatment leads to an intracellular accumulation of dUMP, in part a compensatory attempt to override the block in the methylation of dUMP to dTMP. This accumulation has been shown to lead to the misincorporation of 2'-deoxyuridine-5'-triphosphate (dUTP) into DNA, which is followed by DNA degradation resulting from a futile attempt at cellular repair [381,382]. So, MTX administration induces cellular death at the level of the DNA macromolecule: the false incorporation of dUTP stimulates DNA excision-repair [383]; the depletion of dTTP and purine nucleotides prevents both cell division and DNA repair; and the excessive accumulation of unrepaired DNA strand breaks leads to cell death. Unlike the case with most antineoplastic nucleoside analogs, MTX does not require metabolic activation to exert its cytotoxic effects; however, these cytotoxic effects are sustained by conversion of MTX to an anionic form that enhances its intracellular retention. Moreover, these effects are also responsible for a number of toxicities, which could be reduced by co-administration of MTX and folic acid [384].

Newer thymidylate synthase inhibitors include compounds such as pemetrexed and raltitrexed [385], which are used in anticancer chemotherapy. ZD1694 (Raltrixed), a folate-related compound, is effective in clinical therapy against colorectal cancer [386]. Pemetrexed is an antifolate antineoplastic agent, methotrexate analogous that can enter cells via different folate transporters, RFC and PCFT [387, 388]. Upon entry to the cytoplasm, the drug is converted to a polyglutamated form, which has an increased retention half-life within malignant cells. Thus in the cytosol, pemetrexed can inhibit a variety of folate dependent enzymes to interrupt DNA replication and other folate dependent reactions [389]. Results from phase II trials with

pemetrexed in platinum-resistant OC patients [390] were encouraging, but pemetrexed is still not widely used with platinum-resistant OC patients. Even a pemetrexed and bevacizumab combination phase II study in patients with recurrent/persistent epithelial ovarian showed promising results. Overall, the combination was felt to be highly active and warrants further investigation with phase III trials [391].

Other molecules with folate-related structures have been synthesized, including CB3717, GW1843, AG337 and LY21514 [387]. These molecules have different metabolism according to how well they can be transported into the cells through the RFC system, or other transporter mechanisms. They can be polyglutamylated by folylpolyglutamyl synthetase (FPGS), which increases their intracellular concentration, and their affinity for the enzyme. Drug resistance mechanisms include changes in FPGS or in TS itself [392].

Several other novel thymidylate inhibitors, including BGC 945 [393] and ZD-9331, or raltitrexed [394], are in the early phase of development. BGC 945 is a thymidylate synthase inhibitor, which is preferentially transported into FR $\alpha$ -overexpressing tumours and is inherently potent due to the presence of modified glutamate ligands, which stabilize it against enzymatic hydrolysis. BGC 945 has been shown to inhibit thymidylate synthase in established ovarian cancer cell lines [393]. An early phase clinical trial evaluating BGC 945 in the context of ovarian cancer is on going [395]. ZD-9331 is an inhibitory tetrahydrofolate (THF) analogue that works as a thymidylate synthase inhibitor [394]; it is unable to form polyglutamates based on its chemical structure and is thought to potentially target more resistant tumours and provide more predictable toxicity [396]. Recently, a phase II trial evaluated ZD-9331 on monotherapy or in combination with topotecan to platinum-resistant ovarian cancer patients: results are expected soon [397]. In general, no selectivity between tumour TS and normal cell TS has yet been established, and the driving force of the inhibition is the higher intracellular level of TS in hyper-proliferative tissues, so that a higher inhibitor concentration can be achieved in tumour cells.

#### **1.8.1.5. A novel Folate Cycle Inhibitors: Peptide inhibitors of TS dimeric interface**

Recent studies on peptides specifically designed to bind the monomer–monomer interface of the enzyme, LR (LSCQLYQR) and its derivatives, enlightened a novel allosteric inhibition mechanism of TS.

In particular, LR binds at the interface of the two subunits stabilizing the dimeric inactive form of the protein. The novel mechanism for these peptides was demonstrated through a combination of biophysical and computational approaches (Figure 1.8.1.5.1.) [385].

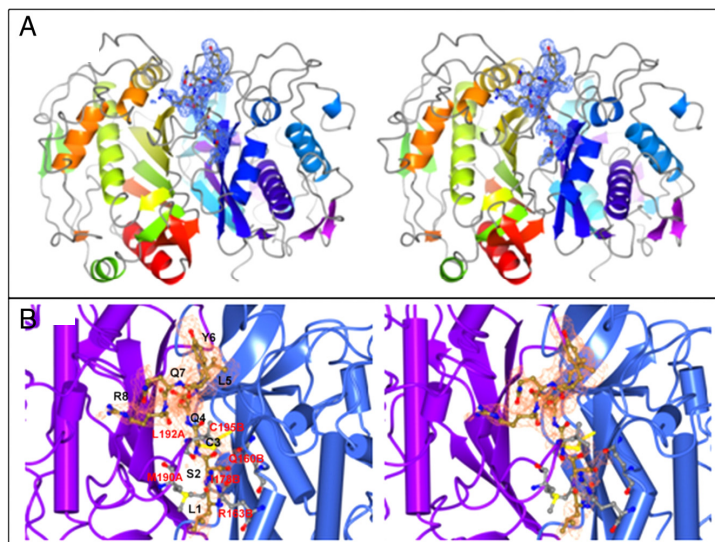


**Figure 1.8.1.5.1.** X-ray crystal structure of the hTS-LR complex.

LR inhibits TS in cell extracts and cDDP sensitive-resistant ovarian cancer cell growth, without contemporary causing TS overexpression. LR also induces a decrease in the level of dihydrofolate reductase (DHFR), another important enzyme of the folate pathway. This effect might result from a decrease of the dihydrofolate levels due to hTS inhibition. Since the peptides alone cannot enter the cells, to test their effects on cell growth a suitable delivery system (Saint PhD delivery system) has been used. The innovative mechanism of action and potency of the mentioned compounds suggested advancing in the discovery process, and the expectation of promising results [385].

## 1.8.2. Folic Acid and folate receptors

Folic acid, also known as folate, vitamin B<sub>9</sub>, pteroyl-L-glutamic acid, and pteroyl-L-glutamate, is a member of the B class of vitamins and a essential nutrient required by all living cell for the normal cellular metabolism [387]. Folate (FA) is composed of the aromatic pteridine ring linked to para-aminobenzoic acid (PABA) and one or more glutamate residues (Figure 1.8.2.1.).

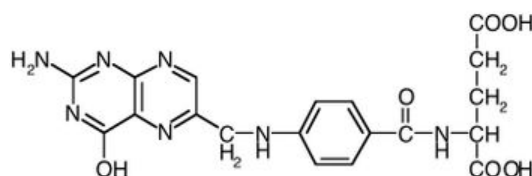


**Figure 1.8.2.1.** Structure of folic acid.

Humans cannot synthesize folate *de novo*; therefore, it has to be supplied through the diet to meet the daily requirements. Folic acid itself is not biologically active, but its biological importance is due to tetrahydrofolate and other derivatives after its conversion to dihydrofolic acid in the liver [398]. Food folate is a mixture of many derivatives; they differ by the state of oxidation, one-carbon substitution of the pteridine ring and by the number of polyglutamate residues. These differences are associated with different physicochemical properties, which, together with certain food constituents, could influence folate bioavailability. Folate instability results from balance among exposure to air oxidation, heat, the acid-peptic milieu of the stomach, and protect agents presence in foods, antioxidants such as ascorbic acid and reduced thiols [399]. Moreover, bioavailability of polyglutamyl folate is lower than the bioavailability of monoglutamyl folate, so polyglutamyl folates must be hydrolyzed by intestinal hydrolases to the respective monoglutamyl derivatives before being absorbed by the intestine. Indeed, this process could be inhibited by the constituents of some foods and by glutamate chain length [400]. The major circulating form of the vitamin in the serum is 5-methyltetrahydrofolate (5-mTHF, 30 nM). It is involved in essential one-carbon transfer reactions, including *de novo* biosynthesis of nucleotides catalyzed by thymidylate synthase (*see section 1.8.1.1.*) and plays a key role in metabolic processes involved in DNA and RNA synthesis and replication, epigenetic processes, cellular division, growth and survival. Folate deficiency is therefore associated with many diseases, including fetal neural tube defects, cardiovascular disease and cancers [401]. Folate deficiency is associated with misincorporation of uracil instead of thymine into DNA leading to increased chromosomal strand breaking [402], abnormal methylation reactions [403], which are implicated in cancer [404].

#### **1.8.2.1. Folic acid uptake**

The hydrophilic and anionic nature of folates at physiologic pH precludes their passive diffusion through the plasma membrane, so cellular folate uptake involves several system, such as reduced folate carrier (RFC) protein couple folate transporter (PCFT) and folate receptor (FR) [405,406].

They present differences in both cellular-tissue distribution and folic acid/folic acid derivatives affinity.

Reduced folate carrier, the predominant system, is a transmembrane anion exchanger ubiquitously expressed in normal adult tissues, despite significant variability in its expression levels among tissue types [405]. RFC binds natural folates such as 5-methyl or 5-formyl tetrahydrofolate (THF), and classical antifolates, such as methotrexate (MTX) and pemetrexed, and can control folate uptake in a bi-directional manner [406]. It is unable to bind folic acid and has a dissociation constant for 5 methyltetrahydrofolate in the micromolar range. Although it operates suboptimally in the physiologic concentration range of folate, the transport is rapid and generally adequate to supply the cellular stores of the co-enzyme [405]. Although the expression status and functional roles of RFC remain largely unknown, its reduction is significantly associated with shorter overall and disease-free survival, suggesting that RFC may be considered as a marker for good prognosis in ovarian cancer patients. Moreover, among patients with high FR $\alpha$  expressing ovarian cancers, the overall and disease-free survival is significantly better in those with high RFC expression than those without, implicating the protective role of RFC for patients with these tumours [407].

Proton-coupled high-affinity folate transporter (PCFT) has been shown to be the major transporter of folate in low pH environments and appears to be the principal transporter in the intestinal absorption of folate [408]. Moreover it is involved in the folate transport from the endosome into the cytoplasm after FR- mediated endocytosis [409].

#### **1.8.2.2. Folate receptors (FRs)**

Folate receptors (FR $\alpha$ ,  $\beta$  and  $\gamma$ ) are a family of cystein rich cell surface glycosyl phosphatidylinositol (GPI)-anchored glycopolypeptides of apparent MW in the range of 38,000–45,000 [410] (Figure 1.8.2.2.1.). They characteristically binds folic acid as well as 5-methyltetrahydrofolate with a dissociation constant in the subnanomolar range and transport them by a non-classical endocytotic mechanism (*see section 1.6.3.1.*) [411] under conditions of limiting dietary folate [412]. Inside of the cell, the acidic environment of the endosome promotes the release of folate from receptors, which is then transported into the cytoplasm by proton-coupled folate transporter [388].

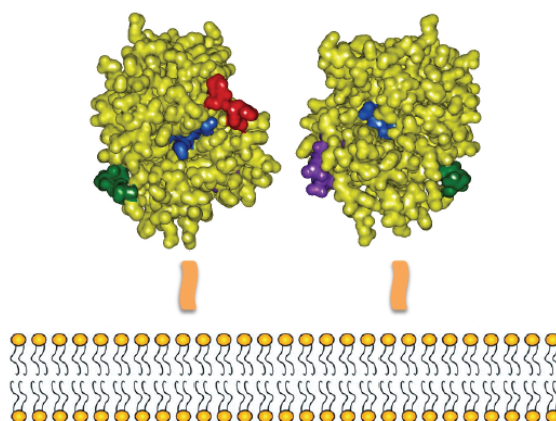


**Figure 1.8.2.2.1.** X-ray crystal structure of folate receptor (FR).

The isoforms can differ in tissue expression, function, and biochemical properties [413]. FR $\alpha$  and FR $\beta$  are both membrane-bound GPI-anchored proteins, unlike the FR $\gamma$  secretory protein that lacks an efficient signal for GPI modification [414] and it is constitutively secreted [415]. Even soluble extracellular forms of FR $\alpha$  and FR $\beta$  are present and produced by different mechanisms, including the action of membrane or intracellular proteases and phospholipase [416]. A polymorphism in the FR $\gamma$  gene is caused by a nonsense mutation that results in a carboxyl-terminal truncation of the protein [414]. Thus, multiple putative disulfide bonds and the relatively heavy N-glycosylation presumably contribute to the stability of the protein [417]. Indeed, N-glycosylation is important for proper protein folding, intracellular trafficking, and functional FR expression but not for folate binding. FR $\alpha$  has three N-glycosylation sites whereas FR- $\beta$  has only two [418].

FRs are significantly different in their relative affinities for folate compounds and antifolates in the range of 2-fold to 100-fold, ( $K_d \sim 0.1$  to 1 nmol/L) [419]. Although they bind folic acid with a uniformly high affinity, FR- $\alpha$  and FR- $\beta$  have opposite stereospecificities for reduced folate compounds [420]. This functional divergence may be attributed to only a couple of single amino acid residue differences between the two FR isoforms [421]. The functional differences between FR isoforms should allow an added level of selectivity in tissue-targeted and FR-mediated drug delivery.

Distribution of FR $\alpha$  in normal and malignant tissues has been examined by several methods including immunohistochemistry using specific mouse monoclonal antibodies [422], measurement of relative mRNA levels by PCR [423], quantitative in situ hybridization and [405] flow cytometry. Radioligand binding assays quantitatively determine FR $\alpha$  in tissues by assessing the receptor's ability to functionally bind folic acid [424].

FR $\beta$  is expressed in later stages of normal myelopoiesis and in placenta, spleen and thymus [425, 426] and granulocytes, neutrophils, activated macrophage express the highest levels of FR- $\beta$  [427, 428]. FR $\beta$  is also a neutrophilic lineage marker, and its levels increase with neutrophil maturation [429].

### **1.8.3. Folate receptor and FR $\alpha$ target strategy**

#### **1.8.3.1. Folate receptor $\alpha$ (FR $\alpha$ )**

FR $\alpha$  is expressed in adult normal tissues, in particular at the luminal surface of polarized epithelial cells including proximal kidney tubules, type I and II pneumocytes in the lungs, choroids plexus, ovary, fallopian tube, uterus, epididymis, submandibular salivary and bronchial glands and trophoblasts in placenta, as well as the basolateral membrane of retinal pigment epithelial cells [430]. Indeed, it is over-express in 90% of non mucinous ovarian carcinoma, but display a high degree of heterogeneity in FR- $\alpha$  expression within the tumour and among similar tumours from different patients over a range of two orders of magnitude [431], implying that it may promote tumour growth [432, 433]. Increased growth and folate accumulation by cancer cells with elevated FR $\alpha$  suggest this may be one mechanism by which folate could progress carcinomas in humans [358]. Several other malignant tumours are known to overexpress FR $\alpha$ , as uterus and cervix, testicular choriocarcinoma, ependymal brain tumours and malignant pleural mesothelioma, while in other malignant types of cancer such as breast, colon and renal, FR- $\alpha$  expression is less frequent [359, 432, 434]. Significant correlations have been observed between the FR $\alpha$  expression level in FR $\alpha$  positive cancer cells and the grade and differentiation status of the tumour. Moreover, the highest expression of FR $\alpha$  is associated with poorly differentiated and more aggressive tumours [431], as well as with promotion of the development of platinum-therapy resistance and poor prognosis [435]. A soluble FR $\alpha$  has been found into the circulation, and a low or undetected level has been reported in normal human serum [436]. So it may provide a means of controlling the expression of FR $\alpha$  at the surface of the cells [433] and may also be a potential serum marker for FR $\alpha$ -positive tumours [436]. This soluble form of FR $\alpha$  with the folate-binding site intact arises from proteolytic cleavage of the membrane-associated FR $\alpha$  precursor by the combined action of a membrane-associated protease and GPI-specific phospholipases C and D, which are abundant in plasma [437].

Indeed, the over expression of FR $\alpha$  has been found to compensate for a defect in RFC function in cells in vitro and could be significant in supporting the growth of tumours resistant

to methotrexate by virtue of defective RFC function [405]. Michelle K. Y et al. demonstrated a mechanistic link between folate, FR $\alpha$ , RFC and E-cadherin [407]. Folate and FR $\alpha$  contribute to the progression and growth of ovarian cancer cells through the regulation of ovarian cancer cell proliferation, migration and invasion, possibly through the down-regulation of cell-cell adhesion molecule E-cadherin in FR $\alpha$ -positive SKOV-3 cells [438]. In fact, patients with such loss of E-cadherin expression were found to have significantly shorter survival [439]. In contrast to FR $\alpha$ , RFC can serve as a balancing partner of FR $\alpha$  and seems to exert a protective role in ovarian cancer patients, conferring longer survival among patients with cancers that showed a high FR $\alpha$  expression status. Moreover, FR $\alpha$  amplification and RFC promoter methylation correlated with mRNA expression in ovarian cancers and suggested that up-regulation of FR $\alpha$  (a putative oncogenic folate transporter) and down-regulation of RFC (a putative tumour suppressor type folate transporter) were controlled genetically and epigenetically respectively during ovarian cancer development. It is tempting to speculate that FR may have a role in regulating tumour growth either by influencing the folate status of the tumour or through raft associated signalling functions that are yet to be characterized. However, at this time there is no clear physiological evidence for either of these roles for FR in malignant tissues [407].

Thus, although folate may prevent cancer initiation, once the neoplasia is established, it appears to enhance cancer progression. For these reasons, the beneficial effect of folate supplement among cancer patients, in particular for patients who suffer from long-term illnesses, is controversial [440].

*FR $\alpha$  non-classical endocytic mechanism.* FR $\alpha$  binds folic acid (FA) with extremely high affinity and it was demonstrated to efficiently internalize both FA analogues and folate-conjugated molecules [441] through a unique non-clathrin mediated pathways (non-classical endocytotic mechanism) that exploits GPI anchors (Figure 1.8.3.1.1.). Lipid rafts are believed to serve as platforms for signal transduction as well as units of protein recycling between the cell surface and endocytic compartments [442]. They are thought of as liquid-ordered plasma membrane microdomains that are characterized by a concentration of cholesterol, glycosphingolipids, GPI-anchored proteins and signalling proteins and that may, in some cells, cover up to half of the cell surface [443]. After the internalization of FR $\alpha$ -folic acid complex, the acidic environment of the endosome promotes the release of folate from receptors, which is then transported into the cytoplasm by proton-coupled folate transporter [409].

Kamen and colleagues established that in MA104 (monkey kidney epithelial) cells, the GPI-anchored FR quantitatively recycles, within minutes, between the cell surface and intracellular compartments [444,445]. FR $\alpha$  recycling has obvious implications in FR-mediated drug delivery to tumour cells. In the FR $\alpha$  over-expressing KB nasopharyngeal epidermoid carcinoma cells, several studies have demonstrated that fluorescent folate-labelled proteins and radiolabeled folate-conjugates are internalized into endosomal compartments and the Golgi complex by FR [446], and at least a small fraction of the internalized macromolecules were released into the cytoplasm [447]. Therefore the rate, the efficiency and the path of internalization of FR-targeted drugs are likely to be determined by cell type and the nature of the drug delivery system.

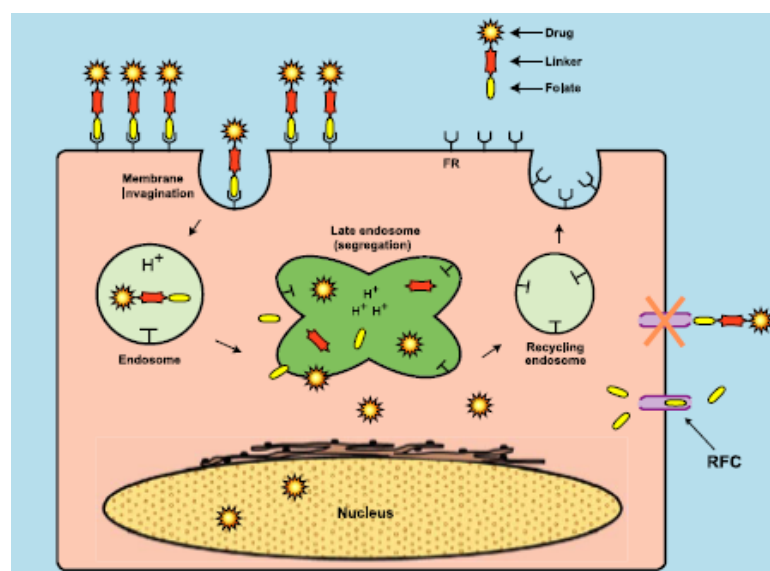


**Figure 1.8.3.1.1.** Representation of FR $\alpha$  endocytic mechanism.

### 1.8.3.2. FR $\alpha$ target strategy

For all these reasons, FR $\alpha$  has become the ideal molecular target for the development of many cancer therapeutics, including anti-FR $\alpha$  antibodies, high-affinity anti-folates, folate-based imaging agents and folate-conjugated drugs and toxins, high-affinity antifolate drugs and immunological agents (anti-FR $\alpha$  antibodies), and folate-based imaging agents [448, 449].

Although the mechanism how FR binds to folate and folate-conjugated drugs is unknown, the FR $\alpha$ -folic acid complex structure in Figure 10 illustrates how the receptor assumes a deep folate-binding pocket that is formed by conserved residues across all receptor subtypes and provides detailed insights into how folic acid interacts with its receptors. Together, these observations establish a rational foundation for designing specific drugs targeting the folate

receptor system [450]. Moreover this strategy promises to expand the therapeutic windows of drugs by improving their cellular internalization and thus biological-biochemical effects decreasing the levels of toxicity in normal tissues [424,441].

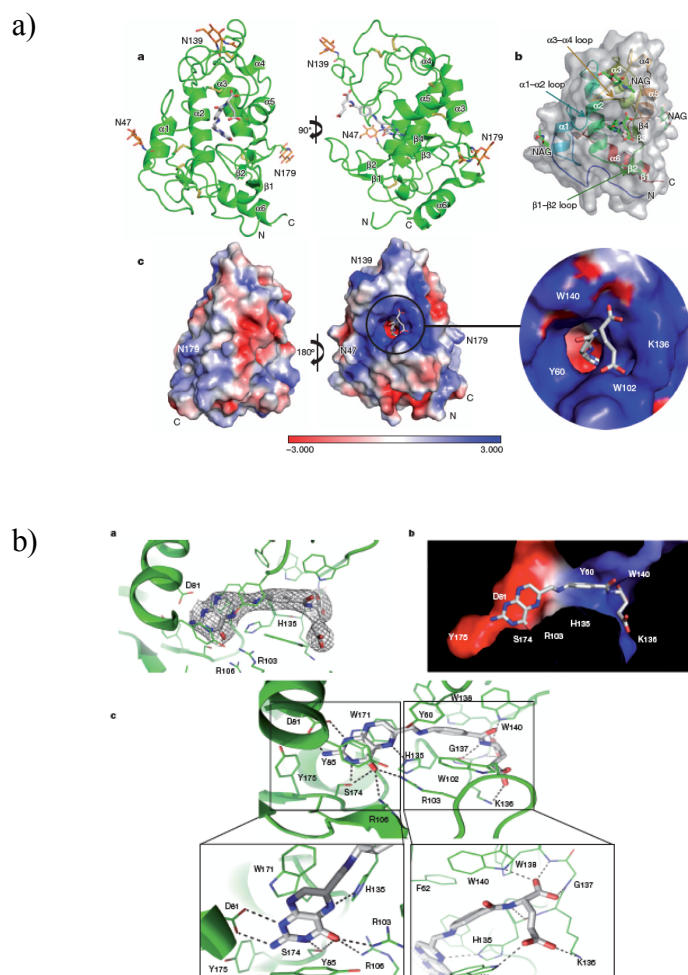


**Figure 1.8.3.2.1.** a) Structure of FR $\alpha$  bound to folic acid and b) structural and biochemical analysis of FR $\alpha$ -folic acid interactions.

*Monoclonal antibody FR $\alpha$  targeted antagonists.* Monoclonal antibodies (MoAbs) are a method of targeting the folate receptor and several have been generated for both diagnostic and therapeutic purposes. Farletuzumab (MORAb-003, Morphotek) is an improved humanized version of LK26 and preclinical studies showed strong affinity for FR $\alpha$  and very limited cross-reactivity to normal tissue [451]. It induced cells expressing FR $\alpha$  to death by antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity immune mechanisms (CDC) [452]. Although results of phase II study with farletuzumab were promising in platinum sensitive ovarian cancer in their first relapse [453,454], phase III combination studies failed to meet the pre-specified criteria for continuation [455, 456].

*Folic acid conjugated FR agents.* Folate can be linked to various therapeutic agents that target FR $\alpha$  overexpressing cancer cells. Folate is amenable to chemical conjugation with other molecules through its  $\gamma$ -carboxyl group without decreasing its binding affinity to FR $\alpha$  [441]. Vintafolide (EC145; Endocyte, Inc., West Lafayette, IN, USA) is a small molecule drug conjugate (SMDC) consisting of folic acid linked to the vinca alkaloid desacetylvinblastine hydrazide (DAVLBH), which is a potent microtubule destabilizing agent (Figure 1.8.3.2.1.) [457]: it targets directly FR $\alpha$  with a different mechanism of action than either pemetrexed or farletuzumab [458]. An advantage of EC145 is that the linker mechanism of the SMDC allows for drug stability in the blood, and the active DAVLBH is released upon endocytosis [459]. It showed promising results in phase 2 trials with both monotherapy [460] and in combination with PLD in platinum-resistant OC (PRECEDENT study) [461]. A phase III study (PROCEED) is currently recruiting patients with platinum-resistant ovarian cancer to study vintafolide and PLD combination [462].

Moreover, etarfolatide ( $^{99m}\text{Tc}$ -EC20, Endocyte, Inc., West Lafayette, IN, USA) fluorescent agent folate conjugated could improve localization of the ovarian cancer implants at the time of surgery [463] and improve PFS after vintafolide treatment [464, 465].

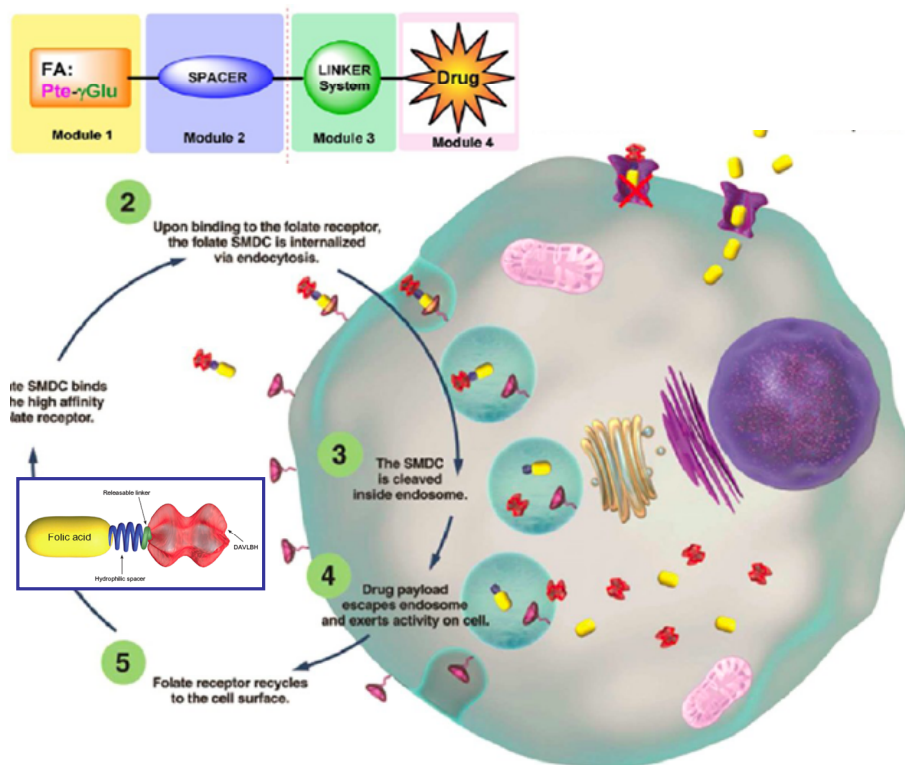


**Figure 1.8.3.2.2.** Chemical structure of Vintafolide and overview of its mechanism of action.

## 1.11. References

- [1] Ledermann J. A., et al., *Ann. Oncol.*, **2013**, 24(6) 24-32;
- [2] EUCAN Cancer Factsheets: Ovary. <http://eu-cancer.iarc.fr/EUCAN/CancerOne.aspx?Cancer=27&Gender=2> (11 July **2013**, date last accessed).
- [3] Weiderpass E., et al., *Saf. Health Work*, **2012**, 3, 166-80;
- [4] Roett M. A., et al., *Am. Fam. Physician*, **2009**, 80(6), 609-616;
- [5] Alsop K., et al., *J. Clin. Oncol.*, **2012**, 30, 2654-2663;
- [6] Burges A., et al., *Dtsch. Arztebl. Int.*, **2011**, 108(38), 635-641;
- [7] Heintz A. P., et al., *Int. J. Gynaecol. Obstet.*, **2006**, 95(1), 161-192;
- [8] Cannistra S. A., *N. Engl. J. Med.*, **2004**, 351, 2519-2529;
- [9] Raja F. A., *Ann. Oncol.*, **2012**, 23, 118-127;
- [10] Armstrong D. K., *N. Engl. J. Med.*, **2006**, 354, 34-43;
- [11] Chen V. W., et al., *Cancer*, **2003**, 97(10), 2631-42;
- [12] Scully R. E., et al., *Histological Typing of Ovarian Tumours*. Berlin, Heidelberg, New York: Springer **1999**;
- [13] Averette HE., *American Cancer Society textbook of Clin. Oncol.*, 2nd ed. Atlanta (GA), **1995**, 552-79;
- [14] Prat J., et al., *Ann. Oncol.*, **2012**, 23(10), 111-117;
- [15] McCluggage W. G., et al., *Pathology*, **2011**, 43, 420-432;
- [16] Kobel M., et al., *Int. J. Gynecol. Pathol.*, **2010**, 29, 203-211;
- [17] Bell D., et al., *Nature*, **2011**, 474, 609-615;
- [18] Diaz-Padilla I., et al., *Gynecol. Oncol.*, **2012**, 126, 279-285;
- [19] Schmeler K. M., et al., *Gynecol Oncol*, **2008**, 108, 510-514;
- [20] Wiegand K. C., et al., *N. Engl. J. Med.*, **2010**, 363, 1532-1543;
- [21] Mackay H. J., et al., *Int. J. Gynecol. Cancer*, **2010**, 20, 945-952;
- [22] International Federation of Gynecology and Obstetrics, *Gynecol. Oncol.*, **1986**, 25, 383-385;
- [23] Shimizu Y., et al., *Cancer*, **1998**, 82, 893-901;
- [24] Han L. Y., et al., *Oper. Tech. Gen. Surg.*, **2007**, 9(2), 53-60;
- [25] <http://flipper.diff.org/app/items/info/3629>;
- [26] Mol B.W., et al., *Gynecol. Oncol.*, **2001**, 80, 162-167;
- [27] Rauh Hain J. A., et al., *Rev. Obstet. Gynecol.*, **2011**, 4 (1), 15-21;
- [28] Greene F. L., et al. *AJCC Cancer Staging Manual*. 6th ed. New York, NY, Springer; **2002**, 275-284
- [29] U.S. Preventive Services Task Force, *Ann. Intern. Med.*, **2005**, 143(5), 355-61. Erratum in: *Ann. Intern. Med.*, **2005**, 143(7), 547;
- [30] Hennessy B. T., et al. *Lancet*, **2009**, 374, 1371-1382;
- [31] Grody W. W., et al., *Henry's Clinical Diagnosis and Management by Laboratory Methods*. Philadelphia, Pa.: Saunders; **2007**: 1334-1335;
- [32] Prat J, et al., *Hum. Pathol.*, **2005**, 36, 861-870;
- [33] Vo C., et al., *Obstet. Gynecol. Clin. North. Am.*, **2007**, 34(4), 687-700;
- [34] Lukanova A., et al., *Cancer Epidemiol. Biomarkers Prev.*, **2005**, 14, 98-107;
- [35] Coglianò V. J., et al., *J. Natl. Cancer Inst.*, **2011**, 103, 1827-1839;
- [36] Beral V., et al., *Lancet*, **2008**, 371, 303-314;
- [37] Whitman D. C., et al., *Cancer Epidemiol. Biomarkers Prev.*, **2003**, 12(1), 42-46;

- [38] Morch L. S., et al., *JAMA*, **2009**, 302(3), 298–305;
- [39] Crum C. P., et al., eds. *Robbins and Cotran Pathologic Basis of Disease*. 7th ed. Philadelphia, Pa., Elsevier, Inc; 2005
- [40] Takeshima N., et al., *Gynecol. Oncol.*, **2005**, 99(2),427-431;
- [41] Barney S. P., et al., *Med. Clin. North. Am.*, **2008**, 92(5),1143-1161;
- [42] American College of Obstetricians and Gynecologists, *Obstet Gynecol*, 2007,110(1),201-214;
- [43] du Bois A., et al., *Der Frauenarzt*, **2005**, 46 (7), 60-567;
- [44] Lerner J. P., et al., *Am. J. Obstet. Gynecol.*, **1994**, 170, 81–85;
- [45] Sevinc A., et al., *Lancet Oncol.*, **2007**, 8(12), 1054-1055;
- [46] Komatsu T., et al., *Radiology*, **1996**, 198(1), 109-115;
- [47] Kinkel K., et al., *Radiology*, **2005**, 236(1), 85-94;
- [48] Salani R., et al., *Gynecol. Oncol.*, **2008**, 108(2), 271–275;
- [49] Pecorrelli S., et al., *J. Epidemiol. Biostat.*, **1998**, 3, 75-102;
- [50] Bristow R. E., et al., *J. Clin. Oncol.*, **2002**, 20(5),1248-1259;
- [51] Ahmed F. Y., et al., *J. Clin. Oncol.*, **1996**, 14, 2968-2975;
- [52] Trimbos J. B., et al., *J. Natl. Cancer Inst.*, **2003**, 95,113-125;
- [53] du Bois A., et al., *Cancer*, **2009**; 115(6),1234-44;
- [54] Van der Burg M. E., et al., *N. Engl. J. Med.*, **1995**, 332, 629-634;
- [55] Vergote I., et al., *N. Engl. J. Med.*, **2010**, 363, 943-953;
- [56] Aletti G. D., et al., *J. Am. Coll. Surg.*, **2009**, 208, 614-620;
- [57] du Bois A., et al., *J. Clin. Oncol.*, **2010**, 28, 1733–1739;
- [58] Dowdy S. C., et al., *Gynecol. Oncol.*, **2008**, 109(2), 303-307;
- [59] Panici P. B., et al., *J. Natl. Cancer Inst.*, **2005**, 97, 560-566;
- [60] Winter-Roach B. A., et al., *Cochrane Database Syst Rev*, **2009**, CD004706;
- [61] Swart A. C., et al., *J. Clin. Oncol. (Meeting Abstracts)*, **2007**, 25(18), Abstr 5509;
- [62] Chan J. K., et al., *Gynecol. Oncol.*, **2010**, 116, 301-306;
- [63] Ozols R. F., et al., *J. Clin. Oncol.*, **2003**, 21, 3,194-3200;
- [64] Canetta R., et al., *Cancer Treat. Rev.*, **1988**, 15(B),17-32;
- [65] Ringel I., et al., *J. Natl. Cancer Inst.*, **1991**, 83, 288-291;
- [66] Covens A., et al., *Gynecol. Oncol.*, **2002**, 85(1), 71-80;
- [67] McGuire W. P., et al., *N. Engl. J. Med.*, **1996**, 334, 1-6;
- [68] Bookman M. A., et al., *J. Clin. Oncol.*, **2009**, 27, 1419–1425;
- [69] Bookman M. A., et al., *J. Clin. Oncol. (Meeting Abstracts)*, **2006**, 24, 5002;
- [70] Schiff P. B., et al., *Nature*, **1979**, 277, 665-667;
- [71] Vasey P. A., et al., *J. Natl. Cancer Inst.*, **2004**, 96, 1682-1691;
- [72] Pignata S., et al., *J. Clin. Oncol.*, **2011**, 29, 3628-3635;
- [73] Armstrong D. K., et al., *N. Engl. J. Med.*, **2006**, 354, 34-43;
- [74] Hess L. M., et al., *Int. J. Gynecol. Cancer*, **2007**, 17, 561–570;
- [75] Markman M., et al., *J. Clin. Oncol.*, **2001**, 19, 1001-1007;
- [76] Jaaback K., et al., *Cochrane Database Systemat Rev.*, **2006**, 1,CD005340;
- [77] Rosenberg B., et al., *Nature*, **1969**, 222, 385-386;

- [78] Lippard S., *Pure App. Chem.*, **1987**, 59, 731-742;
- [79] Todd R. C., et al., *Metallomics*, **2009**, 1(4), 280-291;
- [80] Zamble D. B., et al., *Trends in Biochem.l Sci.s*, **1995**, 20(10), 435-439;
- [81] Sedletska Y., et al., *Curr. Med. Chem. AntiCancer Agents*, 5(3), **2005**, 251–265;
- [82] S. Trzaska, et al., *C&EN News* 83, **2005**;
- [83] Jamieson E. R., et al., *Chem. Rev.*, **1999**, 99(9), 2467-2498;
- [84] [ 85 86\*] Eastman A., *Pharmacol. Ther.s*, **1987**, 34(2),155-166;
- [85] [F]raval H. N, et al., *Cancer Res.*, **1979**, 39, 1793-1797;
- [86] Johnson S.W., et al. Cisplatin and Its Analogues. 6th edition. Philadelphia:Lippincott Williams and Wilkins; **2001**,376-388;
- [87] Basu A., et al., *J. Nucleic Acids*, **2010**,1-16, pii: 201367. doi: 10.4061/2010/201367;
- [88] Sorenson C. M. et al., *Cancer Res.*, **1988**, 48, 6703-6707;
- [89] Sorenson C.M., et al., *J. Natl. Cancer Inst.*, **1990**, 82, 749-755;
- [90] Barry M. A., et al., *Biochem. Pharmacol.*, **1990**, 40, 2353-2362;
- [91] Bellon S. F., et al., *Biochemistry*, **1991**, 30, 8026-8035;
- [9294] Chaney S. G., et al., *J. Inorg. Biochem.*, **1999**, 77, 71-81;
- [93] Zdraveski Z. Z., et al., *J. Biol. Chem.*, **2002**, 277, 1255-1260;
- [94] Huang J. C., et al. *Proc. Natl. Acad. Sci. USA*, **1994**, 91, 10394-10398;
- [95] He G. et al., *Proc. Am. Assoc. Cancer Res.*, **2001**, 42, 901;
- [96] Morgan S. E. et al., *Adv. Cancer Res.*, **1997**, 71, 1-25;
- [97] Bullock A. N, et al., *Nat. Rev. Cancer*, **2001**, 1, 68-76;
- [98] Damia G., et al., *Neoplasia*, **2001**, 3, 10-16;
- [99] Zhao H., et al., *Mol. Cell Biol.*, **2001**, 21, 4129-4139;
- [100] Appella E., et al., *Eur. J. Biochem.*, **2001**, 268, 2764-2772.
- [101] Tang D., et al., *J. Biol. Chem.*, **2002**, 277, 12710-12717;
- [102] Zhang Y., et al., *J. Biol Chem.*, **2002**, 277, 3124-3131;
- [103] Dent P. et al., *Clin. Cancer Res.*, **2001**, 7, 775-783;
- [104] Yeh P. Y., et al., *Biochem. Pharmacol.*, **2002**, 63, 1423-1430;
- [105] Jayaraman L., et al., *Genes Dev.*, **1998**, 12, 462-472;
- [106] Delmastro D. A., et al., *Cancer Chemother. Pharmacol.*, **1997**, 39, 245-253;
- [107] Hershberger P.A., et al., *Mol Cancer Ther*, **2002**, 1, 821-829;
- [108] Alarcon-Vargas D., et al., *Carcinogenesis*, **2002**, 23(4), 541-547;
- [109] Wang X., et al., *J. Biol. Chem.*, **2000**, 275, 39435-39443;
- [110] Makin G. W., et al., *EMBO J.*, **2001**, 20, 6306-6315;
- [111] Del Bello B., et al., *Oncogene*, **2001**, 20, 4591-4595;
- [112] Micheau O., et al., *J. Natl. Cancer Inst.*, **1997**, 89, 783-789;
- [113] Muller M., et al., *J Exp Med*, **1998**, 188, 2033-2045;
- [114] Eischen C. M., et al., *Blood*, **1997**, 90, 935-943;
- [115] Ferreira C. G., et al., *Clin. Cancer Res.*, **2000**, 6, 203-212;
- [116] Fan S., et al., *Cancer Res.*, **1995**, 55, 1649-1654;
- [117] Fan S., et al., *Oncogene*, **1997**, 14, 2127-2136;

- [118] O'Connor P. M., et al., *Prog. Cell Cycle Res.*, **1996**, 2, 165-173;
- [119] Kharbanda S., et al., *Mol. Cell Biol.*, **2000**, 20, 4979-4989;
- [120] Sanchez-Prieto R., et al., *Oncogene*, **2002**, 21, 974-979;
- [121] Chu G., et al., *Cancer*, **1993**, 72, 3707-3714;
- [122] Brabec V., et al., *Drug Resist. Update*, **2002**, 5, 147-161;
- [123] Siddik Z.H., *Oncogene*, **2003**, 22, 7265-7279;
- [124] Cole S.P., et al., *Cancer Res.*, **1994**, 54(22), 5902-5910;
- [125] Johnson S.W., et al., *Exp. Cell Res.*, **1996**, 226,133-139;
- [126] Stewart D., et al., *Proc. Am. Assoc. Cancer Res.*, **1998**, Abstract 39: Abstract # 2236;
- [127] Lowe G. D., *Bibl. Haematol.*, **1981**, 47, 247-251;
- [128] Andrews R. J., et al. *Surg. Neurol.*, **1993**, 39, 218-222;
- [129] Laurencot C. M., et al., *Oncol. Res.*, **1995**, 7, 371-379;
- [130] Kuin A., et al., *Br. J. Cancer*, **1999**, 79,793-801;
- [131] Murakami T., et al., *Int. J. Cancer*, **2001**;93:869-74.
- [132] Andrews P, et al. Cisplatin uptake mediated cisplatin-resistance in human ovarian carcinoma cells. In:  
*Nicolini M, editor. Platinum and other metal coordination compounds in cancer chemotherapy. Boston, MA: Martinus Nijhoff Publishing, 1988, 248-254;*
- [133] Stewart D. J., et al., *Proc. Am. Assoc. Cancer Res.*, **1995**; 36, 399;
- [134] Takahashi K., et al., *Jpn J. Cancer Res.*, **1985**, 76, 68-74;
- [135] Melvik J. E., et al., *Br. J. Cancer*, **1992**, 66, 260-265;
- [136] Fracasso M. E., et al., *Drugs Exp. Clin. Res.*, **1987**, 13, 367-372.
- [137] Eastman A., *Cancer Cells*, **1990**, 2(8-9), 275-280;
- [138] Sedletska Y., et al., *Curr. Med. Chem. AntiCancer Agents*, **2005**, 5(3), 251-265;
- [139] Gately D.P., et al., *Br. J. Cancer*, **1993**, 67, 1171-1176;
- [140] Andrews P., et al., *Cancer Cells*, **1990**, 2, 35-43;
- [141] Andrews P. A., et al., *Cancer Res.*, **1991**, 51(14), 3677-3681;
- [142] Hall M. D., et al., *Ann. Rev. Pharmacol. Toxicol.*, **2008**, 48, 495-535;
- [143] Ishida S., et al., *Proc. Natl. Acad. Sci. U.S.A*, **2002**, 99(22), 14298-14302;
- [144] Safaei R., et al., *Crit. Rev. Oncol. Hematol.*, **2005**, 53, 13-23;
- [145] Holzer A. K., et al., *Clin. Cancer Res.*, **2004**, 10(19), 6744-6749;
- [146] Blair B. G., et al., *Clin. Cancer Res.*, **2009**, 15(13), 4312-4321;
- [147] Liang X. J., et al., *Cancer Res.*, **2006**, 66, 2346-53;
- [148] Kelland L., *Nat. Revs. Cancer*, **2007**, 7(8), 573- 584;
- [149] Gately D. P., et al., *Br. J. Cancer*, **1993**, 67(6), 1171-1176;
- [150] Andrews P.A., et al., *Cancer Res.*, **1988**, 48, 68-73;
- [151] Stewart D. J, et al.,. *Proc. Am. Assoc. Cancer Res.*, **1996**; 37, 174.
- [152] Shen D.W., et al., *Br. J. Cancer*, **2004**, 91, 270-276;
- [153] Min J., et al., *Eukaryot Cell*, **2004**, 3, 795-805
- [154] Upadhyay A. K., et al., *Toxicol. Appl. Pharmacol.*, **2006**, 216, 177-185;
- [155] Wang W., et al., *Oncol. Rep.*, **2004**, 12,1365-1370;
- [156] Mann S.C., et al., *Cancer Chemother. Pharmacol.*, **1990**, 25, 236-240;

- [157] Katano K., *Mol. Pharmacol.*, **2003**, 64, 466-473;
- [158] Materna V., et al., *Int. J. Cancer*, **2005**, 115, 393-402;
- [159] Ishikawa T., et al., *J. Biol. Chem.*, **1994**, 269(46), 29085-29093;
- [160] Safaei R., et al., *Mol. Cancer Ther.*, **2005**, 4, 1595-1604;
- [161] Yeh J. J., et al., *Lung*, **2005**, 183, 177-183;
- [162] Wang W., et al., *Oncol. Rep.*, **2004**, 12, 1365-1370;
- [163] Zurita A. J., et al., *Br. J. Cancer*, **2003**, 88, 879-886;
- [164] Ikeda K., et al., *Anticancer Re.s.*, **1998**, 18, 3077-3080;
- [165] Borst P., et al., *Semin. Cancer Bio.l.*, **1997**, 8, 205-213;
- [166] Godwin A. K., et al., *Proc. Natl. Acad. Sci. U.S.A.*, **1992**, 89, 3070-3074;
- [167] Cepeda V., et al. *AntiCancer Agents Med. Chem.*, **2007**, 7(1), 3–18;
- [168] Ishikawa T., et al., *J. Biol. Chem.*, **1993**, 268, 20116-20125;
- [169] Lai G.R., et al., *J. Natl. Cancer Inst.*, **1989**, 81(7), 535-539;
- [170] Kasherman Y., et al., *J. Med. Chem.*, **2009**, 52(14), 4319-4328;
- [171] Chen H. H. W., et al., *Mol. Pharmacol.*, **2008**, 74(3), 697-704;
- [172] Ban N., et al., *Cancer Res.*, **1996**, 56(15), 3577-3582;
- [173] Piaggi S., et al., *Carcinogenesis*, **2010**, 31(5), 804-811;
- [174] Basu A., et al., *Toxicol. Lett.s*, **1990**, 50(2-3), 123-135;
- [175] Andrews P. A., et al., *Cancer Chemother. Pharmacol.*, **1987**, 19( 2), 149-154;
- [176] Smith D. J., et al., *Urology*, **2006**, 67, 1341-1347;
- [177] Surowiak P., et al., *Virchows Archiv.*, **2007**, 450(3), 279-285;
- [178] Karotki A. V., et al., *J Biol Inorg Chem*, **2009**, 14(7), 1129-1138;
- [179] Hidalgo J., *Brain Res. Bull.*, **2001**, 55(2), 133-145;
- [180] Hagerman D., et al., *Drug Metab. Dispos.*, **2003**, 31(7), 916-923;
- [181] Kelley S. L., et al. *Science*, **1988**, 241(4874), 1813-1815;
- [182] Theocharis S. E., et al. *Histopathology*, **2004**, 45(2), 103-118;
- [183] Parker R. J., et al., *J. Clin. Invest.*, **1991**, 87, 772-777;
- [184] Chu G., et al., *Proc. Natl. Acad. Sci. U.S.A*, **1990**, 87, 3324-3327;
- [185] Dabholkar M., et al., *J. Clin. Invest.*, **1994**, 94, 703-708;
- [186] McKay B. C., et al., *Oncogene*, **2001**, 20(46), 6805-6808;
- [187] Welsh C., et al., *Int. J. Cancer Suppl.*, **2004**, 110(3), 352-361;
- [188] Rosell R., et al., *Cancer Control*, **2003**, 10(4), 297-305;
- [189] D'Andrea A. D., *Cell Cycle*, **2003**, 2, 290-292;
- [190] Chen H.Y., et al., *Int. J. Cancer*, **2010**, 126(8), 1944-1954;
- [191] Orelli B., et al., *J. Biol. Chem.*, **2010**, 285(6), 3705-3712;
- [192] Colton S. L., et al., *J Biol Chem*, **2006**, 281(37), 27117-27125;
- [193] Lomonaco S. L., et al., *DNA Cell Biol.*, **2009**, 28(6), 285-294;
- [194] Damsma G. E., et al., *Nat Structl Mol Biol*, **2007**, 14(12), 1127–1133;
- [195] McKay B. C., et al., *Oncogene*, **2001**, 20(46), 6805-6808;
- [196] Borst P., et al., *Cell Cycle*, **2008**, 7(10), 1353-1359;
- [197] Bergoglio V., et al., *Oncogene*, **2001**, 20, 6181-6187;

- [198] Bassett E., et al., *Biochemistry*, **2003**, 42, 14197-14206;
- [199] Nagatani G., et al., *Cancer Res.*, **2001**, 61, 1592-1597;
- [200] Sun C. L., et al., *Mol. Pharmacol.*, **2005**, 67,1307-1314;
- [201] Avdievich E., et al. *Proc Natl Acad. Sci. U.S.A*, **2008**, 105(11), 4247-4252;
- [202] Lin D. P., et al., *Cancer Res.*, **2004**, 64(2), 517-522;
- [203] Aebi S., et al., *Cancer Res.*, **1996**, 56, 3087-3090;
- [204] Lin X., et al., *Mol. Pharmacol.*, **1999**, 56, 390-395;
- [205] Ono Y., et al., *Mol. Urol.*, **2001**, 5, 25-30;
- [206] Lin X., et al., *Mol. Cancer Ther.*, **2006**, 5,1239-1247;
- [207] Minagawa Y., et al., *Jpn J. Cancer Res.*, **1999**, 90, 1373-1379;
- [208] Kigawa J., et al., *Hum. Cell.*, **2001**, 14, 165-171;
- [209] Achanzar W. E., et al., *Prostate.*, **2002**, 52, 236-244;
- [210] Isonishi S., et al., *Hum. Cell*, **2001**, 14, 203-210;
- [211] Cheng J. Q., et al., *Drug Resist. Updat.*, **2002**, 5, 131-146;
- [212] Abedini M. R., et al., *Oncogene* **2004**, 23, 6997-7004;
- [213] Zahedi P., et al., *Exper. Opin. Drug Deliv.*, **2012**, 9(5), 567-583;
- [214] Baird R. D., et al., *Eur. J. Cancer*, **2003**, 39(17), 2450-2461;
- [215] Pliarchopoulou K., et al., *Crit. Rev. Oncology. Hematol.*, **2011**, 79,17-23;
- [216] Friedlander M., et al., *Int. J. Gynecol. Cancer*, **2011**, 21, 771-775;
- [217] Harter P., et al., *Ann. Surg. Oncol.*, **2009**, 16, 1324-1330;
- [218] Zang R. Y., et al., *Br. J. Cancer*, **2011**, 105, 890-896;
- [219] Leamon C. P., et al., *Pharmacogenomics Pers. Med.*, **2013**, 6, 113-125
- [220] Zahedi P., et al., *Mol. Pharm.*, **2011**, 8(1), 260-9
- [221] Vaughan S., et al., *Nat. Rev. Cancer*, **2011**, 11, 719-25;
- [222] Rustin G. J., et al., *J. Clin. Oncol. (Meeting Abstracts)* **2009**; 27(18s), 1;
- [223] Heintz A. P. M., et al., *Int. J. Gynecol. Obstet.*, **2006**, 95(1), S163;
- [224] McGuire W. P., et al., *Ann. Int. Med.*, **1989**, 111(4), 273-279;
- [225] Rowinsky E.K., et al., *N. Engl. J. Med.*, **1995**, 332(15), 1004-1014;
- [226] Gynecologic Oncology Group, Markman M, et al., *Gynecol. Oncol.*, **2006**, 101(3), 436-440;
- [227] Baird R. D., et al., *Nat. Rev. Clin. Oncol.*, **2010**, 7(10), 575-582;
- [228] Rosenberg P., et al., *Acta Oncol.*, **2002**, 41(5), 418-424;
- [229] Ten Bokkel Huinink W., et al., *J. Clin. Oncol.*, **1997**,15(6), 2183-2193;
- [230] Armstrong D.K., *Oncologist*, **2004**, 9(1), 33-42;
- [231] Markman M., et al., *Gynecol. Oncol.*, **2000**, 79(1), 116-119;
- [232] Muggia F. M., et al., *J. Clin. Oncol.*, **1997**,15(3), 987-993;
- [233] Pujade-Lauraine E., et al., *J. Clin. Oncol.*, **2010**, 28, 3323-3329;
- [234] <http://clinicaltrials.gov>
- [235] Monk B. J., et al., *J. Clin. Oncol.*, **2010**, 28, 3107-3114;
- [236] Poveda A., et al., *Ann. Oncol.*, **2011**, 22, 39-48;
- [237] Mutch D. G., et al., *J. Clin. Oncol.*, **2007**, 25(19), 2811-2818;
- [238] Ferrandina G., et al., *J. Clin. Oncol.*, **2008**, 26(6), 890-896;

- [239] Astex Pharmaceuticals, Oral Presentation at AACR Supports Potential of SGI-110 In The Treatment Of Platinum-Resistant Ovarian Cancer , April 10, 2013;
- [240] Singh V., et al., *Curr. Cancer Drug. Targets*, **2013**, 13(4), 379-399;
- [241] Puzanov I., et. al., *Clin. Cancer Res.*, **2011**, 17(11), 3794-3802;
- [242] Satyam A., et al., *J. Med. Chem.*, **1996**, 39(8), 1736-1747;
- [243] Lyttle M. H., et al., *J. Med. Chem.*, **1994**, 37(10),1501-1507;
- [244] Vergote I., et al., *Int. J. Gynecol. Cancer*, **2010**, 20(5), 772-780;
- [245] Rajkumar S.,et. al., *Pharmacophore*, **2011**, 2(2), 104-113;
- [246] Cheetham P., et al., *Cancer J.*, **2013**, 19(1), 59-65;
- [247] Colombo N., et al., *J. Clin. Oncol.*, **2012**, 30(31), 3841-3847;
- [248] De Geest K., et al., *J- Clin. Oncol.*, **2010**, 28(1), 149-153;
- [249] Capasso A., et. al., *Curr. Drug Targets*, **2012**, 13(8), 1065-1071;
- [250] <http://www.basilea.com/Development/BAL101553/>
- [251] Robinson W. R., et al., *Int. J. Gynecol. Cancer*, **2009**, 19(1),124-128;
- [252] Carroll A. R., et al., *Front. Biosci. (Schol. Ed.)*, **2011**, 3, 82-97.
- [253] Ramakrishnan S., et al., *Angiogenesis*, **2005**, 8, 169-182;
- [254] Burger R. A, et al, *Gynecol. Oncol.*, **2011**, 121, 230- 238;
- [255] Dvorak H. F., et al., *J. Clin. Oncol.*, **2002**, 20(21), 4368-4380;
- [256] Folkman J., et al., *N Engl J Med* , **1971**, 285(21), 1182-1186;
- [257] Lafky J.M., et al., *Biochim Biophys Acta*, **2008**, 1785, 232-65;
- [258] Folkman J., *Nat Med*, **1995**, 1(1), 27-31;
- [259] ] Di Blasio A. M, et al.. *Am. J. Obstet. Gynecol.*, **1993**;169,1517-1523.
- [260] Chen H., et al., *Gynecol. Oncol.*, **2004**, 94, 630-635;
- [261] Santin A. D., et al., *Eur. J. Gynecol. Oncol.*, **1999**, 20, 177-181;
- [262] Crickard K., et al., *Gynecol. Oncol.*, **1994**, 55, 277-284;
- [263] Burger R. A., et al., *N. Engl. J. Med.*, **2011**, 365, 2473-2483;
- [264] Perren T. J.; et al., *N. Engl. J. Med.*, **2011**, 365, 2484-2496;
- [265] Aghajanian C., et al., *J. Clin. Onco.l*, **2011**, 29 [Abstract LBA5007] :
- [266] Poveda A. M., et al., *Ann. Oncol.*, **2012**, 23, doi:10.1093/annonc/mds499;
- [267] Campos S.M., et al., *J. Oncol*, **2010**,:149362. doi: 10.1155/2010/149362;
- [268] Monk B. J., et al., *Crit. Rev. Oncol Hematol*, **2013**, 86(2), 161-175;
- [269] Sharma T.; et al., *Mini Rev. Med. Chem.*, **2013**, 13, 530-540;
- [270] Byrne A. T.; et al., *Clin. Cancer Res.*, **2003**, 9, 5721-5728;
- [271] Baffert F., et al., *Am. J. Physiol. Heart Circ. Physiol.*, **2006**, 290, 547-559;
- [272] Holash J., et al., *Proc. Natl. Acad. Sci.U. S. A.*, **2002**, 99, 11393-11398;
- [273] Coleman R. L., et al., *Lancet Oncol.*, **2011**, 12, 1109-1117;
- [274] Ferry A. L. M., et al., *Clin. Cancer Res.*, **2011**, 17, 7156-7163;
- [275] Apte S. M., et al., *Gynecol. Oncol.*, **2004**, 93, 78-86;
- [276] Valve E., et al., *Int. J. Cancer.*, **2000**, 88, 718–725;
- [277] Matei D, et al., *Oncogene*, **2006**, 25, 2060–2069;
- [278] Matei D, et al., *Cancer Biol.Ther* , **2007**, 6,1951-1959;

- [279] Wilczynski S. P., et al., *Hum Pathol*, **2005**, 36, 242-249;
- [280] Schilder R. J., *J. Clin. Oncol.*, **2008**, 26(20), 3418-3425;
- [281] Buchdunger E., et al., *Proc. Natl. Acad. Sci. U.S.A*, **1995**, 92(7), 2558-2562;
- [282] Ledermann J. A., et al., *J. Clin. Oncol.*, **2011**, 29, 3798-3804;
- [283] du Bois A., et al., *Ann. Oncol.*, **2010**, 21, 370-375;
- [284] Friedlander M., et al., *Gynecol. Oncol.*, **2010**, 119, 32-37;
- [285] Matulonis U. A., et al., *J. Clin. Oncol.*, **2009**, 27, 5601-5606;
- [286] Sahade M., et al., *Future Oncol.*, **2012**, 8, 775-781;
- [287] Polcher M., et al., *Cancer Chemother. Pharmacol.*, **2010**, 66, 203-207;
- [288] Kohn E. C., et al., *J. Clin. Oncol.*, **2011**, 29 [Abstract 5019];
- [289] Bauerschlag D. O. et al., *Anticancer Res.*, **2010**, 30, 3355-3360;
- [290] Campos S. M.; et al., *Gynecol. Oncol.*, **2013**, 128, 215-220;
- [291] Schilder R. J., et al., *Gynecol. Oncol.*, **2013**;129(1), 86-91;
- [292] Karlan B. Y.; et al., *J. Clin. Oncol.*, **2012**, 30, 362-371;
- [293] Jubb A. M.; et al., *J Clin Oncol*, **2006**, 24, 217-227;
- [294] Horowitz N. S.; et al., *Clin. Ovarian Cancer Other Gynecol. Malig.*, **2011**, 4, 26-33;
- [295] De Graeff P., et al., *Br. J Cancer*, **2009**, 101, 149-159;
- [296] Xia W., et al., *Mol. Carcinog.*, **2009**, 48, 610-617;
- [297] Bookman M. A., et al., *J. Clin. Oncol.*, **2003**, 21(2), 283-290;
- [298] Gordon M. S., et al., *J. Clin. Oncol.*, **2005**, 23(16S), abstract 5051;
- [299] Seiden M. V., et al., *Gynecol. Oncol.*, **2007**, 104(3), 727-731;
- [300] Posadas E. M., et al., *Cancer*, **2007**, 109(7), 1323-1330;
- [301] Gordon A. N., et al., *Int. J. Gynecol. Cancer*, **2005**, 5(5), 785-792;
- [302] Campos S., et al., *J. Clin. Oncol.*, **2005**, 23(24), 5597-5604;
- [303] Kaye S. B., et al., *J. Clin. Oncol.*, **2008**, 26, abstract 5520;
- [304] Gordon M. S., et al., *J. Clin. Oncol.*, **2006**, 24(26), 4324-4332;
- [305] Schilder R. J., et al., *Clin. Cancer Res.*, **2005**, 11(15), 5539-5548;
- [306] Tanner B., et al., *J. Clin. Oncol.*, **2006**, 24(26), 4317-4323;
- [307] Herbst R. S., et al., *J. Clin. Oncol.*, **2005**, 23(11), 2544-2555;
- [308] Campos S. M., et al., *Br. Gynecological Society Meeting*, **2008**, abstract 93;
- [309] Fridland A., et al., *Cancer Res.*, **1974**, 34, 883-888;
- [310] Ledermann J. A., et al., *Gynecol Oncol*, **2010**, 119, 51-156;
- [311] Wiener J. R., et al., *Gynecol. Oncol.*, **2003**, 88, 73-79;
- [312] Pengetnze Y., et al., *Biochem. Biophys. Res. Commun.*, **2003**, 309, 377-383;
- [313] Chen T., et al., *Mol. Cancer. Ther.*, **2005**, 4, 217-224;
- [314] Han L. Y., et al., *Cancer Res.*, **2006**, 66, 8633-8639;
- [315] Secord A.A, et al., *Clin Cancer Res*, **2012**, 18(19), 5489-5498.
- [316] Bendell J. C, et. al., *Clin. Cancer Res*, **2014**, 20(2), 480-489;
- [317] Brenner A. J., et al., *Clin. Cancer Res.*, **2013**, 19, 3996-4007;
- [318] Buikhuisen W. A., et al., *Lancet Oncol.*, **2013**;14(6), 543-551;
- [319] Sierra J. R., et al., *Ther. Adv. Med. Oncol.*, **2011**, 3(S1), 21-35;

- [320] Bowles D., et al., *Drugs of Today*, **2011**, 47(11), 857-868;
- [321] Akinleye A., et al., *J. Hematol. Oncol.*, **2013**, 6(27), doi: 10.1186/1756-8722-6-27
- [322] Tate C., et al., *J. Biol. Chem.*, **2013**, 288(9), 6743-6753
- [323] Jiang B. H., *Biochim Biophys Acta.*, **2008**, 1784(1),150-158;
- [324] Gera J. F., et al., *J. Biol. Chem.*, **2004**, 279(4), 2737-2746;
- [325] Mabuchi S., et al., *Clin. Cancer Res.*, **2007**, 13(14), 4261-4270;
- [326] Berndt N., et al., *Cell. Death. Differ.*, **2010**, 17(11), 1795-1804;
- [327] Baserga R., et al., *Expert Opin. Ther. Targets.*, **2005**, 9, 753-768;
- [328] Beltran P. J, et al., *Proc. Am. Soc. Clin. Oncol.*, **2007**, 207 [abstr];
- [329] Cazzaniga M., et. al., *J. Biomed. Biotechnol.*, **2012**, 172897; doi: 10.1155/2012/172897
- [330] Carrassa L., et. al., *Cell Cycle*, **2012**, 11(13), 2507-2517;
- [331] Keen N., et al., *Nat. Rev. Cancer.*, **2004**, 4 (12), 927- 936;
- [332] Landen C. N. Jr, et al., *Clin. Cancer Res.*, **2007**, 13(14), 4098-4104;
- [333] Lin Y. G., et al., *Clin. Cancer Res.*, **2008**, 14(17), 5437- 5446;
- [334] Scharer C. D, et al., *J. TranslMed*, **2008**, 6, (79), doi: 10.1186/1479-5876-6-79
- [335] Manfredi M. G., et al., *Proc. Natl. Acad. Sci. U.S.A*, **2007**, 104(10), 4106-4111;
- [336] Do T. V., et al., *Oncogene* , **2013**, doi: 10.1038/onc.2012.632
- [337] Collins I., et al., *Curr. Opin. Pharmacol.*, **2005**, 5, 366-373;
- [338] Tutt A. N. J., et al., *Cold Spring Harb. Symp. Quant. Biol.*, **2005**, 70, 139-148;
- [339] Patel A. G, et al., *Proc. Natl. Acad. Sci. U.S.A*, **2011**, 108, 3406- 3411;
- [340] Lord C. J., et al. *Curr. Opin. Pharmacol.*, **2008**, 8(4) , 363–369;
- [341] Konstantinopoulos P.A., et al., *J Clin Oncol*, **2012**, 30, 347-350;
- [342] Press J. Z., et al., *BMC Cancer*, **2008**, 8:17. doi: 10.1186/1471-2407-8-17
- [343] Rottenberg S., et al., *Proc. Natl. Acad. Sci. U.S.A*, **2008**, 105, 17079-17084;
- [344] Yang D., et al., *JAMA*, **2011**, 306, 1557-1565;
- [345] Ledermann J. A., et al., *N. Engl. J. Med.*, **2012**, 366(15), 1382-1392;
- [346] Tentori L. , et al., *Eur J Cancer*, **2007**, 43(14), 2124-2133;
- [347] Liu J., et al. (ASCO Meeting Abstracts), *J. Clin. Oncol.*, **2011**, 29(15), 5028;
- [348] Jones P., et al., *J. Med. Chem.*, **2009**, 52(22), 7170-7185;
- [349] Drew Y., et al., (ASCO Meeting Abstracts), *J. Clin. Oncol.*, **2011**, 29(15), 3104;
- [350] Lepeak L. M.; et al., *Drugs of the Future*, **2010**, 35(10), 815-822;
- [351] Mukhopadhyay A., et al., *Cancer Res.*, **2012**, 72(22), 5675–5682;
- [352] Allensworth J. L., *Breast Cancer Res. Treat.*, **2013**, 137(2), 359-371.;
- [353] Zhang M. M, et. al., *Gynecol Oncol*, **2007**, 105, 194-198;
- [354] Lorusso D., et al., *Br. J. Cancer*, **2012**, 107(1), 37-42;
- [355] Lu H., et. al, *Clin Cancer Res.*, **2012**, 18, 499-509;
- [356] Tse B. W. C, et. al., *Ann. Oncol.*, **2013**, 00, 1–9;
- [357] Liu X., et.al., *Blood*, **2010**, 115(17), 3520-3530;
- [358] Antony A.C., *Ann. Rev. Nutr.*, **1996**, 16, 501-521;
- [359] Bueno R., et al., *J. Thorac. Cardiovasc. Surg.*, **2001**, 121(2), 225-233.
- [360] Kelemen L. E, et al., *Cancer Epidemiol. Biomarkers Prev.*, **2010**, 19, 1822-1830.

- [361] Markert S., et al., *Anticancer Res*, **2008**, 28, 3567-3572;
- [362] Costi M. P., et al., *Curr. Drug Targets* 2, **2001**, 135- 166;
- [363] Costi M. P., et al., *Curr. Med. Chem.*, **2005**, 12, 763-771;
- [364] Chu E., et al., *BioEssay*, , **1996**, 18(3)191-198;
- [365] Houghton J. A., et al., *Clin. Cancer Res.*, **1995**, 1, 723;
- [366] Lenz H. J., et al., *J. Clin. Oncol.*, **1995**, 14, 176;
- [367] Forsthoefel A. M., et al., *Biochemistry*, **2004**, 4, 1972;
- [368] Rahman L., et al., *Cancer cell*, **2004**, 5, 341-351.
- [369] Chu E, et al., *Proc. Natl. Acad. Sci. U.S.A*, **1991**, 88, 8977-8981;
- [370] Schiffer C. A., *Biochemistry*, **1995**, 34(50),16279-16287;
- [371] Barman H. M., et al., *Nucl. Acids Res.*, **2000**, 28, 235;
- [372] Perry K., *Proteins*, **1990**, 8, 315,.
- [373] Montfort W. R., et al., *Biochemistry*, , **1990**, 29, 6977 ().
- [374] Genovese F., et al., *Protein Sci*, **2010**, 19, 1023- 1030;
- [375] Doroshow J., et al., *Proc. Am. Soc. Clin Oncol.*,**1986**, 5, 117;
- [376] El-Hajj HH, et al., *J. Bacteriol.* , **1992**, 174, 4450-4456;
- [377] Berg J. M., et al., *Biochemistry*,**2007**, 6<sup>th</sup> Ed, New York,, W. H. Freeman.
- [378] Berger S. H, *Biochim. Biophys. Acta*, **2004**, 1696, 15-22;
- [379] Farber S., et al., *N. Engl. J. Med.*, **1948**, 238, 787-793;
- [380] Hertz R., et al., *Proc. Soc. Exp. Biol. Med.*, **1956**, 93, 361-366;
- [381] Fridland A., et al., *Cancer Res.*,**1974**, 34, 883-888;
- [382] Li J. C. et al., . *Proc. Natl. Acad. Sci. U.S.A.*, **1984**, 81, 5694-5698;
- [383] Ingraham H. A., et al., *Molec. Pharm.*, **1982**, 21, 211-216;
- [384] Cronstein B. N., *Pharmacol. Rev.*, **2005**, 57(2)163-172;
- [385] Cardinale D, et al., *Proc. Natl. Acad. Sci. U.S.A.*, **2011**, 108, 542-549 ,
- [386] Christen L., et al.. *Gynecol. Oncol.*, **2013**, 131, 493-498;
- [387] Antony A. C., et al., *Blood*, **1992**, 79, 2807-2820;
- [388] Zhao R, et al., *Cancer Metastasis Rev.*, **2007**, 26(1), 129-139;
- [389] Mendelsohn L. G., et al., *Semin. Oncol.*, **1999**, 26(2 Suppl 6), 42-47;
- [390] Miller D. S., et al., *J. Clin. Oncol.*, **2009**, 27(16), 2686-2691.
- [391] Hagemann A. R., et al., *J. Clin. Oncol*, **2012**, 30(Suppl. abstr 5013);
- [392] Phan J., et al., *J. Biol. Chem.*, **2001**, 276, 14170-14177 ;
- [393] Gibbs D. D., et al., *Cancer Res.*, **2005**, 65, 11721-11728;
- [394] Celeste L. R. , et al., *Protein Sci.* , **2012**, 21, 219-228;
- [395] Clinicaltrials.gov. NCT00787514, **2008**, Royal Marsden NHS Foundation Trust;
- [396] Plummer R., et al., *Clin. Cancer Res.*, **2003**, 9, 1313-1322;
- [397] Clinicaltrials.gov. ZD9331, AstraZeneca, 2011
- [398] Bailey S.W., et al., *Proc. Natl. Acad. Sci. U S A.*, **2009**, 106(36),15424-15429;
- [399] Salazar M. D. et al., *Cancer Metastasis Rev.*, **2007**, 26(1),141-152;
- [400] Seyoum E., et al, *J. Nutr.*, **1998**, 128, 1956-1960;
- [401] Stover P. J., *Nutr. Rev.*, **2004**, 62 (6 Pt2), S3–12, discussion S13.

- [402] Blount B. C., et al. *Proc. Natl. Acad. Sci. U.S.A.*, **1997**, 94, 3290–3295;
- [403] Stern L. L., et al., *Cancer Epidemiol. Biomark Prev.*, **2000**, 9,849-853;
- [404] Strathdee G., et al., *Am. J. Pathol.*, **2001**, 158(3), 1121-1127;
- [405] Matherly L. H., et al., *Vitam. Horm.*, **2003**, 66, 403-456
- [406] Matherly L. H., et al., *Cancer Metastasis Rev.*, **2007**, 26(1), 111- 128.
- [407] Siu M. K., et. al, Plos One, **2012**, 7(11), e47201. doi: 10.1371/journal.pone.0047201
- [408] Qiu A., et al., *Cell*, **2006**, 127(5), 917-928;
- [409] Zhao R. et al., *J. Biol. Chem.*, **2009**, 284(7), 4267-4274;
- [410] Elnakat H., et al., *Adv. Drug Deliv. Rev.*, **2004**, 56(8), 1067-1084;
- [411] Kamen B. A., et al., *Adv. Drug. Deliv. Rev.*, **2004**, 56(8), 1085-1097;
- [412] Piedrahita J. A., et al., *Nat. Genet.*, **1999**, 23(2), 228-232;
- [413] Henderson G. B., *Ann. Rev. Nutr.*, **1990**, 10, 319-335;
- [414] Antony A. C., et al., *J. Biol. Chem.*, **1985**, 260(28), 14911-14917;
- [415] Shen, F., et al., *Biochemistry*, **1995**, 34(16), 5660-5665;
- [416] Wang J., et al., *Biochemistry*, **1997**, 36(47), 14583-14592;
- [417] Zheng X., et al., *Mol. Cell. Biol.*, **2003**, 23(6), 2202-2212;
- [418] Shen F., et al., *Biochem. J.*, **1997**, 327(Pt 3), 759-764;
- [419] Reddy J.A., et al., *Cancer Res.*, **2007**, 67(13), 6376-6382;
- [420] Wang X., et al., *Biochem. Pharmacol.*, **1992**, 44(9), 1898-1901;
- [421] Maziarz K. M., et al., *J. Biol. Chem.*, **1999**, 274 (16), 11086-11091;
- [422] Smulders Y. M., et al., *Semin. Vascular. Med.*, **2005**, 5(2), 87-97;
- [423] Moat S. J., et al., *J. Nutr. Biochem.*, **2004**, 15(2), 64-79;
- [424] Parker N., et al., *Anal. Biochem.*, **2005**, 338(2), 284-293;
- [425] Wang H., et al., *Blood*, **2000**, 96(10), 3529-3536;
- [426] Ross J. F., et al., *Cancer*, **1999**, 85(2), 348-357;
- [427] Nakashima-Matsushita N., et al., *Arthritis Rheum.*, **1999**, 42(8), 1609-1616;
- [428] Shen F., et al., *Biochemistry*, **1994**, 33(5), 1209-1215;
- [429] Low M.G., *Biochim. Biophys. Acta*, **1989**, 988(3), 427-454;
- [430] Chancy C. D., et al., *J. Biol. Chem.*, **2000**, 275(27), 20676-20684;
- [431] Wu M., et al., *Cancer Epidemiol. Biomarkers. Prev.*, **1999**, 8(9), 775-782;
- [432] Ross J.F., et al., *Cancer*, **1994**, 73, 2432-2443;
- [433] Campbell I. G., et al., *Cancer Res.*, **1991**, 51, 5329-5338;
- [434] Weitman S. D., et al., *J. Neuro-oncology*, **1994**, 21(2), 107-112;
- [435] Toffoli G., et al., *Int. J. Cancer (Pred. Oncol.)*, **1998**, 79, 121-126;
- [436] Tran T., et al., *Cancer Res.*, **2005**, 65, 4431-4441;
- [437] Brown D., et al., *J. Am. Soc. Nephrol.*, **1992**, 3, 895-906;
- [438] Veatch A. L., et al., *Int. J. Cancer*, **1994**, 58, 393-399;
- [439] Darai E., et al., *Hum. Pathol.*, **1997**, 28, 922-928;
- [440] Kim Y. I., *Cancer Epidemiol. Biomarkers Prev.*, **2008**, 17(9), 2220-2225.
- [441] Leamon C. P., et al., *Adv. Drug. Deliv. Rev.*, **2004**, 56(8), 1127-1141
- [442] Sabharanjak S., et al., *Dev. Cell*, **2002**, 2(4), 411-423;

- [443] Edidin M., *Annu. Rev. Biophys. Biomol. Struct.*, **2003**, 32, 257-283.
- [444] Kamen B. A., et al., *J. Biol. Chem.*, **1988**, 263, 13602-13609;
- [445] Kamen B. A., et al., *J. Clin. Invest.*, **1989**, 84(5), 1379-1386;
- [446] Leamon C. P., et al., *Proc. Natl. Acad. Sci. U.S.A.*, **1991**, 88(13), 5572-5576;
- [447] Turek J. J., et al., *J. Cell Sci.*, **1993**, 106(1), 423-430;
- [448] Leamon C. P., et al., *Drug Discov. Today*, **2001**, 6, 44-51;
- [449] Reddy J. A., et al., *Curr. Pharm. Biotechnol.*, **2005**, 6, 131-150;
- [450] Chen C., et al., *Nature*, **2013**, 500, 486-490;
- [451] Ebel W., et al., *Cancer Immun.*, 2007, 7:6.
- [452] Kalli K. R., *Curr. Opin. Investig. Drugs*, **2007**, 8(12), 1067-1073;
- [453] White A. J., et al., *J. Clin. Oncol.*, **2010**, 28(15); 2010 ASCO Annual Meeting Abstracts 2010: 5001;
- [454] Clinicaltrials.gov. Effectiveness of MORAb-003 in women with ovarian cancer who have relapsed after platinum-based chemotherapy. clinicaltrials.gov: Morphotek, 2012.
- [455] Clinicaltrials.gov. An efficacy and safety study of MORAb-003 in platinum-resistant or refractory relapsed ovarian cancer (FAR-122). clinicaltrials.gov:Morphotek, 2012.
- [456] Morphotek.. Exton, PA, 1-10-2013.
- [457] Vlahov I. R., et al., *Bioorg. Med. Chem. Lett*, **2006**, 16(19), 5093-5096;
- [458] Dosio F., et al., *Curr. Opin. Investig. Drugs*, **2010**, 11(12), 1424-1433;
- [459] Vlahov I. R., et al., *Bioconjug. Chem.*, **2012**, 23(7), 1357-1369;
- [460] Naumann R., et al., *16th International Meeting of the European Society of Gynaecological Oncology*, October 11-14, **2009**, Belgrade, Serbia;
- [461] Naumann R. W., et al., *J. Clin. Oncol.*, **2013**, 31(35):4400-6. doi: 10.1200/JCO.2013.49.7685;
- [462] Clinicaltrials.gov. Study for women with platinum resistant ovarian cancer evaluating EC145 in combination with Doxil® (PROCEED). Washington University School of Medicine; 2012.
- [463] Leamon C. P., et al., *Bioconjug. Chem.*, **2002**, 13, 1200-1210;
- [464] Fisher R. E., et al., *J. Nucl. Med.*, **2008**, 49(6), 899-906;
- [465] Symanowski J. T., et al., *J. Clin. Oncol.*, **2010**, 28(15).

### 1.10. The AIRC-DROC Targeting drug resistance in ovarian cancer Project

The AIRC-DROC “*Targeting drug resistance in ovarian cancer*” project has as main objective the optimization of peptides that inhibit hTS, in order to overcome the phenomenon of drug resistance in ovarian cancer.

Ovarian cancer, as previously described in *section 1.1.*, is particularly difficult to treat, and represents the most common cause of death from gynaecologic cancer in all-ages women in the Western World. This primarily reflects the fact that there are no early warning signs, and most patients with epithelial ovarian cancer present advanced stages (FIGO III and IV) at the time of diagnosis. Moreover, resistance to the first-line chemotherapy for ovarian cancer, based on different platinum/taxane combinations chemotherapy (*see section 1.1.5.*), often rapidly occurs. The mechanisms behind acquired resistance to cisplatin and its derivatives have not been elucidated yet, but it seems clear that it is a multifactorial process, which includes enhanced DNA repair (*see section 1.2.*). A common feature of DDP-resistant ovarian cancer cell lines, which was associated with cross-resistance to 5-FU and methotrexate, seems to be the over-expression of thymidylate synthase (TS), that is accompanied by an increase in the intracellular pools of 5, 10-methylenetetrahydrofolate and tetrahydrofolate.

The main clinical problem in OC treatment remains the therapy of platinum- resistant patients and efforts to improve survival focus on the development of more effective systemic and targeted therapies, as described in the *section 1.3*. In particular, as showed in *section 1.8.3.*, interest in exploiting the folate receptor (FR) for drug targeting applications has rapidly increased, since folate receptor (FR) represents both a biomarker and a potential target for tumour-selective drug delivery. In fact, targeted drug delivery systems promise to expand the therapeutic windows of drugs by increasing delivery to the target tissue as well as the target–non-target tissue ratio. In particular, using folate as the ligand, a wide variety of drug payloads can be delivered to FR-positive cells.

Recent studies on peptides specifically designed to bind the monomer–monomer interface of the enzyme (LR and its analogues), showed in *section 1.8.1.5.*, enlightened a novel mechanism of TS inhibition, since they stabilize the dimeric inactive form of the protein and inhibit cancer cell growth, without contemporary causing TS over-expression. The novel inhibition mechanism for these peptides was demonstrated through a combination of biophysical and computational approaches.

The innovative mechanism of action and potency of the mentioned compounds, suggested advances in the discovery process and the expectation of promising results. To forward the most active compounds to pre-clinical evaluation, two main strategies could be adopted: i) peptide optimization and development of peptidomimetic from the active peptides, ii) conjugation of the peptide/peptidomimetic with folic acid carrier, to deliver specifically (targeting) the candidate to the Folate Receptors (FR) membrane protein overexpressed in the OC membranes.

The ultimate aim of the AIRC-DROC project is to directly halt the tumour progression and interfere with the development of drug resistance upon treatment with platinum-derived drugs, by specifically inhibiting the protein regulatory function of TS on cancer cells through small molecule cellular perturbation.

Different expertises were combined (computational chemistry for peptide/peptidomimetic design, peptide chemistry, delivery studies, cellular and molecular biology, biophysical and structural approaches) to achieve the project objectives.

In order to overcome the intrinsic problems related to stability and delivery of peptide drugs, the peptides were optimized in peptidomimetics. To accomplish the purpose of delivering our TS –inhibitory peptides/peptidomimetics using a receptor–targeted drug delivery strategy, pterooate/folate-peptide conjugates were synthesized, purified, characterized and tested both in test tubes and in cell culture. To study a possible mechanism of action of LR peptides, a profiling of the effect of the peptide/peptidomimetic conjugation system on folate related pathway through Mass Spectrometry was performed. Finally, it was also performed a profiling of the biological effect of the designed conjugated system, on both sensitive and platinum-resistant OC cells.

### **1.11. Objectives of my PhD work**

My research work has been performed within the AIRC-DROC project. In particular I wanted to achieve the following objectives:

1. To study the biological efficacy of LR peptides on EOC cell lines;
2. To validate the proteomic profile obtained after the treatment of EOC cell lines with LR and its more active derivative;
3. To evaluate the FR $\alpha$  expression on EOC cell lines.

### **1.12. Work plan**

To achieve these aims I performed the following activities:

1. I tested the LR and its D isomer [DGln<sup>4</sup>]LR (LSC<sub>q</sub>LYQR ) derivative on 2008, C13\* cell lines;
2. I assessed the modulation of the profile of selected proteins on A2780, A2780/CP and IGROV1 cell lines;
3. Furthermore, to evaluate the efficiency of the FR $\alpha$ -targeted strategy for selectively delivering our peptides within ovarian cancer cells, a first-generation pteroyl-nonapeptide conjugated (PNPC) has been synthesised by the collaborator team of Ferrara University (Dr. Remo Guerrini and colleagues). In this context, I performed:
  1. the characterization of a panel of OC cell lines ( A2780, A2780/CP, 2008, C13\*, IGROV1, TOV112D, COV504, OAW28) for FR $\alpha$  expression and FA binding;
  2. the preliminary evaluation of the biological activity of the PNPC on ovarian cancer cells IGROV1, TOV112D, COV504 and OAW28.

## SECTION 2.

In this section I'll present the work performed in order to study the biological efficacy of LR peptides on EOC cell lines (*see section 1.11.1.*) and to validate the proteomic profile obtained after the treatment of EOC cell lines with LR peptides, (*see section 1.11.2.*). In this contest I tested the LR and its D isomer [DGln<sup>4</sup>]LR (LSCqLYQR) derivative on 2008, C13\* cell lines (*see section 1.12.1.*) and I assessed the modulation of the profile of selected proteins on A2780, A2780/CP and IGROV1 cell lines (*see section 1.12.2.*).

The results of the first objective are reported in the following articles:

- 1) *Optimization of peptides that target human thymidylate synthase to inhibit ovarian cancer cell growth*, Michela Pelà, Puneet Saxena, Rosaria Luciani, Matteo Santucci, Stefania Ferrari, Gaetano Marverti, Chiara Marraccini,<sup>2</sup> Andrea Martello, Silvia Pirondi, Filippo Genovese, Severo Salvadori, Domenico D'Arca, Glauco Ponterini, Maria Paola Costi, Remo Guerrini ; in press in the Journal of Medical Chemistry.
- 2) *An Integrated Proteomics and Bioinformatics Approach to the Definition of the pharmacodynamic profile of a Peptide inhibiting ovarian cancer growth.* ( submitted to Journal of Molecular and Cellular proteomics).

Each manuscript is presented through a n extensive summary, in order to introduce the publication context.

## **2.1. Introduction to the manuscript: Optimization of peptides that target human thymidylate synthase to inhibit ovarian cancer cell growth**

### **2.1.1. Background**

In the following article, in press in the Journal of Medical Chemistry, an alternative therapeutic approach for the treatment of ovarian cancer has been explored.

The first line treatment of this disease is based on platinum drugs (Pt drugs), that has a high percentage of response but also a high frequency of relapse due to the development of a Pt-resistant phenotype (*see section 1.1.5.*). In particular, since the over-expression of folate-dependent, as human thymidylate synthase (hTS) and dihydrofolate reductase (DHFR), is frequently observed in ovarian cancer cells resistant to platinum compounds, this treatment regimen may trigger the development of cross-resistance to anti-hTS drugs. Therefore, OCs that are resistant to Pt-drugs are also less sensitive to anti-hTS drugs. These conventional antifolate drugs bind the active site, inhibiting hTS activity and perturbing the feedback regulation of TS mRNA, which results in TS over-expression (*see sections 1.8.1.2. and 1.8.1.5.*). New drug candidates targeting hTS must utilize a different mechanism of action to prevent, or circumvent, resistance. Since hTS is an enzyme of the folate metabolic pathway, which exists in different states within the cell, including its active/inactive and dimeric/monomeric forms, peptides that specifically target the protein-protein interface of hTS to stabilize the inactive form of the enzyme have been designed. The inhibition mechanism for these peptides was elucidated through a combination of biophysical and computational techniques. Previous experiments demonstrated that the LR peptide inhibits intracellular hTS activity and cell growth without contemporary causing hTS over-expression, when administered to OC cells with a mechanism alternative to classical antifolates (*see section 1.6.1.5.*).

### **2.1.2. Aims**

The main objective of the present work was the development of more effective peptides, starting from a lead (LR) that showed a novel allosteric inhibition mechanism of hTS. In order to improve peptides interaction and allosteric inhibition of the protein target hTS, as well as the anticancer activity against OC cells, LR peptide optimization has been performed. LR-derived peptides were developed through a structure-activity-relationship (SAR) approach. A wide range of modifications has been performed, including site-specific mutation and truncation.

### 2.1.3. Results

The SAR study gave us many fundamental about the effect of peptide LR modification. For example, it has been observed that the C-terminal carboxylate group is essential for bioactivity, while the inversion of chirality at position 4 generated peptide [DGln<sup>4</sup>] LR showed increased activity compared to LR at the cellular level. Moreover, the Circular Dichroism (CD) analysis revealed that these mutations also affect the overall conformation of the peptide. In addition, Molecular dynamics (MD) studies, which highlighted the importance of secondary structure in inducing peptidic activities against human TS enzyme, agreed with CD experiments. MD studies indicated that for the activity it is important to maintain the structural rigidity of the peptide along with optimal geometry. In particular, the observation from these studies revealed that [DGln<sup>4</sup>] LR has higher structural rigidity, compared to peptides LR and [DCys<sup>3</sup>] LR, suggesting the importance of this conformational property to achieve higher affinity for the protein. In fact, hTS enzymatic assay showed that [DGln<sup>4</sup>] LR derivative has an increased potency than LR, in terms of inhibition of the activity. Moreover, among the screened peptides, only [DGln<sup>4</sup>] LR exhibited noticeable growth-inhibitory activity against four human ovarian carcinoma cell lines (2008; the about 13-fold cisplatin (cDDP)-resistant C13\* sub-line; A2780 and the about 10-fold cDDP-resistant parent cell line A2780/CP). The effectiveness of this peptide against cisplatin-resistant cells A2780/CP and C13\* was similar to the activity of LR and 5-FU, while [DGln<sup>4</sup>] LR displayed superior activity against the cDDP-sensitive A2780 and 2008 cells. These results suggest that [DGln<sup>4</sup>] LR also affects the cDDP-resistant phenotype.

In addition to the improved biological activity compared to LR, [DGln<sup>4</sup>] LR exhibited the same modulation of the folate-dependent proteins, suggesting similar cellular behavior for the two peptides. In particular, immunoblot analysis on 2008 and C13\* ovarian cancer cell lines, after 48 h treatment with the two peptides, showed no significant changes in TS protein levels. In contrast, the total hTS content increased in both cell lines after treatment with 5-FU. Furthermore, [DGln<sup>4</sup>] LR induced a decrease in the DHFR protein level, similarly to LR. Therefore, quantitative assessment of the TS mRNA levels in these OC cells, treated with both peptides, was performed after a 48 h treatment. Results showed that TS mRNA increased in the sensitive cells after treatment with 5-FU and also, to a lesser extent, with LR and [DGln<sup>4</sup>] LR. In resistant cells, instead, TS mRNA levels remained nearly constant after treatment with 5-FU and with both peptides. Both LR and [DGln<sup>4</sup>] LR inhibited TS activity by approximately 30–50% at the concentrations evaluated, particularly in the sensitive 2008 cell line; these results were in agreement with previously published observations. This finding

is consistent with the new mode of action proposed for these peptides: unlike 5-FU, the peptides do not cause the formation of an inactive ternary complex with the protein. Thus, our results suggest the same mechanism of action to LR and [DGln<sup>4</sup>] LR, which differs from the mechanism of action of the classical antifolate.

#### **2.1.4. Conclusions**

In the present work, the LR lead optimization is combined with the evaluation of the intracellular modulation of two proteins: the target, TS, and an associated protein, DHFR (Figure 2.1.4.1.). In order to improve LR interaction with the protein target hTS and its anticancer activity against OC cells, its peptidic derivatives were modified through a structure-activity-relationship approach. In particular, the molecular dynamics studies were in agreement with circular dichroism experiments, indicating that [DGln<sup>4</sup>] LR higher structural rigidity is an important conformational property to achieve higher affinity for the protein. In fact, cytotoxicity studies against ovarian cancer cell lines demonstrated the general improved effectiveness of [DGln<sup>4</sup>] LR compared to LR and 5-FU. Indeed, western blot analysis showed that the intracellular TS and DHFR modulation pattern is the same for [DGln<sup>4</sup>] LR and LR, and both differ from 5FU's one. Studies are ongoing to elucidate the mechanism of action for these peptides at the cellular level.

Studies on chemical and biological stability were carried out by colleagues of University of Modena and Reggio Emilia. In particular, a quantitative LC-MS/MS method for high sensitivity LR peptide quantitation in cell lysates has been developed (manuscript in preparation). Briefly, results demonstrated that LR peptide is stable in both aqueous solution and cell lysates for at least 8 hours; moreover, the method allowed the detection and quantification of the peptide within the cells after different incubation time sets. Best results in terms of quantity were obtained incubating LR-Saint PhD complex with C13\*ovarian cancer cells lines for four hours at 37°C. Thus, these findings were fundamental for a deeper understanding of LR peptide biological effect.

Experimental procedures and more detailed information have been described in detail in the full version of the article reported later in *section 2.1.5*.

During my PhD work, I contributed to understand the biological activity of LR peptide and [DGln<sup>4</sup>]LR derivative in ovarian cancer cell lines by cell growth assay, Western Blot Analysis and qPCR evaluation of TS mRNA after treatment with LR peptides.

A panel (Figure 2.1.4.1.) is reported below as a conceptual summary of the results.

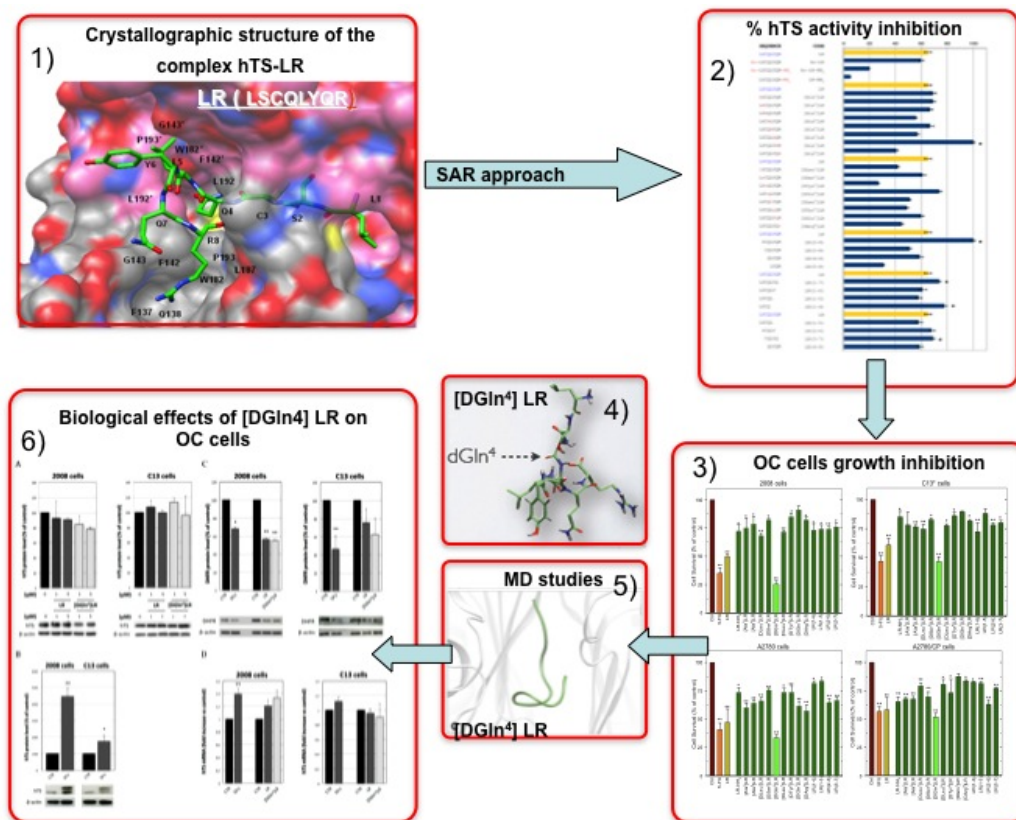


**Figure 2.1.4.1.** In this panel, the main results of the studies on the peptide LR derivatives obtained by a SAR study are described. Crystallographic structure of the complex hTS-LR, shown in Figure 1, was the starting point for the development of a panel of derivatives of the LR peptide. All novel compounds were tested using the hTS enzymatic assay, shown in Figure 2, for primary screening. Next, peptide cytotoxicity, shown in Figure 3, was evaluated in four human ovarian carcinoma cell lines: these studies demonstrated that [DGln<sup>4</sup>] LR, which presents an inversion of the chirality at position 4, has a general improved effectiveness compared to LR and 5-FU (% of inhibition cell growth average at 10  $\mu$ M : was about 55% for 5-FU, 50% for LR and 60% for [DGln<sup>4</sup>] LR). Furthermore, molecular dynamics (MD) studies were performed to analyse the conformational properties of LR derivatives. As shown in Figure 4, Molecular dynamics predictions suggest that the higher structural rigidity of [DGln<sup>4</sup>] LR, compared to LR, determines also a higher affinity for the protein.

Within these premises, I assessed the biological effect of LR and [DGln<sup>4</sup>] LR. Evaluation of TS/DHFR expression in cells treated with [DGln<sup>4</sup>] LR (Figure 6) showed that the TS protein levels did not significantly vary and the DHFR protein levels decreased. So, the intracellular TS and DHFR modulation pattern is the same for [DGln<sup>4</sup>] LR and LR, and both differ from 5FU's one.

# Optimization of Peptides That Target Human Thymidylate Synthase to Inhibit Ovarian Cancer Cell Growth

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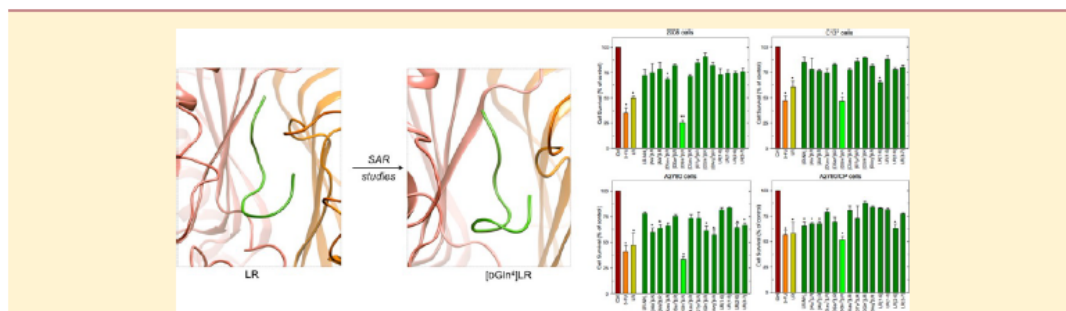
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13 **S** Supporting Information



**ABSTRACT:** Thymidylate synthase (TS) is a target for pemetrexed and the prodrug 5-fluorouracil (5-FU) that inhibit the protein by binding at its active site. Prolonged administration of these drugs causes TS overexpression, leading to drug resistance. The peptide lead, LR (LSCQLYQR), allosterically stabilizes the inactive form of the protein and inhibits ovarian cancer (OC) cell growth with stable TS and decreased dihydrofolate reductase (DHFR) expression. To improve TS inhibition and the anticancer effect, we have developed 35 peptides by modifying the lead. The D-glutamine-modified peptide displayed the best inhibition of cisplatin-sensitive and -resistant OC cell growth, was more active than LR and 5-FU, and showed a TS/DHFR expression pattern similar to LR. Circular dichroism spectroscopy and molecular dynamics studies provided a molecular-level rationale for the differences in structural preferences and the enzyme inhibitory activities. By combining target inhibition studies and the modulation pattern of associated proteins, this work avenues a concept to develop more specific inhibitors of OC cell growth and drug leads.

## INTRODUCTION

Human thymidylate synthase (hTS) plays a key role in DNA synthesis and is a good target for cancer treatment because it is the only de novo source of 2'-deoxythymidine-5'-monophosphate. In many cancers, including colorectal cancer, mesothelioma, and ovarian cancer, hTS is overexpressed. Therefore, hTS has become a drug target for several clinically relevant anticancer drugs, such as raltitrexed, pemetrexed, and the prodrug 5-fluorouracil (5-FU).<sup>1</sup> This protein is part of the folate metabolic pathway, in which folate metabolites may function as substrates or cofactors.<sup>2</sup> hTS exists in different states within the cell, including its active/inactive and dimeric/monomeric forms. As a dimer, hTS catalyzes the reaction

converting 2'-deoxyuridine-5'-monophosphate (dUMP) to 2'-deoxythymidine-5'-monophosphate (dTMP) and is assisted by N<sup>5</sup>,N<sup>10</sup>-methylenetetrahydrofolate (MTHF) as a cofactor. In addition to its catalytic role, hTS has been shown to regulate protein synthesis by interacting with its own mRNA as well as the mRNAs of several other proteins involved in the cell cycle.<sup>3–6</sup> The regulatory function of hTS as an mRNA-binding protein has been shown to be maximal when the protein is ligand-free.<sup>7</sup> This observation, together with the observation that cancer cells resistant to anti-hTS drugs showed increased

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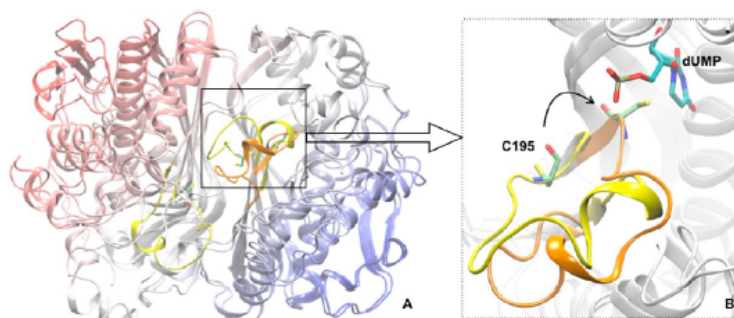


**Figure 1.** Homodimeric forms of hTS enzyme. The figure describes the structural changes incorporated by the protein during binding with the substrate molecules. In yellow is the inactive state of the enzyme (PDB id: 1HW3) whereas in orange is the active form (PDB id: 1HVV). On the right is the flexible loop running from residue Ala181–Ala197 (highlighted in orange/yellow). The loop turns approximately  $180^\circ$  while interacting with the substrate. This topographical change makes the catalytic Cys195 move into its new location by a distance of around 10 Å. One letter code for the residues is shown to maintain the clarity of the image.

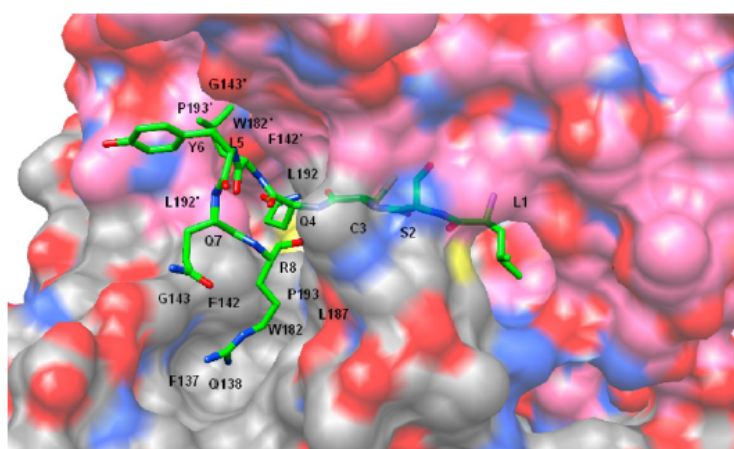


**Figure 2.** Crystallographic structure of the complex hTS–LR. Protein is represented as surface; LR peptide is represented in stick. All residues are colored by atom: O atom in red; N atom in blue; S atom in yellow; C atom, respectively, in gray in monomer A of hTS, in pink in monomer B of hTS, in green in LR peptide. One letter code for the residues is shown to maintain the clarity of the image.

47 levels of hTS, led to the suggestion that the overexpression of  
48 hTS is correlated with the loss of mRNA regulatory capacity  
49 when the protein is bound to its inhibitors.<sup>4,6,8,9</sup> In the case of  
50 5-FU, a covalent hTS inhibitor, high hTS protein levels were  
51 attributed to both mRNA regulation and a decrease in enzyme  
52 degradation efficiency caused by inhibitor binding.<sup>10,11</sup> These  
53 studies were performed on colon cancer cells, but clinical and  
54 biochemical observations suggest that a similar behavior occurs  
55 in other cancer types, such as ovarian cancer.

56 Usually anticancer drug design aims at searching for broad-  
57 spectrum anticancer agents; however, some intrinsically less  
58 sensitive cancer types such as ovarian cancer<sup>12</sup> are often  
59 neglected and become “orphan diseases” (<http://www.orpha.net/>; <http://rarediseases.info.nih.gov/gard>). At variance with  
61 this approach, in our work, we focused on ovarian cancer. Its  
62 first line treatment is based on platinum drugs (Pt drugs) that  
63 may cause the development of a Pt drugs resistant phenotype  
64 with increased concentration of hTS and other related folate-  
65 dependent proteins, such as dihydrofolate reductase (DHFR);

therefore, this treatment regimen may trigger the development  
66 of cross-resistance to anti-hTS drugs.<sup>13</sup> Consequently, ovarian  
67 cancers (OCs) that are resistant to Pt drugs are also less  
68 sensitive to anti-hTS drugs.<sup>14</sup> New drug candidates targeting  
69 hTS must utilize a different mechanism of action to prevent or  
70 circumvent resistance. Therefore, we have designed peptides  
71 that specifically target the protein–protein interface of hTS to  
72 stabilize the inactive form of the enzyme.<sup>15</sup> The inhibition  
73 mechanism for these peptides was elucidated through a  
74 combination of biophysical and computational techniques.<sup>15</sup>  
75 The X-ray crystal structure of LR (primary sequence: LSCQLYQR)  
76 peptide in complex with hTS unambiguously  
77 confirms that LR binds at the monomer–monomer interface.  
78 The two enzyme monomers must move apart to allow peptide  
79 binding. Unlike the above quoted drugs that target hTS, these  
80 peptides inhibit intracellular hTS and cell growth without  
81 causing hTS overexpression when administered to OC cells.<sup>15</sup>  
82 Crystallographic studies reported in the Protein Data Bank  
83 (PDB id: 1HVV) indicate that the enzyme is homodimeric and  
84

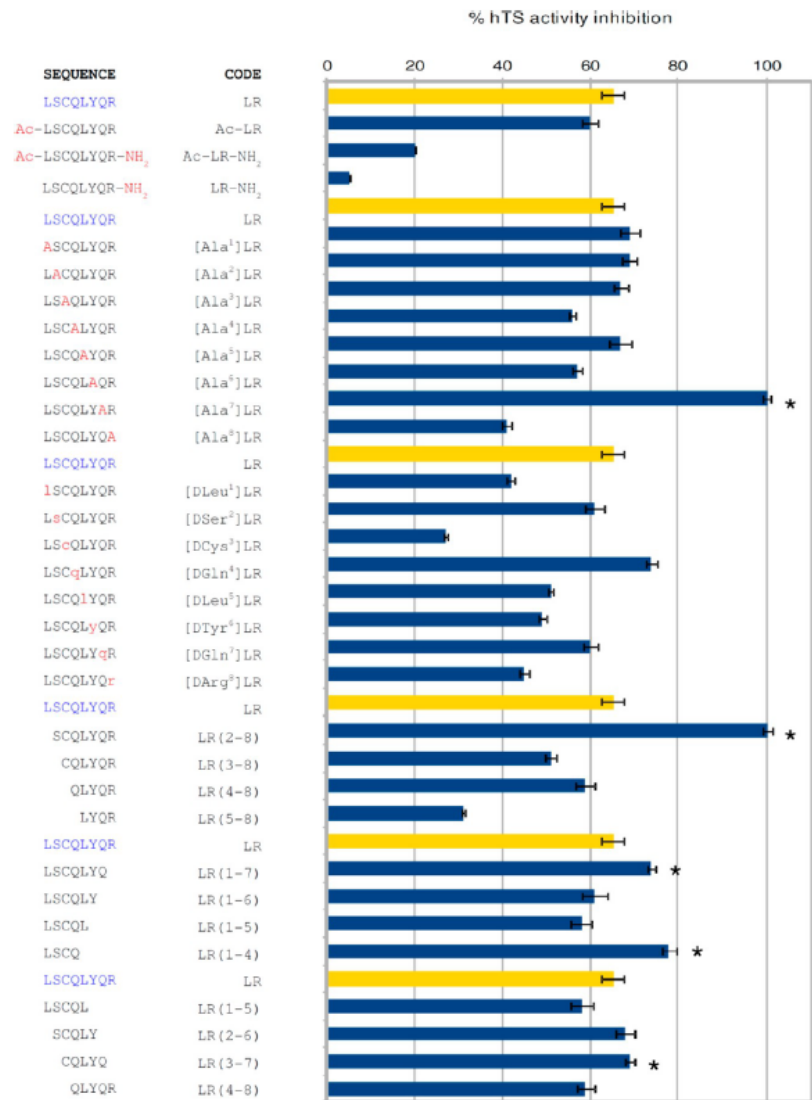


**Figure 3.** hTS activity inhibition percentages (I%) values measured at 100  $\mu$ M concentration of inhibitor. I% values are expressed as mean  $\pm$  SEM;  $n = 3$ . Reference peptide (LR) is colored in yellow. \*Data extrapolate from I% = 60 at 50  $\mu$ M for [Ala<sup>7</sup>]LR; I% = 54% at 50  $\mu$ M for LR(2–8); I% = 56% at 75  $\mu$ M for LR(1–7); I% = 59% at 75  $\mu$ M for LR(1–4); I% = 52% at 75  $\mu$ M for LR(3–7).

that it is in the inactive form when unbound and in the active form when bound to a substrate<sup>16,17</sup> (Figure 1). In its crystallographic binding site, LR binds in an inverted-cone-shaped cleft at the interface between the two monomers. The LR-peptide residues, Leu<sup>1</sup>-Cys<sup>3</sup>, are buried by the Met190, Ala191, and Leu192 side chains of subunit A, which participates in hydrophobic interactions with the Phe142' and Val158' of subunit B in the free protein dimer. The LR-peptide residues Gln<sup>4</sup>, Leu<sup>5</sup>, and Arg<sup>8</sup> bind in lipophilic pockets, whereas Tyr<sup>6</sup> and Gln<sup>7</sup> from the LR-peptide are oriented toward the outside of the protein into the solvent (Figure 2).<sup>15</sup>

On the basis of previous findings, we began a discovery program to explore LR-derived peptides and examined the main structural features responsible for the peptide–hTS binding interaction and the resulting allosteric inhibition. For this, we started with a structure–activity relationship (SAR) study of the peptides. Structural studies were performed on the most interesting peptides by circular dichroism (CD) spectroscopy. Molecular dynamics (MD) studies, carried out on the peptide–hTS complex, provided a rationale for the conformational preferences observed by CD as well as a molecular-level interpretation of differences in inhibitory activity.

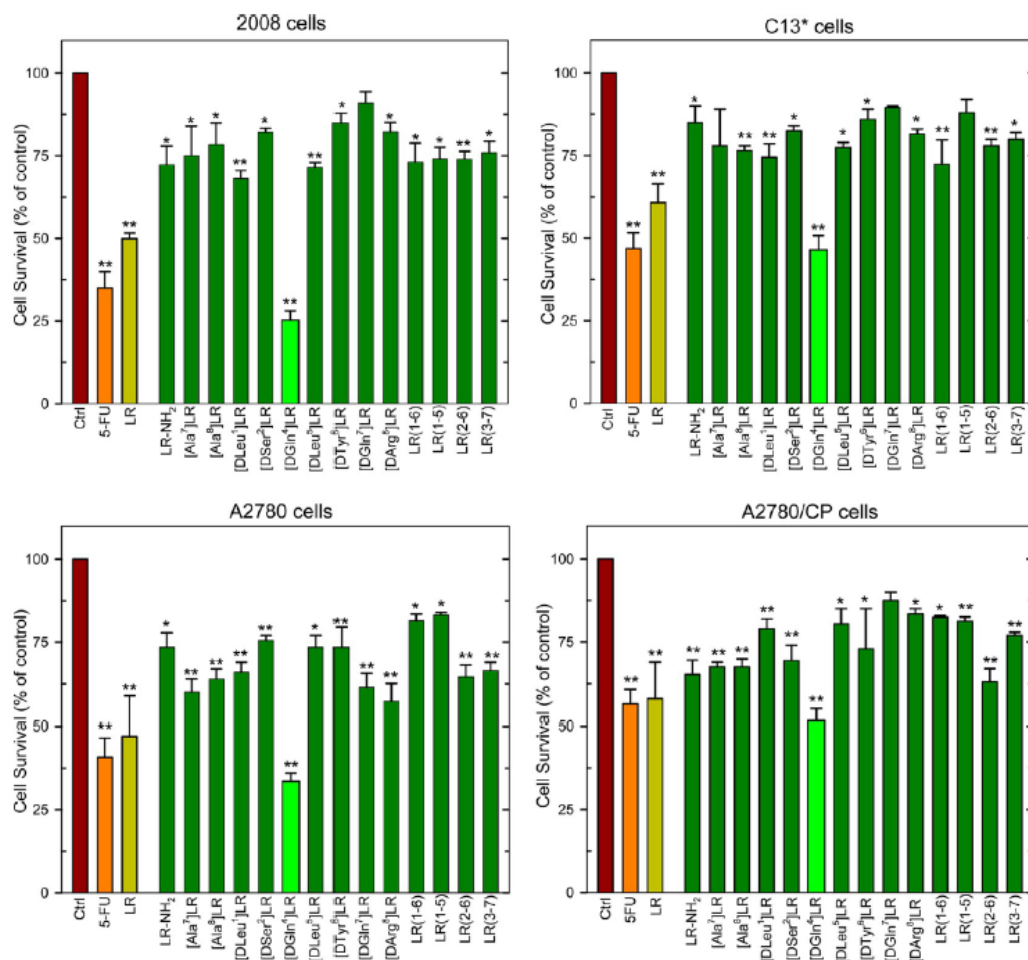


**Figure 4.** Inhibition of cell growth by the LR peptide and its derivatives for cisplatin-sensitive and -resistant cells. The peptides were transfected into 2008 and C13\* cells (upper panels) and A2780 and A2780/CP cells (lower panels) via a peptide delivery system. The effects of 10  $\mu$ M peptides was compared to the same concentration of 5-FU. Cell survival percentages are the mean  $\pm$  SEM of at least three separate experiments performed in duplicate. Statistical significance was estimated by ANOVA followed by Dunnett's post hoc multiple comparison test; a difference was considered to be significant at \* $P$  < 0.05 or \*\* $P$  < 0.01.

The peptides were tested against four cisplatin-sensitive and -resistant OC cell lines. Of the compounds synthesized, [DGln<sup>1</sup>]<sub>1</sub>LR demonstrated superior biological activity relative to the initial lead and exhibited the same modulation of the folate-dependent proteins, suggesting similar cellular behavior.

## RESULTS AND DISCUSSION

**Design of the LR-Peptide Derivatives.** A SAR study of the parent LR peptide was performed with the aim of finding peptides with improved affinity over LR. This study utilized general strategies based on structural modifications to the peptides<sup>18</sup> such as N-terminal acetylation, C-terminal amidation, N- and C-terminal truncation, and pentapeptide scanning to identify the minimal bioactive peptide sequence; additionally, D- and alanine scanning were applied to the entire peptide sequence. N- and C-terminal masking provides information

regarding whether the integrity of the carboxylate and amino terminal functionalities is necessary for bioactivity. Alanine scanning consists of replacing one residue at a time from the primary peptide sequence with a neutral Ala side chain. Likewise, the replacement of each residue in the sequence by its optical isomer (D-scanning) reportedly promotes changes in the peptide conformation and can provide valuable information regarding the specific stereochemical requirements at each position of the peptide sequence as well as the sites that contribute to the preferred conformations. Finally, N- and C-terminal truncation was performed by deleting amino acid residues; pentapeptide scanning studies were performed by sequentially reducing the lead peptide length to five residues and can assist in identifying the minimal bioactive peptide sequence<sup>19</sup> (Figure 3).

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All novel compounds were tested using the hTS enzymatic assay for primary screening. N-terminal acetylation (Ac-LR) was less detrimental than C-terminal amidation (LR-NH<sub>2</sub>) (Figure 3, Table 1S, Supporting Information), suggesting that the C-terminal carboxylic function was important for bioactivity. Alanine scanning from [Ala<sup>1</sup>]LR to [Ala<sup>6</sup>]LR (Figure 3, Table 1S, Supporting Information) generated LR analogues with potencies comparable to that of the lead compound (the enzymatic activity inhibition percentages (1% at 100 μM were 56–69% compared to 65% for unmodified LR). [Ala<sup>7</sup>]LR exhibited higher activity, while a decrease in bioactivity was shown by [Ala<sup>8</sup>]LR ([Ala<sup>7</sup>]LR 1% at 50 μM was 60% and [Ala<sup>8</sup>]LR 1% at 100 μM was 41%).

The D-scanning data reported in Figure 3 (Table 1S, Supporting Information) indicates that the chirality of many residues was important for bioactivity. All derived peptides except [DGln<sup>4</sup>]LR were less active than LR (1% values at 100 μM were 74% for [DGln<sup>4</sup>]LR and 27–61% for the other D-modified peptides). The inversion of chirality at position 3, [DCys<sup>3</sup>]LR, drastically reduced hTS inhibition (1% at 100 μM was 27%). Replacing L-amino acids with D-amino acids at positions 2 and 7 (1% values at 100 μM were 61% and 60%, respectively) generated derivatives that were only slightly less active than LR. Therefore, the chirality of these residues was not critical to the bioactivity. However, inverting the chirality at position 4 to yield [DGln<sup>4</sup>]LR increased the potency relative to LR (1% at 100 μM was 74%). In addition, the enantiomer of LR (containing only D-amino acids, LR-D, see Table 1S, Supporting Information) did not inhibit hTS at 100 μM, confirming that a stereoselective interaction occurs between LR and hTS.

The data obtained from the N- and C-terminal truncation approach (Figure 3 and Table 1S, Supporting Information) indicated that shortening LR was well tolerated at both the N and C termini. The deletion of three amino acids from either the N or C terminus (see Figure 3; for example, LR(4–8) and LR(1–5)) were well tolerated for the active LR analogues. In addition, pentapeptide fragments from the middle portion of the LR (LR(2–6) and LR(3–7)) inhibited hTS with potency comparable to that of the full-length peptide. The deletion of Leu<sup>1</sup> in LR(2–8) generated a more potent analogue than LR (LR(2–8), (see Figure 3). Collectively, the in vitro data suggest that the full-length peptide was unnecessary for LR-promoted hTS inhibition. However, no evidence was obtained that the shorter peptides utilized the same binding site as LR. C-terminal amidation and the inversion of chirality at positions 3 and 4 induced the most dramatic change in the hTS activity with the smallest chemical modifications.

**OC Cell Growth Inhibition.** Peptide cytotoxicity was evaluated in four human ovarian carcinoma cell lines: 2008; the about 13-fold cisplatin (cDDP)-resistant C13\* subline; A2780; and the about 10-fold cDDP-resistant parent cell line A2780/CP. The cytotoxicity values were calculated by comparing cultures exposed to the drug to untreated (control) cultures. After treatment for 72 h, the cell growth inhibitory activity exhibited by the peptides was determined using a crystal violet dye assay; the dye extracted from the assay was proportional to the cellular biomass. The tested compounds were administered to cancer cells by exploiting a specific peptide delivery system that did not alter cell growth.<sup>15</sup>

The effectiveness of the peptides was compared to the activity of a known hTS inhibitor, 5-FU, and the lead LR peptide.<sup>15</sup> Most peptides inhibited cell growth less than 5-FU

and LR. However, while some peptides significantly reduced the survival of some cell lines, only [DGln<sup>4</sup>]LR exhibited notable growth-inhibitory activity against all four cell lines when compared to untreated control cells (Figure 4 and Table 2S, Supporting Information). The effectiveness of this peptide against A2780/CP and C13\* was similar to the activity of LR and 5-FU; [DGln<sup>4</sup>]LR displayed superior activity against the cDDP-sensitive A2780 and 2008 cells. Slightly less than 50% of the resistant cells survived the treatment with [DGln<sup>4</sup>]LR, while only 25–30% of the sensitive parental lines survived, suggesting that [DGln<sup>4</sup>]LR also affects the cDDP-resistant phenotype.

**Structural Studies of the LR Derivatives. CD Spectroscopy.** We have previously shown on a set of eight octapeptides that the propensity to assume a secondary structure correlated with the inhibitory activity versus hTS.<sup>15</sup> Such a correlation is usually interpreted as connected with an increased affinity of ordered ligands versus proteins as compared with disordered ones due to decreased entropy loss in binding.<sup>20–25</sup> In order to assess the intrinsic propensity of the peptides to arrange in a secondary-structural motif, we measured their CD spectra in water and in the structure-inducing solvent, 2,2,2-trifluoroethanol (TFE). This information was then compared with the findings of MD experiments.

For these studies, we selected LR, its inactive C-terminal amidated analogue LR-NH<sub>2</sub>, and the two D mutants that exhibited the largest variations in activity with respect to LR; the D mutants were [DCys<sup>3</sup>]LR, which exhibited a decreased 1% value (1% at 100 μM was 27% compared to 65% for LR), and [DGln<sup>4</sup>]LR, which displayed the best 1% value (1% at 100 μM was 74%). LR and [DGln<sup>4</sup>]LR were also selected because they were the most potent inhibitors of OC cell growth. The CD spectra (Figure 5) of LR and LR-NH<sub>2</sub> in water display negative bands with minima at 195–196 nm and similar Δε values near −4 M<sup>−1</sup> cm<sup>−1</sup>, indicating that these peptides are largely unordered in water. However, [DCys<sup>3</sup>]LR exhibits a preference for a secondary structure in water, as indicated by a weaker, blue-shifted (minimum at 191–192 nm) negative band. This

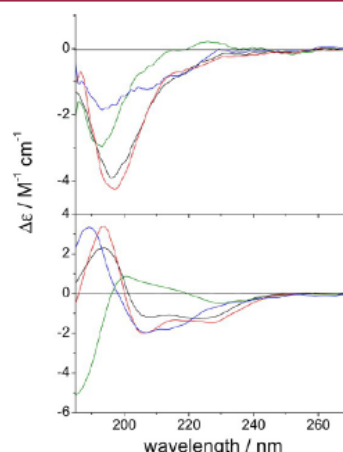


**Figure 5.** CD spectra of peptides LR (black), LR-NH<sub>2</sub> (red), [DCys<sup>3</sup>]LR (green), and [DGln<sup>4</sup>]LR (blue) in water (top) and in 2,2,2-trifluoroethanol. Δε = ε<sub>L</sub> − ε<sub>R</sub>; M = moles of amino acids per liter.

spectral change suggests that some of the [DCys<sup>3</sup>]LR molecules feature a class B spectrum,<sup>26</sup> which is characterized by a positive CD in the 200–225-nm region.

Class B CD spectra are frequently,<sup>26,27</sup> but not exclusively,<sup>28</sup> assigned to type II  $\beta$  turns (a structural characterization of  $\beta$  turns can be found in the literature<sup>28</sup>). This secondary structure preference is demonstrated more clearly by [DCys<sup>3</sup>]LR in TFE (lower panel in Figure 5). In water, [DGln<sup>4</sup>]LR also exhibits a perturbation in unordered peptide spectral features, which is consistent with the presence of a significant fraction of structured molecules. However, for this compound, the pronounced compensation of the negative band from the parent LR below 205 nm suggests an  $\alpha$ -helical contribution. The spectrum of this peptide in TFE indicates that it adopts an  $\alpha$ -helical structure. The same conclusion applies to both LR and LR-NH<sub>2</sub> in TFE. The spectra of these peptides and the data for a typical  $\alpha$  helix differ only slightly; a shift in the positive band between 190 and 195 nm and an unusually shaped negative band in the 220–230 nm region are observed. These perturbations may arise from different contributions from the high-energy transitions of the tyrosine side chain in the CD spectrum.<sup>29</sup> The CD spectra indicate that different conformational behavior occurs in the [DCys<sup>3</sup>]LR and [DGln<sup>4</sup>]LR peptides; the former prefers a  $\beta$ -turn conformation, and the latter prefers an  $\alpha$ -helical conformation.

**Conformational Characterization of the Peptides by MD Experiments.** According to the results generated through the D-scanning of the LR peptide, changing the enantiomeric form of some of the constituent amino acids affects the overall conformation of the peptide and therefore modulates its inhibition potency against hTS. To investigate the structural/dynamic consequences of L-to-D mutation at Cys<sup>3</sup> and Gln<sup>4</sup>, we performed MD studies on hTS binary complexes with the LR, [DCys<sup>3</sup>]LR, and [DGln<sup>4</sup>]LR peptides.

When complexed with hTS, the latter two peptides adopted different conformations (Figure 6). Due to the inverted-cone geometry of the dimeric interface region in hTS, the N-terminal residues (Leu<sup>1</sup>-Ser<sup>2</sup>-Cys<sup>3</sup>) of three peptides pack tightly into the top of the narrow cavity, while the C-terminal residues (Gln<sup>4</sup>-Leu<sup>5</sup>-Tyr<sup>6</sup>-Gln<sup>7</sup>-Arg<sup>8</sup>) remain in the broader portion of the cavity. Therefore, the C terminus is more exposed to the

solvent and relatively more mobile. Furthermore, the hydrophobic residues (Leu<sup>5</sup>-Tyr<sup>6</sup>) induce the formation of a prominent turn (or bend) in this region. During the MD trajectory analysis for the unbound LR peptide, the backbone atoms of the residues do not engage in hydrogen bonding, and the peptide adopts an expanded geometry (Figure 7D). The MD simulations for [DCys<sup>3</sup>]LR and [DGln<sup>4</sup>]LR revealed the formation of hydrogen bonds between Gln<sup>4</sup>-Gln<sup>7</sup> ( $n, n + 3$ ) in the former and Gln<sup>4</sup>-Arg<sup>8</sup> ( $n, n + 4$ ) in the latter; these types of features are characteristic of  $\beta$  and  $\alpha$  turns, respectively.<sup>30,31</sup> These conclusions were further validated using the DSSP program<sup>31</sup> that determines the secondary structure based on the existing hydrogen bonds (Table 1).<sup>32</sup> The results obtained from the MD are thus consistent with the CD results and explain the different CD behaviors observed in the three peptides. Specifically, the analysis of the relevant dihedral angles<sup>28</sup> obtained via the MD simulation indicate that the [DCys<sup>3</sup>]LR peptide prefers a type II'  $\beta$ -turn conformation. Other structural components, such as the radius of gyration ( $R_g$ ) and the conformational rigidity, were examined to understand the dynamic behavior of the three peptides on the nanosecond time scale. To estimate the compactness of the three peptides, we calculated the  $R_g$  for each peptide using the g\_gyrate utility and observed the differences between the  $R_g$  values that were directly related to the conformations adopted by the peptides. The LR peptide had the largest  $R_g$  value corresponding to the least compact structure; this value was validated by the absence of intramolecular hydrogen bonding. The expanded geometry of the LR peptide was maintained throughout the dynamics experiment (Figure 7D). However, large fluctuations in the  $R_g$  value were observed for [DCys<sup>3</sup>]LR and [DGln<sup>4</sup>]LR. The plotted  $R_g$  values (Figure 7D) indicate that [DCys<sup>3</sup>]LR (Figure 7B) has a more compact geometry than [DGln<sup>4</sup>]LR because the  $\beta$  turn (four residues involved) of the former is smaller than the  $\alpha$  turn (five residues involved) of the latter.

To examine the rigidity of the three peptides, we calculated the root-mean-square deviation (rmsd) of the backbone  $\alpha$  atoms from the residues forming the 8-mer peptides during 5 ns simulations. While the rmsd of the LR drifted gradually (Figure 8A), the rmsd of the [DCys<sup>3</sup>]LR exhibited rapid fluctuations, especially at approximately 2000 ps (Figure 8B). For [DGln<sup>4</sup>]LR (Figure 8C), the rmsd became more stable after an initial rise at approximately 200 ps. The calculated interaction energies between the turn-governing residues in [DCys<sup>3</sup>]LR and [DGln<sup>4</sup>]LR (highlighted in Figure 6) suggests that the binding strength between the turn-forming residues directly impacts the conformational stability of the entire peptide. The data in Figure 8D indicate that the Gln<sup>4</sup>-Arg<sup>8</sup> interaction energy in [DGln<sup>4</sup>]LR reaches a stable value (−40 kJ/mol) in the same interval required to form an  $\alpha$  helix. Conversely, the interaction energy between Gln<sup>4</sup> and Gln<sup>7</sup> of [DCys<sup>3</sup>]LR drops to −10 kJ/mol while the  $\beta$  turn is destabilized.

From the MD study, we observed that [DGln<sup>4</sup>]LR adopts a conformation more suitable for embedding into the interface region and maintains its structural rigidity throughout the dynamics experiment. Such a structural feature of [DGln<sup>4</sup>]LR might provide an explanation to its better activity with respect to both [DCys<sup>3</sup>]LR and LR, where the former attains a shrunken geometry while the latter is more expanded.

**Role of the C-Terminal Carboxylic Group of Arg<sup>8</sup>.** Analysis of the LR-peptide interactions with the hTS interface residues

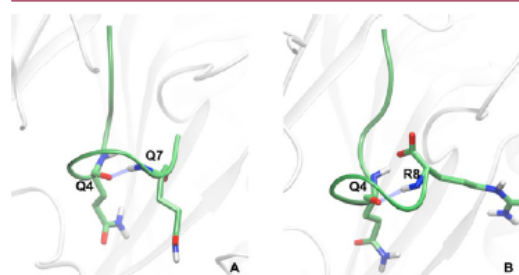


**Figure 6.** View of the tendency of the peptide (drawn in New cartoon, in lime color) amino acids (colored by atom with in licorice; C atoms in lime color) to indulge in the hydrogen-bond formation. (A) In [DCys<sup>3</sup>]LR, the carboxyl group of Gln<sup>4</sup> makes a hydrogen bond with the NH group of Gln<sup>7</sup>, displaying  $n, n + 3$  conformations leading to a  $\beta$  turn. (B) In [DGln<sup>4</sup>]LR, Gln<sup>4</sup> is again found to hydrogen bond but with Arg<sup>8</sup>, showing the existence  $n, n + 4$  conformation of an  $\alpha$  turn. One letter code for the residues is shown to maintain the clarity of the image. The H bonds are shown as blue lines.

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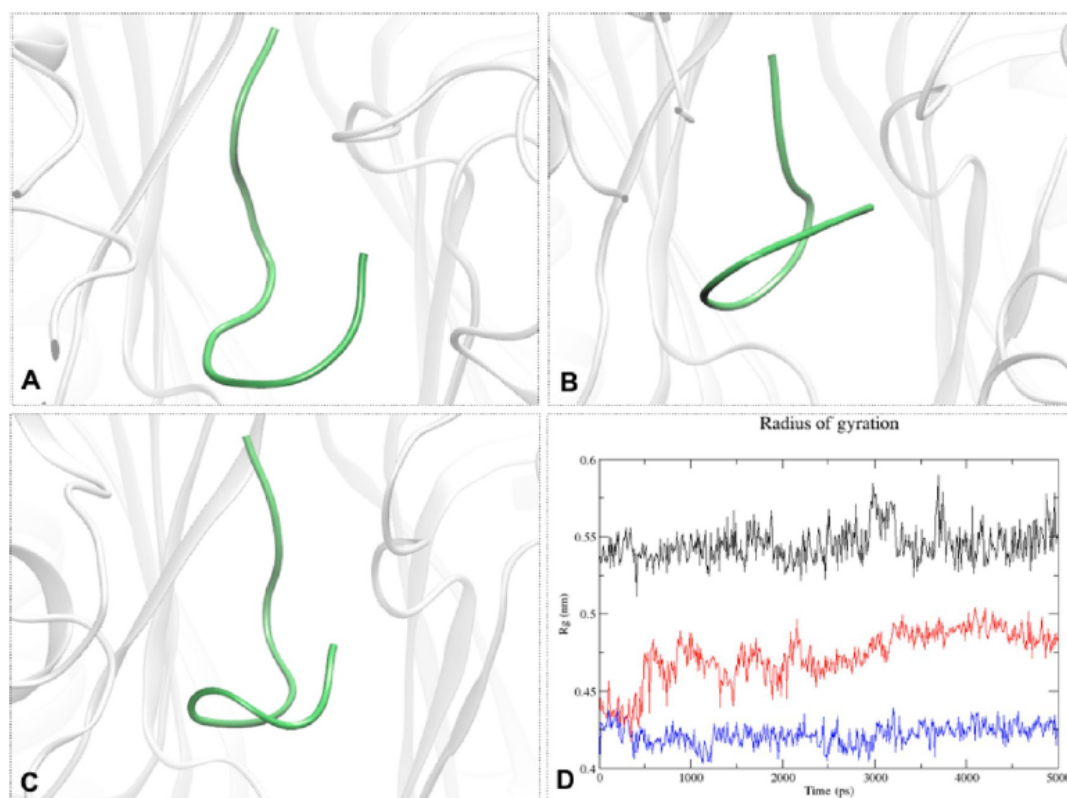


**Figure 7.** Pictorial representation of the structures obtained by the peptides (in lime) from 5 ns MD run. (A) LR attains the stretched-out geometry at the interface of the dimeric protein, whereas (B) [DCys<sup>3</sup>] LR collapses into a close-packed turn due to a hydrogen bond between Gln<sup>4</sup> and Gln<sup>7</sup>. (C) In the case of [DGN<sup>4</sup>]LR, the turn is uniform and comparatively broader (of four residues) lying between Gln<sup>4</sup> and Arg<sup>8</sup>. The  $R_g$  values for the LR peptide in black, [DGN<sup>4</sup>]LR in red, and [DCys<sup>3</sup>]LR in blue are plotted in (D), which further encourage the deductions drawn from the trajectory analysis.

**Table 1. Sequence Characterization Performed by DSSP Program of the Three Peptides: LR, [DCys<sup>3</sup>]LR, [DGln<sup>4</sup>]LR<sup>a</sup>**

|            | peptide |   |   |   |   |   |   |   |                        |   |   |   |   |   |   |   |                        |   |   |   |   |   |   |   |
|------------|---------|---|---|---|---|---|---|---|------------------------|---|---|---|---|---|---|---|------------------------|---|---|---|---|---|---|---|
|            | LR      |   |   |   |   |   |   |   | [DCys <sup>3</sup> ]LR |   |   |   |   |   |   |   | [DGln <sup>4</sup> ]LR |   |   |   |   |   |   |   |
|            | L       | S | C | Q | L | Y | Q | R | L                      | S | C | Q | L | Y | Q | R | L                      | S | C | Q | L | Y | Q | R |
| amino acid |         |   |   |   |   |   |   |   |                        |   |   |   |   |   |   |   |                        |   |   |   |   |   |   |   |
| sec struct |         |   |   |   | S | S | S |   | S                      |   |   | S | T | T | S |   | S                      | T | T | T | T | T | T | T |
| H-bond pos |         |   |   |   |   |   |   |   |                        |   |   | > | 3 | 3 | < |   |                        |   |   | > | 4 | 4 | 4 | 4 |

"S" = bend; "T" = turn; ">" denotes backbone CO of this residue makes H bond ( $i, i + n$ ); "<" denotes backbone NH of this residue makes H bond ( $i, i - n$ ); "3" "4" = residues bracketed by H bond; sec struct = secondary structure; H-bond pos = hydrogen-bond position.

revealed that the carbonyl oxygen of the Gln<sup>7</sup> side chain forms a hydrogen bond with the amide nitrogen of the catalytic loop residue (Leu192) in monomer B early in the dynamics experiment (Figure 9A). However, the Gln<sup>4</sup> amine group is oriented roughly perpendicular to the indole ring of the Trp182 within the catalytic loop of monomer A (Figure 9B). Because the configuration of the LR peptide is extended, the carbonyl oxygen of Arg<sup>8</sup> forms a hydrogen bond with the amide group on Leu192, while its side chain interacts with the Leu187 on monomer A (Figure 9B). For [DCys<sup>3</sup>]LR, the Gln<sup>4</sup> side chain adjusts to form a hydrogen-bond network with the catalytic Cys195 in monomer B and the peptidic Arg<sup>5</sup>; Arg<sup>8</sup> forms

additional hydrogen bonds to the catalytic Cys195 in monomer A (Figure 9C). Although this network of hydrogen bonds did not persist throughout the dynamics experiments, clearly, [DCys<sup>3</sup>]LR might engage the active Cys195 on each monomer. Finally, an analysis of the 5 ns trajectories of the [DGLn<sup>4</sup>]LR demonstrated that the carbonyl oxygen of Gln<sup>4</sup> forms a hydrogen bond with the amide group of Arg<sup>8</sup> (Figure 9D). By replacing L-Gln<sup>4</sup> with D-Gln<sup>4</sup>, the side chain of this residue interacts with the catalytic Cys195 on the B monomer. Additionally, the upward orientation of the free carboxylate head of the Arg<sup>8</sup> forms a strong interaction with the Trp182 of the A monomer. The formation of this hydrogen-bond network

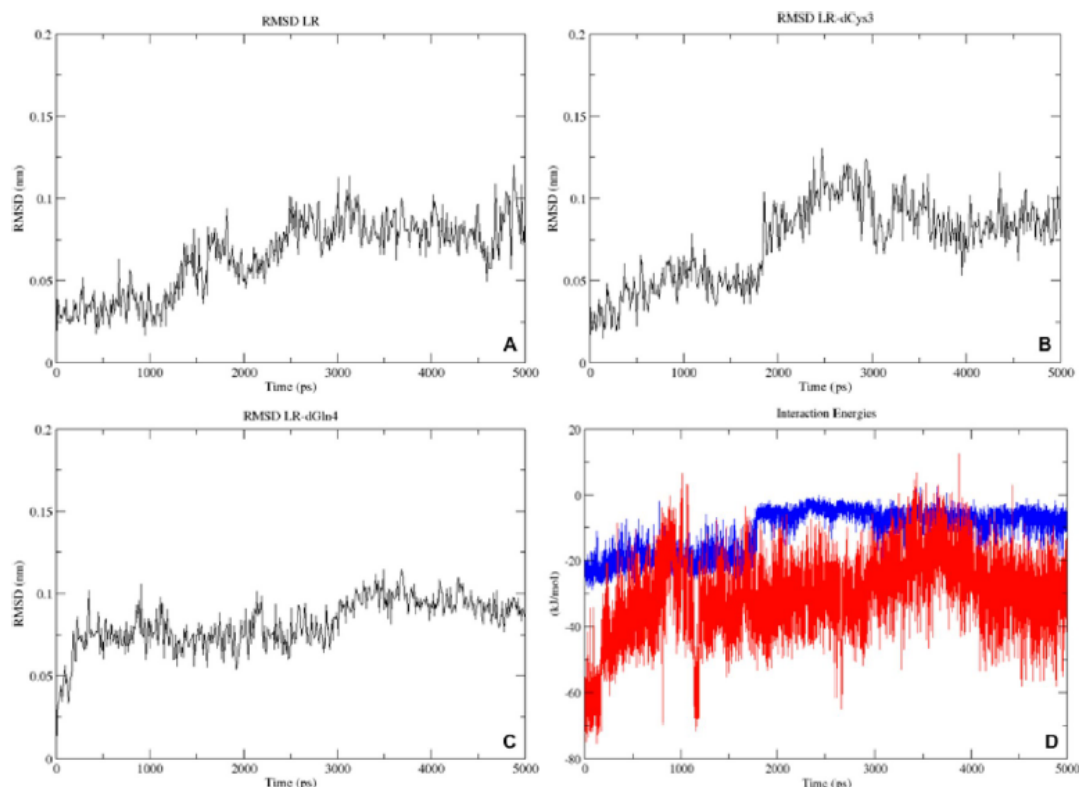


**Figure 8.** Calculated rmsd values for C $\alpha$  carbon atoms for (A) LR-peptide, (B) [DCys<sup>3</sup>]LR, and (C) [DGln<sup>4</sup>]LR peptide residues. (D) Interaction energies calculated between Gln<sup>4</sup> and Gln<sup>7</sup> (of [DCys<sup>3</sup>]LR), shown in blue and between Gln<sup>4</sup> and Arg<sup>8</sup> (of [DGln<sup>4</sup>]LR) drawn in red color. The trend in the energy values nicely corresponds to the fluctuation in the stability of the peptides: [DCys<sup>3</sup>]LR and [DGln<sup>4</sup>]LR.

involves both hTS monomers and was favored by the relatively broad conformation of [DGln<sup>4</sup>]LR.

In all three studied systems, the carboxy terminal of Arg<sup>8</sup> is critical for peptide binding to the hTS. Throughout the trajectories, the upward orientation of the Arg<sup>8</sup> free carboxylate group favors an interaction with the nearby catalytic-loop residues through an amide group projected toward the interface, namely, Trp182, Leu192, and Cys195 of monomer A (Figure 9). The C-terminal amidation of the peptides precludes these hydrogen bonds. Therefore, this finding provides a structural rationale for the observed loss of activity in LR after C-terminal amidation.

**Biological Effects of [DGln<sup>4</sup>]LR on 2008 and C13\* OC Cell Models.** As reported above, treatment with LR did not induce a significant increase in hTS protein expression levels in the OC cell lines;<sup>15</sup> therefore, we expected that compounds exhibiting the same inhibition mechanism against the recombinant protein (hTS) might trigger the same effect in cells. Consequently, we examined whether [DGln<sup>4</sup>]LR would have the same effect as LR by performing an immunoblot analysis using protein extracts from both the 2008 and C13 OC cell lines after 48 h treatments with the two peptides.

When treated with [DGln<sup>4</sup>]LR, no significant changes were observed in the TS protein levels (Figure 10A). In contrast, the total hTS content increased in both cell lines after treatment

with 5-FU due to the formation of an inactive ternary complex (Figure 10B). Furthermore, as illustrated in Figure 10C, [DGln<sup>4</sup>]LR induced a decrease in the DHFR protein level, similar to LR. A significant reduction was observed in the sensitive cell line and was comparable to the reduction observed after 5-FU treatment; in the C13\* resistant cell line, DHFR was decreased to a lesser extent.

To understand the relationships between the TS mRNA levels and TS protein expression, a quantitative assessment of the TS mRNA levels in the 2008 and C13\* cells treated with LR and [DGln<sup>4</sup>]LR was performed after 48 h of treatment. The TS mRNA increased in the sensitive cells after treatment with 5-FU and, to a lesser extent, also with LR and [DGln<sup>4</sup>]LR (Figure 10D, left). In the resistant cells, the TS mRNA levels remained nearly constant after treatment with 5-FU and both peptides (Figure 10D, right).

Both LR and [DGln<sup>4</sup>]LR inhibited the TS activity by approximately 30–50% at the concentrations evaluated, particularly in the sensitive 2008 cell line (data not shown); these results were similar to previous observations.<sup>15</sup> This finding is consistent with the new mode of action proposed for these peptides: unlike 5-FU, these peptides did not cause the formation of an inactive ternary complex with the protein.

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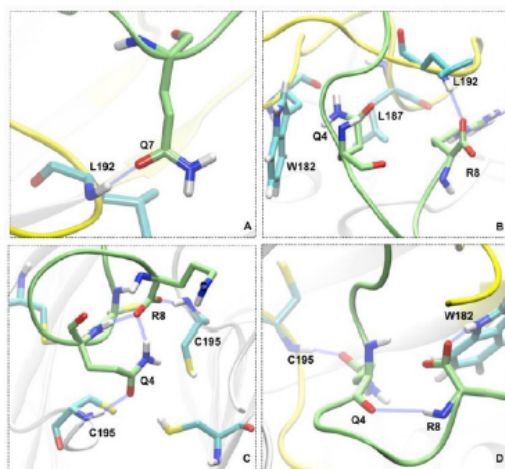


**Figure 9.** (A, B) Pictorial representation of the key interactions shown by LR peptide residues (in lime, licorice representation) with the catalytic loop (in yellow) residues (in cyan, licorice representation). (A) Carboxyl group of the Gln<sup>7</sup> side chain makes a H bond with the backbone of the Leu192 residue. (B) Steric interaction between the Gln<sup>4</sup> side chain and the Trp182 cyclic ring and the COO<sup>-</sup> group of Arg<sup>8</sup> making an H bond with Leu192 while its side chain is interacting with Leu187. (C) In the case of [DCys<sup>3</sup>]LR, cross-network bonding is shown where the peptide residues, Gln<sup>4</sup> and Arg<sup>8</sup> interacts among themselves as well as simultaneously fetch interactions from the catalytic Cys195 from both monomers. (D) In the case of [DGln<sup>4</sup>]LR, the peptide residues Gln<sup>4</sup> and Arg<sup>8</sup> are shown to indulge in hydrogen-bond network formation between them as well as catalytic Cys195 (on one monomer) and Trp182 (of another monomer), simultaneously. The backbone of the peptides are shown in lime color in New cartoon mode. One letter code for the residues is shown to maintain the clarity of the image. The H bonds are shown as blue lines.

#### 414 ■ CONCLUSIONS

415 In the present work, we tackle the problem of developing new,  
416 more effective peptides starting from a lead that showed a novel  
417 allosteric inhibition mechanism of hTS. The focus is on OC, a  
418 cancer type particularly difficult to treat. The tumor  
419 heterogeneity makes the understanding of the tumor biology  
420 difficult; as a result, effective drug targets cannot be easily  
421 identified. Indeed, no new drugs specifically developed against  
422 OC are yet available.

423 We have adopted an approach in which the lead optimization  
424 is combined with the evaluation of the intracellular modulation  
425 of two proteins: the target, TS, and an associated protein,  
426 DHFR.

427 In particular, through a SAR approach, we have modified the  
428 LR peptide to improve its interaction with the protein target  
429 hTS and its anticancer activity against OC cells. A wide range of  
430 modifications have been performed including site-specific  
431 mutation and truncation. The SAR study suggested the  
432 following: (i) the C-terminal carboxylate group is essential for  
433 bioactivity; (ii) the inversion of chirality at position 4 generated  
434 peptide [DGln<sup>4</sup>]LR, which showed increased activity compared  
435 to LR at the cellular level; and (iii) shortening LR is tolerated at  
436 both the N and C termini. The MD studies were in agreement  
437 with CD experiments indicating that the conformational  
438 behaviors of the [DCys<sup>3</sup>]LR and [DGln<sup>4</sup>]LR peptides differed:

the former preferred a  $\beta$ -turn conformation, while the latter was  $\alpha$ -helical in TFE solution and arranged in a five-residue  $\alpha$  turn when bound to hTS. The observation from MD studies that [DGln<sup>4</sup>]LR has higher structural rigidity compared to peptides LR and [DCys<sup>3</sup>]LR suggests this conformational property to be important to achieve higher affinity for the protein. This information might prove valuable for the rational design of cyclic LR analogues and "constrained analogues". Cytotoxicity studies against four cisplatin-sensitive and -resistant OC cell lines demonstrated the general improved effectiveness of [DGln<sup>4</sup>]LR compared to LR and 5-FU. Evaluation of TS/DHFR expression in the cells treated with [DGln<sup>4</sup>]LR showed that the TS protein levels did not vary significantly and the DHFR protein levels decreased. So, the intracellular TS and DHFR modulation pattern is the same for [DGln<sup>4</sup>]LR and LR, and both differ from that of 5FU. Studies are ongoing to elucidate the mechanism of action for these peptides at the cellular level.

#### ■ EXPERIMENTAL SECTION

**General.** Amino acid derivatives, reagents, and solvents were purchased from Sigma Aldrich (Steinheim, Germany) or Bachem (Bubendorf, Switzerland). The purity of the tested compounds has been assessed by reverse-phase high-performance liquid chromatography (RP-HPLC). All compounds showed >95% purity. Mass spectrometry (MS) analyses were performed on a ESI-Micromass ZMD 2000. Exact mass was recorded with an Agilent ESI-Q-TOF 6520 instrument.

**Peptide Synthesis.** All peptides were synthesized with an automatic solid-phase peptide synthesizer Syro II (Biotage, Uppsala Sweden) using Fmoc/tBu chemistry.<sup>32</sup> A preloaded 4-benzyloxymethyl alcohol resin (Wang resin) was used as a solid support for the synthesis of all peptides except for the peptide LR-NH<sub>2</sub> that was obtained using the resin 4-(2',4'-dimethoxyphenyl)-Fmoc-aminomethyl-phenoxycetamido-norleucyl-MBHA (Rink amide MBHA resin). First, the Fmoc protecting group on the resin was removed by treatment with 40% piperidine/DMF. The Fmoc-amino acids (4-fold excess) were activated by mixing with the coupling reagent *N,N'*-diisopropylcarbodiimide/ethyl 2-cyano-2-(hydroxyimino)acetate (DIPCDI/Oxima pure) (1:1) (4-fold excess), and the resin mixed at room temperature for 20 min. Cycles of deprotection of Fmoc and coupling with the subsequent amino acids were repeated until the desired peptide-bound resin sequence was completed. To improve the analytical profile of the crude peptide, capping with acetic anhydride (0.5 M/DMF) with the presence of *N*-methylmorpholine (0.25 M/DMF) (3:1 v/v; 2 mL/0.2 g of resin) was performed at any step. At the end of the synthesis, the peptide resin was washed with methanol and dried in vacuo to yield the protected LR-resin derivatives. The protected peptide-resin was treated with reagent B<sup>33</sup> (trifluoroacetic acid/H<sub>2</sub>O/phenol/triisopropylsilane 88:5:5:2; v/v; 10 mL/0.2 g of resin) for 1.5 h at room temperature. After filtration of the resin, the solvent was concentrated in vacuo, and the residue was triturated with ether. The crude peptide was purified by preparative RP-HPLC to yield a white powder after lyophilization.

**Peptide Purification and Analytical Determinations.** Crude peptides were purified by preparative RP-HPLC using a Water Delta Prep 3000 system with a Jupiter column C<sub>18</sub> (250 mm × 30 mm, 300 Å, 15  $\mu$ m spherical particle size). The column was perfused at a flow rate of 20 mL/min with a mobile phase containing solvent A (5%, v/v, acetonitrile in 0.1% TFA) and a linear gradient from 0 to 50% of solvent B (60%, v/v, acetonitrile in 0.1% TFA) over 25 min for the elution of peptides. Analytical HPLC analyses were performed on a Beckman 116 liquid chromatograph equipped with a Beckman 166 diode array detector. Analytical purity of the peptides were determined using a Luna C<sub>18</sub> column (4.6 mm × 100 mm, 3  $\mu$ m particle size) with the above solvent system (solvents A and B) programmed at a flow rate of 0.5 mL/min using a linear gradient from 0% to 80% B over 25 min.

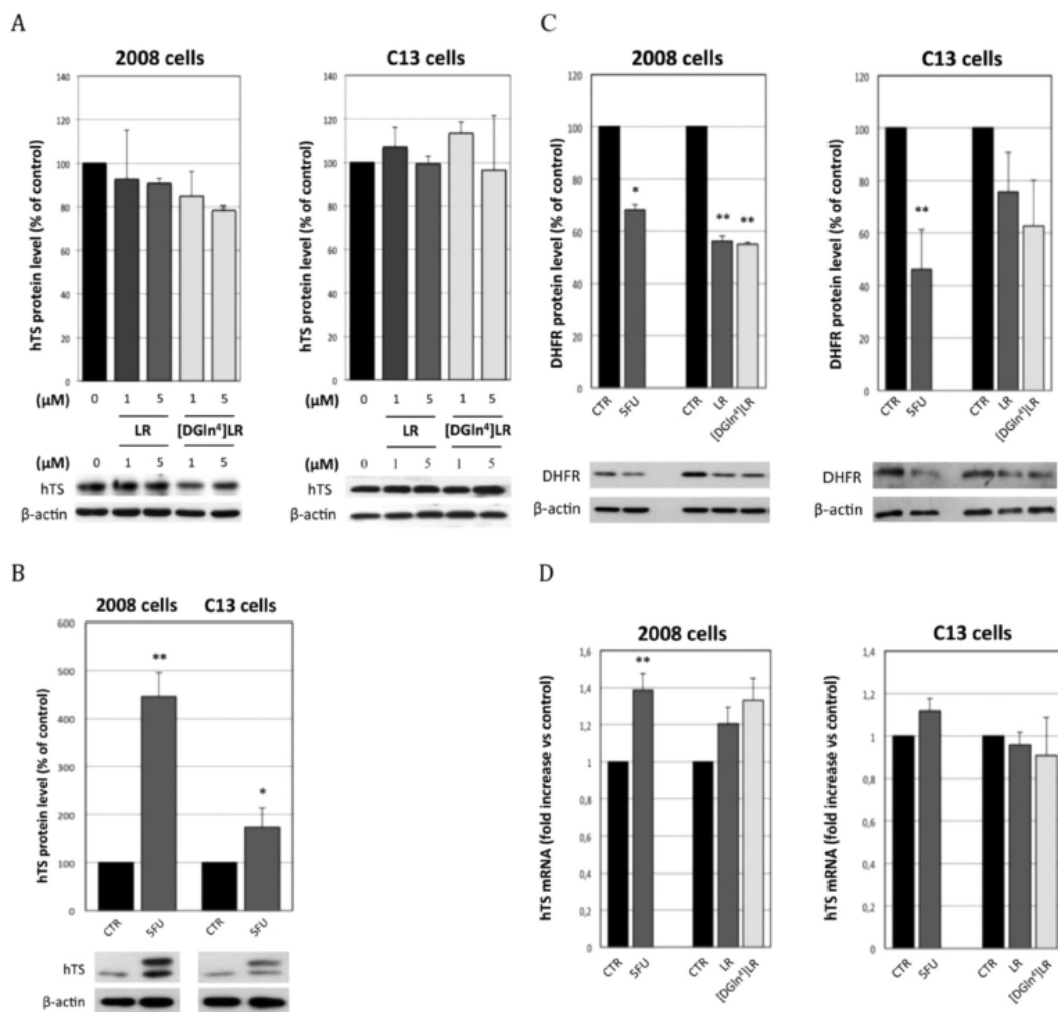


**Figure 10.** (A) Western blot analysis and their respective densitometric analyses normalized by  $\beta$ -actin (bar graphs) of hTS protein levels in 2008 and C13 cell lines treated for 48 h with 1 and 5  $\mu$ M LR (dark gray) and [DGLn<sup>4</sup>]LR (light gray). The experiments were carried out three times, and a representative result is shown. (B) The 2008 and C13 cell lines were treated with 5  $\mu$ M 5-FU, and the total amount of free hTS and of inactive ternary complex (hTS–FdUMP–mTHF) was determined using western blotting analysis. Quantification normalized by  $\beta$ -actin is shown in the graph, and bars indicate mean  $\pm$  SEM;  $n = 3$ . (C) Immunoblot and relative quantitation (bar graphs) of DHFR protein levels treated with 5  $\mu$ M 5-FU, 5  $\mu$ M LR, and 5  $\mu$ M [DGLn<sup>4</sup>]LR; bars indicate mean  $\pm$  SEM;  $n = 3$ . (D) Quantitative real time polymerase chain reaction (qPCR) of hTS mRNA levels. hTS expression in 2008 (left) and C13 (right) cells was determined after treatment with 5  $\mu$ M LR, [DGLn<sup>4</sup>]LR, and 5-FU for 48 h. The amount of hTS mRNA was normalized with GAPDH mRNA, and bars indicate mean  $\pm$  SEM;  $n = 3$ . Student's  $t$  test was performed for both western blot and qPCR analyses to evaluate statistical significance (\*,  $p < 0.05$ ; \*\*,  $p < 0.02$ ).

505 min. All analogues showed  $\geq 95\%$  purity when monitored at 220 nm.  
 506 Accurate mass of final compounds was determined using an Agilent  
 507 6520 Q-TOF mass spectrometer, and the data obtained was reported  
 508 in the Supporting Information.

509 **CD Spectroscopy.** The CD spectra of LR, LR-NH<sub>2</sub>, [DCys<sup>3</sup>]LR,  
 510 and [DGLn<sup>4</sup>]LR peptides were measured with a Jasco spectropolarim-  
 511 eter. Each spectrum was the average of 4–10 acquisitions. The cell  
 512 plus solvent contributions were measured and subtracted from the  
 513 averaged spectra. Concentrated peptide solutions were prepared in  
 514 TFE and deionized water and were diluted to the concentrations

required for the measurements (ca. 70  $\mu$ M) by adding suitable  
 515 volumes of water and/or TFE so that the needed solvent mixtures  
 516 were obtained. All samples were checked by UV–visible (UV–vis)  
 517 absorption spectroscopy using a Varian Cary 100 spectrophotometer.  
 518 Cuvettes (1 mm) were employed, and measurements were performed  
 519 at 20–22 °C about 20 min after sample preparation.  
 520

**MD Simulations.** The work was initiated by using the X-ray  
 521 crystallographic structure of hTS with LR peptide as the starting  
 522 model. The remaining two complexes hTS with [DCys<sup>3</sup>]LR and hTS  
 523 with [DGLn<sup>4</sup>]LR were obtained by replacing L-Cys (at position 3 of LR 524

peptide) with D-Cys and L-Gln (at position 4 of LR peptide) by D-Gln, respectively. MD simulations were carried out by using GROMACS (version 4.0.5) software package and the GROMOS'96 atom force field.<sup>34</sup> All three complexes were solvated with SPC water molecules in a cubic box of dimension 20 Å edge length. To make the systems electrostatically neutral, 3 Cl<sup>-</sup> ions were added to each system using the genion GROMACS utility.

In the initial step, all three respective systems were subjected to minimization protocol (steepest descent followed by conjugate gradient) until the maximum force is less than 100 kJ/(mol·nm). During this process, the atoms of the protein and peptide were held fixed with a force constant of 1000 kJ/(mol·nm<sup>2</sup>), and the solvent molecules were allowed to relax. The minimized systems were then imported for the equilibration phase for a 1 ns run, time-step ( $\delta t$ ) of 1 fs (femtoseconds), with no restraints applied, followed by production run for 10 ns, without any restraints, with a  $\delta t$  of 1 fs.

The MD simulations were conducted at a constant temperature of 300 K and a constant pressure of 1 atm, which was controlled by Berendsen algorithm. Periodic boundary condition was also implemented in all three systems. The long-range interactions were treated with the particle-mesh Ewald (PME) method<sup>35</sup> within a radius of 15 Å, while the short-range interactions were calculated with the Leannard-Jones potential with a cutoff of 15 Å.

For the system, hTS with LR peptide, default parameter files of GROMACS were used, whereas for hTS with [D-Cys]<sup>3</sup>LR and hTS with [D-Gln]<sup>4</sup>LR, a new set of parameter files was created that contained the necessary definitions (such as improper torsion angles) for D-Cys and D-Gln, respectively (see the Gromacs parameter files for D-amino acids section of the Supporting Information).

For analyzing the msd and the radius of gyration, g\_rms and g\_gyrate utilities, provided by the GROMACS package were used, respectively. The resulting trajectories from the simulations were visualized by VMD software (version 1.0.9).<sup>36</sup> The hydrogen bonds were explored by using the default parameters of HBonds utility available in the software.

Secondary structure analysis of the resulting peptides was performed by the DSSP program that determines it on the basis of hydrogen bonds present.<sup>31</sup>

**Enzymatic Assay.** hTS was purified as previously reported.<sup>15</sup> Enzyme solution was thawed the day of the experiment and the enzyme concentration was determined by UV-vis spectroscopy using  $\epsilon_{280} = 89,000$  and  $M_r = 74,229$ . Thawed protein solution was kept constantly at 4 °C. In this condition, the enzyme was able to reproduce normal kinetic activity values ( $K_M^{\text{dUMP}} = 10\text{--}12\ \mu\text{M}$ ,  $K_M^{\text{mTHF}} = 4\text{--}6\ \mu\text{M}$ ,  $k_{\text{cat}} = 0.8\text{--}0.9\ \text{s}^{-1}$ ). Peptide solution was mixed or vortexed in order to help solubilization; the precise concentration was determined through UV-vis spectroscopy. A stock solution of mTHF was prepared in carbonate buffer. The stock was prepared in the range 5–6 mM. A stock solution of dUMP was prepared in bidistilled water in the range 10–12. Peptide inhibitors were incubated for 1 h with hTS in phosphate buffer, pH 6.9, at 25 °C. A 1 mL amount of assay buffer consists of 50 mM TES, pH 7.4, containing 25 mM MgCl<sub>2</sub>, 6.5 mM HCHO, 1 mM EDTA, 75 mM  $\beta$ -mercaptoethanol ( $\beta$ -ME), 0.6 mM dUMP, and 0.15 mM mTHF. Following the addition of the enzyme and inhibitor mixture, the absorbance was monitored at 340 nm in a UV-vis spectrophotometer for 3 min. In the control sample, an equal aliquot of enzyme alone was added. hTS activity inhibition percentages (%) values were measured at 50–100  $\mu\text{M}$  concentration of inhibitor. I % values are expressed as mean  $\pm$  SEM;  $n = 3$ .

**Cell Lines.** The 2008 cell line is an ovarian carcinoma established from a patient with serous cystadenocarcinoma of the ovary.<sup>37</sup> The cDDP-resistant variant C13\* cell line, derived from the parent 2008 cell line, is about 13-fold resistant to cDDP and was developed by monthly exposure to cDDP, followed by chronic exposure to stepwise increases in cDDP concentration.<sup>38</sup> The A2780/CP human ovarian carcinoma cells are 9- to 12-fold resistant to cDDP and derived from the parent A2780 cell line.<sup>39</sup> The cell lines were grown as monolayers in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and 50  $\mu\text{g}/\text{mL}$  gentamycin sulfate.

All cell media and serum were purchased from Lonza (Verviers, Belgium). Cultures were equilibrated with humidified 5% CO<sub>2</sub> in air at 37 °C. All studies were performed in *Mycoplasma* negative cells, as routinely determined with the MycoAlert Mycoplasma detection kit (Lonza, Walkersville, MD, USA). Protein content in the various assays was estimated by the method of Lowry,<sup>40</sup> unless otherwise indicated.

**Cell Growth Assay.** Cell growth was determined using a modified crystal violet assay.<sup>41</sup> On selected days, the tissue culture medium was removed, and the cell monolayer was fixed with methanol and stained with 0.2% crystal violet solution in 20% methanol for at least 30 min. After being washed several times with distilled water to remove excess dye, the cells were left to dry. The incorporated dye was solubilized in acidified isopropanol (1 N HCl/2-propanol, 1:10). After appropriate dilution, the absorbance was determined spectrophotometrically at 540 nm. The extracted dye was proportional to the cell number. The percentage of cytotoxicity was calculated by comparing the absorbance of cultures exposed to the drug to unexposed (control) cultures. Statistical significance was estimated by ANOVA followed by Dunnett's post hoc multiple comparison test.

**Treatment with LR and [D-Gln]<sup>4</sup>LR in the Presence of SAINT PhD Delivery System.** Treatment with each peptide was performed according to the standard transfection protocol of the SAINT PhD delivery system (Synvolux Therapeutics, NL). Complexes of peptide ( $\mu\text{g}$ ) with SAINT PhD ( $\mu\text{L}$ ) were prepared at a ratio of 1.6  $\mu\text{g}/20\ \mu\text{L}$  (corresponding to approximately 1  $\mu\text{M}$  final) or 8  $\mu\text{g}/20\ \mu\text{L}$  (which corresponds to a concentration of approximately 5  $\mu\text{M}$  final). For each protein sample, complexes were prepared as follows: for the treatment of 6-well plates, the appropriate amount of each peptide was diluted in 120  $\mu\text{L}$  of HBS, and then, 80  $\mu\text{L}$  of SAINT PhD was pipetted to the solution without vortexing; the mixture was incubated for 5 min at room temperature and then filled up to 500  $\mu\text{L}$  with serum-free medium. For the treatment of 32 mm plates, 80  $\mu\text{L}$  of SAINT PhD was used preserving the same ratio with peptide and HBS. The culture medium was aspirated from the cells, and the SAINT PhD/peptide complex was added to the wells and incubated for 4 h (37 °C; 5% CO<sub>2</sub>). After this time, complete RPMI was added to reach the appropriate volume for maintaining the cell culture 48 h before performing the experiments (2 mL for the 12-well plates and 4 mL for the 6-well plates).

**Protein Extraction and Western Blot Analysis.** Cells were washed twice in ice-cold PBS, harvested, and resuspended in RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 1  $\mu\text{g}/\text{mL}$  leupeptin). Cells were then lysed by three freeze/thaw sequences and centrifuged at 14,000 rpm for 30 min to remove debris. Protein concentrations were determined using the Bradford protein assay reagent (Sigma Aldrich). A 40  $\mu\text{g}$  amount of each protein sample was resolved by SDS-PAGE according to the method of Laemmli.<sup>42</sup> Gels were blotted onto PVDF membranes (Hybond-P, Amersham). Antibody staining was performed with a chemiluminescence detection system (ECL Plus, Amersham), using a 1:500 dilution of the mouse TS106 monoclonal primary antibody (Abnova), 1:250 dilution of DHFR (A-4) mouse monoclonal antibody (Santa Cruz Biotechnology, Inc.), and 1:2000 dilution of  $\beta$ -actin mouse antibody (Santa Cruz Biotechnology, Inc.) in TBS-T with 5% dry milk, in conjunction with a 1:5000 dilution of a horseradish peroxidase-conjugated sheep antimouse secondary antibody (Amersham). Densitometric quantification of the immunoblot bands was performed using an Epson perfection 2580 photo scanner (Epson, Long Beach, CA) and QuantityOne densitometry software. For each sample, densitometry values of three replicates expressed in arbitrary units were acquired and evaluated for statistical significance with a Student's *t* test.

**RNA Extraction and qPCR Evaluation of TS and DHFR mRNA.** Cell lines were tested for mRNA expression by RT-PCR. Cells were harvested, and total RNA was isolated from cells using InnusOLV RNA reagent (Steroglass). Reverse transcription was performed with 0.5 or 1  $\mu\text{g}$  of total RNA using SuperScript first-strand, synthesis for RT-PCR (Invitrogen). The cDNA strand generated was used for PCR amplification in a total volume of 20  $\mu\text{L}$ . RT-PCR was performed with 663

664 100 ng of cDNA using the Power SYBR green PCR master mix  
665 (Applied Biosystems).<sup>15</sup> Samples were amplified by 40 cycles at 95 °C  
666 for 10 min, 94 °C for 15 s, 60 °C for 1 min, with a final cycle at 95 °C  
667 for 2 min, 60 °C for 15 s, and 95 °C for 15 s (dissociation curve), in  
668 Applied Biosystems 7900HT fast real-time PCR system. The amplified  
669 were analyzed by the ABI Prism 7900 HT SDS version 2.2 software  
670 (Applied Biosystems). For each sample, three replicates were acquired,  
671 and the Student's *t* test was performed to evaluate statistical  
672 significance.

673 The amount of target expressed was normalized with GAPDH and  
674 detected by the 2<sup>-ΔΔC<sub>t</sub></sup> method. TS target primer forward (3hTS2 fw):  
675 5'-CAGATTATTACAGACAGGAGTT-3' (Sigma Genosys 596,  
676 8460-065), reverse (3hTS2 rw): 5'-CATCAGAGGAAGATCTCT-  
677 TGGATT-3' (Sigma Genosys 596, 8460-059); DHFR target primer  
678 forward (3hDHFR2 fw): 5'-GCAATCATTCTAGGCGAGAAA-3'  
679 (Sigma Genosys 596, 8459-066), reverse (4hDHFR2 rw): 5'-  
680 GGGCTAAGCAGTCACATCATT-3' (Sigma Genosys 596, 8502-  
681 025); GAPDH reference primer forward (hGAPDH1 fw) (Sigma  
682 Genosys 1062 3174-083): 5'-CAAGGTCATCCATGACAACTTGTG-  
683 3', reverse: 5'-GGGCCATCCACAGTCTTCTG-3' (hGAPDH1 rw)  
684 (Sigma Genosys 1062 3174-084). We performed dissociation curve  
685 analysis and agarose gel electrophoresis to confirm the amplification.<sup>43</sup>

686 **TS Catalytic Assay.** TS catalytic assay, conducted essentially  
687 according to a previously reported method,<sup>44</sup> determines the catalytic  
688 activity of TS by measuring the amounts of <sup>3</sup>H release from  
689 [5-<sup>3</sup>H]dUMP during its TS catalyzed conversion to dTMP. Briefly,  
690 the assay consisted of enzyme suspensions, 650 μM 5,10-  
691 methylenetetrahydrofolate in a final volume of 50 μL. The reaction  
692 was started by adding [5-<sup>3</sup>H]dUMP (1 μM final concentration, specific  
693 activity 5 mCi/mol), incubated for 60 min at 37 °C, and stopped by  
694 adding 50 μL of ice-cold 35% trichloroacetic acid. Residual  
695 [5-<sup>3</sup>H]dUMP was removed by the addition of 250 μL of 10% neutral  
696 activated charcoal. The charcoal was removed by centrifugation at 14  
697 000g for 15 min at 4 °C, and a 150 μL sample of the supernatant was  
698 assayed for tritium radioactivity by liquid scintillation counting in the  
699 liquid scintillator analyzer Tri-Carb 2100 (Packard).

## 700 ■ ASSOCIATED CONTENT

### 701 ■ Supporting Information

702 Analytical HPLC profiles and HRMS spectra of final  
703 compounds; table of hTS activity inhibition percentages (I  
704 %); table of cell survival (% of control) of human OC cell lines;  
705 and Gromacs parameter files for D-amino acids. This material is  
706 available free of charge via the Internet at <http://pubs.acs.org>.

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### 713 Notes

714 The authors declare no competing financial interest.

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## 718 ■ ABBREVIATIONS USED

719 hTS, human thymidylate synthase; OC, ovarian cancer; CD,  
720 circular dichroism; MD, molecular dynamics; SAR, structure-  
721 activity relationship; mnsd, root-mean-square deviation; RP-  
722 HPLC, reverse-phase high-performance liquid chromatography;  
723 HPLC, high-performance liquid chromatography; MS, mass  
724 spectrometry; HBS, HEPES-buffered saline; PBS, phosphate-  
725 buffered saline; RIPA, radioimmuno precipitation assay buffer

## ■ REFERENCES

- (1) Metzger, R.; Leichman, C. G.; Danenberg, K. D.; Danenberg, P. 727  
V.; Lenz, H. J.; Hayashi, K.; Groshen, S.; Salonga, D.; Cohen, H.; 728  
Laine, L.; Crookes, P.; Silberman, H.; Baranda, J.; Konda, B.; 729  
Leichman, L. ERCC1 mRNA levels complement thymidylate synthase 730  
mRNA levels in predicting response and survival for gastric cancer 731  
patients receiving combination cisplatin and fluorouracil chemo- 732  
therapy. *J. Clin. Oncol.* **1998**, *16*, 309–316. 733
- (2) Garg, D.; Beribisky, A. V.; Ponterini, G.; Ligabue, A.; Marverti, 734  
G.; Martello, A.; Costi, M. P.; Sattler, M.; Wade, R. C. Translational 735  
repression of thymidylate synthase by targeting its mRNA. *Nucleic 736  
Acids Res.* **2013**, *41*, 4159–4170. 737
- (3) Chu, E.; Allegra, C. J. The role of thymidylate synthase as an 738  
RNA binding protein. *Bioessays* **1996**, *18*, 191–198. 739
- (4) Chu, E.; Koeller, D. M.; Casey, J. L.; Drake, J. C.; Chabner, B. A.; 740  
Elwood, P. C.; Zinn, S.; Allegra, C. J. Autoregulation of human 741  
thymidylate synthase messenger RNA translation by thymidylate 742  
synthase. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 8977–8981. 743
- (5) Chu, E.; Voeller, D.; Koeller, D. M.; Drake, J. C.; Takimoto, C. 744  
H.; Maley, G. F.; Maley, F.; Allegra, C. J. Identification of an RNA 745  
binding site for human thymidylate synthase. *Proc. Natl. Acad. Sci.* 746  
*U.S.A.* **1993**, *90*, 517–521. 747
- (6) Liu, J.; Schmitz, J. C.; Lin, X.; Tai, N.; Yan, W.; Farrell, M.; Bailly, 748  
M.; Chen, T.; Chu, E. Thymidylate synthase as a translational 749  
regulator of cellular gene expression. *Biochim. Biophys. Acta* **2002**, 750  
*1587*, 174–182. 751
- (7) Ju, J.; Pedersen-Lane, J.; Maley, F.; Chu, E. Regulation of p53 752  
expression by thymidylate synthase. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, 753  
*96*, 3769–3774. 754
- (8) Ozasa, H.; Oguri, T.; Uemura, T.; Miyazaki, M.; Maeno, K.; Sato, 755  
S.; Ueda, R. Significance of thymidylate synthase for resistance to 756  
pemetrexed in lung cancer. *Cancer Sci.* **2010**, *101*, 161–166. 757
- (9) Chu, E.; Callender, M. A.; Farrell, M. P.; Schmitz, J. C. 758  
Thymidylate synthase inhibitors as anticancer agents: From bench to 759  
bedside. *Cancer Chemother. Pharmacol.* **2003**, *52* (Suppl 1), 80–89. 760
- (10) Berger, S. H.; Berger, F. G.; Lebiola, L. Effects of ligand binding 761  
and conformational switching on intracellular stability of human 762  
thymidylate synthase. *Biochim. Biophys. Acta* **2004**, *1696*, 15–22. 763
- (11) Kitchens, M. E.; Forsthoefel, A. M.; Rafique, Z.; Spencer, H. T.; 764  
Berger, F. G. Ligand mediated induction of thymidylate synthase 765  
occurs by enzyme stabilization. Implications for autoregulation of 766  
translation. *J. Biol. Chem.* **1999**, *274*, 12544–12547. 767
- (12) Bast, R. C., Jr.; Hennessy, B.; Mills, G. B. The biology of ovarian 768  
cancer: New opportunities for translation. *Nat. Rev. Cancer* **2009**, *9*, 769  
415–428. 770
- (13) Scanlon, K. J.; Kashani-Sabet, M. Elevated expression of 771  
thymidylate synthase cycle genes in cisplatin-resistant human ovarian 772  
carcinoma A2780 cells. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 650–653. 773
- (14) Marverti, G.; Ligabue, A.; Paglietti, G.; Corona, P.; Piras, S.; 774  
Vitale, G.; Guerrieri, D.; Luciani, R.; Costi, M. P.; Frassinetti, C.; 775  
Moruzzi, M. S. Collateral sensitivity to novel thymidylate synthase 776  
inhibitors correlates with folate cycle enzymes impairment in cisplatin- 777  
resistant human ovarian cancer cells. *Eur. J. Pharmacol.* **2009**, *615*, 17– 778  
26. 779
- (15) Cardinale, D.; Guaitoli, G.; Tondi, D.; Luciani, R.; Henrich, S.; 780  
Salo-Ahen, O. M.; Ferrari, S.; Marverti, G.; Guerrieri, D.; Ligabue, A.; 781  
Frassinetti, C.; Pozzi, C.; Mangani, S.; Fessas, D.; Guerrini, R.; 782  
Ponterini, G.; Wade, R. C.; Costi, M. P. Protein-protein interface- 783  
binding peptides inhibit the cancer therapy target human thymidylate 784  
synthase. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, E542–E549. 785
- (16) Phan, J.; Koli, S.; Minor, W.; Dunlap, R. B.; Berger, S. H.; 786  
Lebiola, L. Human thymidylate synthase is in the closed conformation 787  
when complexed with dUMP and raltitrexed, an antidotal drug. 788  
*Biochemistry* **2001**, *40*, 1897–1902. 789
- (17) Phan, J.; Steadman, D. J.; Koli, S.; Ding, W. C.; Minor, W.; 790  
Dunlap, R. B.; Berger, S. H.; Lebiola, L. Structure of human 791  
thymidylate synthase suggests advantages of chemotherapy with 792  
noncompetitive inhibitors. *J. Biol. Chem.* **2001**, *276*, 14170–14177. 793

- (18) Hruby, V. J.; Balse, P. M. Conformational and topographical considerations in designing agonist peptidomimetics from peptide leads. *Curr. Med. Chem.* **2000**, *7*, 945–970.
- (19) Haubner, R.; Finsinger, D.; Kessler, H. Stereoisomeric peptide libraries and peptidomimetics for designing selective inhibitors of the  $\alpha v \beta 3$  integrin for a new cancer therapy. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 1374–1389.
- (20) Chang, Y. P.; Chu, Y. H. Using surface plasmon resonance to directly determine binding affinities of combinatorially selected cyclopeptides and their linear analogs to a streptavidin chip. *Anal. Biochem.* **2005**, *340*, 74–79.
- (21) Dathe, M.; Nikolenko, H.; Klose, J.; Bienert, M. Cyclization increases the antimicrobial activity and selectivity of arginine- and tryptophan- containing hexapeptides. *Biochemistry* **2004**, *43*, 9140–9150.
- (22) Dumez, E.; Snaith, J. S.; Jackson, R. F.; McElroy, A. B.; Overington, J.; Wythes, M. J.; Withka, J. M.; McLellan, T. J. Synthesis of macrocyclic, potential protease inhibitors, using a generic scaffold. *J. Org. Chem.* **2002**, *67*, 4882–4892.
- (23) Kang, J. H.; Kim, S. Y.; Lee, L.; Marquez, V. E.; Lewin, N. E.; Pearce, L. V.; Blumberg, P. M. Macrocyclic diacylglycerol-bis-lactones as conformationally constrained analogues of diacylglycerol-lactones. Interactions with protein kinase C. *J. Med. Chem.* **2004**, *47*, 4000–4007.
- (24) Khan, A. R.; Parrish, J. C.; Fraser, M. E.; Smith, W. W.; Bartlett, P. A.; James, M. N. Lowering the entropic barrier for binding conformationally flexible inhibitors to enzymes. *Biochemistry* **1998**, *37*, 16839–16845.
- (25) Nam, N. H.; Ye, G.; Sun, G.; Parang, K. Conformationally constrained peptide analogues of pTyr-Glu-Glu-Ile as inhibitors of the Src SH2 domain binding. *J. Med. Chem.* **2004**, *47*, 3131–3141.
- (26) Woody, R. W. Circular dichroism. *Methods Enzymol.* **1995**, *246*, 34–71.
- (27) Vass, E.; Holly, S.; Majer, Z. S.; Samu, J.; Laczkó, I.; Hollósi, M. FTIR and CD spectroscopic detection of H-bonded folded. *J. Mol. Struct.* **1997**, *408/409*, 47–56.
- (28) Smith, J. A.; Pease, L. G. Reverse turns in peptides and proteins. *CRC Crit. Rev. Biochem.* **1980**, *8*, 315–399.
- (29) Sreerama, N.; Manning, M. C.; Powers, M. E.; Zhang, J.-X.; Goldenberg, D. P.; Woody, R. W. Tyrosine, phenylalanine, and disulfide contributions to the circular dichroism of proteins: Circular dichroism spectra of wild-type and mutant bovine pancreatic trypsin inhibitor. *Biochemistry* **1999**, *38*, 10814–10822.
- (30) Chou, K.-C. Prediction of tight turns and their types in proteins. *Anal. Biochem.* **2000**, *286*, 1–16.
- (31) Kabsch, W.; Sander, C. Dictionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* **1983**, *22*, 2577–2637.
- (32) Benoiton, N. L. *Chemistry of Peptide Synthesis*; Taylor & Francis: London, 2005; pp 125–154.
- (33) Solé, N. A.; Barany, G. Optimization of solid-phase synthesis of [Ala<sup>8</sup>]-dynorphin. *J. Org. Chem.* **1992**, *57*, 5399–5403.
- (34) Hess, B.; Kutzner, C.; van der Spoel, D.; Lindahl, E. GROMACS 4: Algorithms for highly efficient, load-balanced, and scalable molecular simulation. *J. Chem. Theor. Comput.* **2008**, *4*, 435–447.
- (35) Darden, T.; York, D.; Pedersen, L. Particle mesh Ewald: an  $N \log(N)$  method for Ewald sums in large systems. *J. Chem. Phys.* **1993**, *98*, 10089–10092.
- (36) Humphrey, W.; Dalke, A.; Schman, K. VMD-visual molecular dynamics. *J. Mol. Graphics* **1996**, *14*, 33–38.
- (37) DiSaia, P. J.; Sinkovics, J. G.; Rutledge, F. N.; Smith, J. P. Cell-mediated immunity to human malignant cells. A brief review and further studies with two gynecologic tumors. *Am. J. Obstet. Gynecol.* **1972**, *114*, 979–989.
- (38) Andrews, P. A.; Murphy, M. P.; Howell, S. B. Differential potentiation of alkylating and platinating agent cytotoxicity in human ovarian carcinoma cells by glutathione depletion. *Cancer Res.* **1985**, *45*, 6250–6253.
- (39) Andrews, P. A.; Jones, J. A. Characterization of binding proteins from ovarian carcinoma and kidney tubule cells that are specific for cisplatin modified DNA. *Cancer Commun.* **1991**, *3*, 1–10.
- (40) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folic phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.
- (41) Kueng, W.; Siber, E.; Eppenberger, U. Quantification of cells cultured on 96-well plates. *Anal. Biochem.* **1989**, *182*, 16–19.
- (42) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.
- (43) Arocho, A.; Chen, B.; Ladanyi, M.; Pan, Q. Validation of the 2-DeltaDeltaCt calculation as an alternate method of data analysis for quantitative PCR of BCR-ABL P210 transcripts. *Diagn. Mol. Pathol.* **2006**, *15*, 56–61.
- (44) van Triest, B.; Pinedo, H. M.; van Hensbergen, Y.; Smid, K.; Telleman, F.; Schoenmakers, P. S.; van der Wilt, C. L.; van Laar, J. A. M.; Noordhuis, P.; Jansen, G.; Peters, G. J. Thymidylate synthase level as the main predictive parameter for sensitivity to 5-fluorouracil, but not for folate-based thymidylate synthase inhibitors, in 13 non selected colon cancer cell lines. *Clin. Cancer Res.* **1999**, *5*, 643–654.

## Supporting Information

Table 1S: hTS activity inhibition percentages (I%) values measured at 100  $\mu$ M concentration of inhibitor. I% values are expressed as mean  $\pm$  S:E:M;n=3.

| sequence                          | code                   | I%                         |
|-----------------------------------|------------------------|----------------------------|
| <b>LSCQLYQR</b>                   | LR                     | 65 $\pm$ 2.6               |
| <b>Ac-LSCQLYQR</b>                | Ac-LR                  | 60 $\pm$ 1.8               |
| <b>Ac-LSCQLYQR-NH<sub>2</sub></b> | Ac-LR-NH <sub>2</sub>  | 20 $\pm$ 0.05              |
| <b>LSCQLYQR-NH<sub>2</sub></b>    | LR-NH <sub>2</sub>     | 5 $\pm$ 0.01               |
| <b>LSCQLYQR</b>                   | LR                     | 65 $\pm$ 2.6               |
| <b>ASCQLYQR</b>                   | [Ala <sup>1</sup> ]LR  | 69 $\pm$ 2.2               |
| <b>LACQLYQR</b>                   | [Ala <sup>2</sup> ]LR  | 69 $\pm$ 1.6               |
| <b>LSAQLYQR</b>                   | [Ala <sup>3</sup> ]LR  | 67 $\pm$ 1.7               |
| <b>LSCALYQR</b>                   | [Ala <sup>4</sup> ]LR  | 56 $\pm$ 0.8               |
| <b>LSCQAYQR</b>                   | [Ala <sup>5</sup> ]LR  | 67 $\pm$ 2.6               |
| <b>LSCQLAQR</b>                   | [Ala <sup>6</sup> ]LR  | 57 $\pm$ 1.1               |
| <b>LSCQLYAR</b>                   | [Ala <sup>7</sup> ]LR  | 100 <sup>a</sup> $\pm$ 0.8 |
| <b>LSCQLYQA</b>                   | [Ala <sup>8</sup> ]LR  | 41 $\pm$ 1.1               |
| <b>LSCQLYQR</b>                   | LR                     | 65 $\pm$ 2.6               |
| <b>ISCQLYQR</b>                   | [DLeu <sup>1</sup> ]LR | 42 $\pm$ 0.9               |
| <b>LsCQLYQR</b>                   | [DSer <sup>2</sup> ]LR | 61 $\pm$ 2.1               |
| <b>LScQLYQR</b>                   | [DCys <sup>3</sup> ]LR | 27 $\pm$ 0.5               |
| <b>LSCqLYQR</b>                   | [DGln <sup>4</sup> ]LR | 74 $\pm$ 1.4               |
| <b>LSCQlYQR</b>                   | [DLeu <sup>5</sup> ]LR | 51 $\pm$ 0.7               |
| <b>LSCQLyQR</b>                   | [DTyr <sup>6</sup> ]LR | 49 $\pm$ 0.9               |
| <b>LSCQLYqR</b>                   | [DGln <sup>7</sup> ]LR | 60 $\pm$ 1.6               |
| <b>LSCQLYQr</b>                   | [DArg <sup>8</sup> ]LR | 45 $\pm$ 1.1               |
| <b>lscqlyqr</b>                   | LR-D                   | NI                         |

|                 |                |                        |
|-----------------|----------------|------------------------|
| <b>LSCQLYQR</b> | <b>LR</b>      | 65 ± 2.6               |
| <b>SCQLYQR</b>  | <b>LR(2-8)</b> | 100 <sup>b</sup> ± 1.1 |
| <b>CQLYQR</b>   | <b>LR(3-8)</b> | 51 ± 1.4               |
| <b>QLYQR</b>    | <b>LR(4-8)</b> | 59 ± 2.2               |
| <b>LYQR</b>     | <b>LR(5-8)</b> | 31 ± 0.4               |
| <b>LSCQLYQR</b> | <b>LR</b>      | 65 ± 2.6               |
| <b>LSCQLYQ</b>  | <b>LR(1-7)</b> | 74 <sup>c</sup> ± 0.9  |
| <b>LSCQLY</b>   | <b>LR(1-6)</b> | 61 ± 2.9               |
| <b>LSCQL</b>    | <b>LR(1-5)</b> | 58 ± 2.3               |
| <b>LSCQ</b>     | <b>LR(1-4)</b> | 78 <sup>d</sup> ± 1.7  |
| <b>LSCQLYQR</b> | <b>LR</b>      | 65 ± 2.6               |
| <b>LSCQL</b>    | <b>LR(1-5)</b> | 58 ± 2.5               |
| <b>SCQLY</b>    | <b>LR(2-6)</b> | 68 ± 2.1               |
| <b>CQLYQ</b>    | <b>LR(3-7)</b> | 69 <sup>e</sup> ± 1.2  |
| <b>QLYQR</b>    | <b>LR(4-8)</b> | 59 ± 2.1               |

<sup>a</sup> extrapolate from I%=60 at 50 µM, <sup>b</sup> extrapolate from I%=54% at 50 µM, <sup>c</sup> extrapolate from I%=56% at 75 µM,

<sup>d</sup> extrapolate from I%=59% at 75 µM, <sup>e</sup> extrapolate from I%=52% at 75 µM

Table 2S. Cell survival (% of control) of the 2008, C13\*, A2780 and A2780/CP human ovarian cancer cell lines by 72 h-exposure to peptides (n=2/4) or to 5-FU (n=4).

Cell survival (% of control) of the 2008, C13\*, A2780 and A2780/CP after the treatment with 5-FU was about 45%, 53 after LR and PF 40%

| Peptides                    | 2008 cells<br>(10 µM) | C13* cells<br>(10 µM) | A2780 cells<br>(10 µM) | A2780/CP cells<br>(10 µM) |
|-----------------------------|-----------------------|-----------------------|------------------------|---------------------------|
| <b>5-FU</b>                 | 35.5 ± 5              | 46.5 ± 4              | 41.5 ± 4               | 56.4 ± 4                  |
| <b>LR</b>                   | 49.8 ± 4              | 60.8 ± 8              | 47 ± 11                | 58.1 ± 11                 |
| <b>LR-NH<sub>2</sub></b>    | 62.3 ± 10             | 85.2 ± 5              | 77.8 ± 4               | 65.3 ± 6                  |
| <b>[Ala<sup>7</sup>]LR</b>  | 75.2 ± 9              | 78 ± 11               | 60.6 ± 4               | 67 ± 6                    |
| <b>[Ala<sup>8</sup>]LR</b>  | 78.5 ± 6              | 76.5 ± 4              | 64 ± 3                 | 68.2 ± 9                  |
| <b>[DLeu<sup>1</sup>]LR</b> | 68.6 ± 4              | 74.6 ± 6              | 66.4 ± 5               | 79 ± 7                    |
| <b>[DSer<sup>2</sup>]LR</b> | 82.5 ± 2              | 82.5 ± 8              | 76.6 ± 8               | 69.1 ± 5                  |
| <b>[DGln<sup>4</sup>]LR</b> | <b>25.2 ± 5</b>       | <b>48.2 ± 9</b>       | <b>34.1 ± 3</b>        | <b>53.3 ± 7</b>           |
| <b>[DLeu<sup>5</sup>]LR</b> | 71.5 ± 1              | 77.1 ± 3              | 74.1 ± 7               | 81.1 ± 9                  |
| <b>[DTyr<sup>6</sup>]LR</b> | 85.4 ± 4              | 83.6 ± 6              | 73.6 ± 6               | 73.2 ± 10                 |
| <b>[DGln<sup>7</sup>]LR</b> | 91.2 ± 7              | 90.2 ± 5              | 61.2 ± 6               | 85.8 ± 7                  |
| <b>[DArg<sup>8</sup>]LR</b> | 82.7 ± 5              | 82.4 ± 8              | 56.8 ± 5               | 82.6 ± 9                  |
| <b>LR(1-6)</b>              | 73.4 ± 11             | 65.5 ± 5              | 81.5 ± 3               | 82.2 ± 8                  |
| <b>LR(1-5)</b>              | 74.4 ± 6              | 88.2 ± 4              | 83.6 ± 8               | 81.6 ± 5                  |
| <b>LR(2-6)</b>              | 74.6 ± 5              | 70.2 ± 7              | 65.2 ± 4               | 63.5 ± 8                  |
| <b>LR(3-7)</b>              | 76 ± 7                | 80.1 ± 4              | 67.2 ± 5               | 78.5 ± 6                  |

### Gromacs parameter files for d-amino acids

In *aminoacids.dat*, the three letter codes for respective *d*- amino acids were added.

In *ffgm.x.rtp*, the following lines were added.

for **dCys**:

[ atoms ]

```
N  N -0.280  0
H  H  0.280  0
CA CH1 0.000  1
CB CH2 0.000  2
SG  S -0.064  3
HG  HS 0.064  3
C   C  0.380  4
O   O -0.380  4
```

[ bonds ]

```
N  H
N  CA
CA  C
C   O
-C  N
CA  CB
CB  SG
SG  HG
```

[ impropers ]

```
N  -C  CA  H
-C -CA  N  -O
CA  C   N  CB
```

for **dGln**:

[ atoms ]

```
N  N -0.280  0
H  H  0.280  0
CA CH1 0.000  1
CB CH2 0.000  2
CG CH2 0.000  3
CD  C  0.380  4
OE1 O -0.380  4
NE2 NT -0.830  5
HE21 H  0.415  5
HE22 H  0.415  5
C   C  0.380  6
O   O -0.380  6
```

[ bonds ]

```
N  H
N  CA
CA  C
C   O
-C  N
CA  CB
CB  CG
CG  CD
CD  OE1
CD  NE2
NE2 HE21
NE2 HE22
```

[ impropers ]

```
N  -C  CA  H
-C -CA  N  -O
CA  C   N  CB
CD  OE1  NE2  CG
NE2 HE21 HE22  CD
```

## **2.2. Introduction to manuscript: An Integrated Proteomic and Bioinformatic Approach to the Definition of the pharmacodynamic profile of a Peptide inhibiting ovarian cancer growth**

In the following work (F. Genovese et al., under submission) a preliminary study of the mechanism of action of the LR peptide was performed exploiting mass spectrometry analysis of whole cell proteome. From the integration of proteomics, bioinformatics, and western blot validation emerged a panel of proteins differentially expressed after the treatment with these peptides. This protein profile could be useful to monitor the LR octapeptide efficacy during early drug development process.

We also introduced a known drug as a reference drug, Pemetrexed, an antifolate drug that competes with the natural substrate of TS, DHFR, and other folate proteins, inhibiting their activity. Pemetrexed (PMX) is currently under clinical studies for the treatment of ovarian cancer as second line (*see section 1.8.1.4.* ).

I contributed to this work by evaluating the biological effects of LR peptides on ovarian cancer lines and validating the protein expression of selected proteins in ovarian cancer cell lines, both cisplatin-sensitive and -resistant, after the treatment with LR and its more potent analogue [DGln<sup>4</sup>]LR.

### **2.2.1. Background**

Although several therapies are currently under evaluation in clinical trials, drug discovery remains a difficult and long process, and one of the main issues in late drug development is lack of efficacy. Since most of these candidates may fail in late phases of clinical trials, one way to diagnose poor drug efficacy at an early stage is to monitor the effects of the drug candidates in proper cell disease models, primary culture cells and, finally, in animals. In this context, the most important challenge is to check early phase biomarkers in the drug discovery. They are more predictive and informative activity indicators for the evaluation of cytotoxic effect. Indeed, they are useful both to understand the mechanism of action of the drugs candidates and to monitor the candidate efficacy during the preclinical drug development process (process monitoring). By studying the early phase biomarkers, a candidate drug can also be stopped before entering into the clinical phase if it does not meet the pre-specified criteria for continuation.

### 2.2.2. Aims

The aim of this multidisciplinary approach, based on proteomic and bioinformatics studies, was to define a protein panel of pharmacodynamic profile of LR and its analogue to be used in the early drug discovery process phase as a more predictive and informative activity indicator than the cytotoxic effect.

### 2.2.3. Results

In this study, the biological effect of LR and its analogue [DGln<sup>4</sup>]LR were evaluated on A2780 cisplatin-sensitive, IGROV1 cisplatin-sensitive and A2780 cisplatin-resistant (A2780 CP), ovarian cancer cell lines. Previous cytotoxicity assays showed that LR, an allosteric inhibitors of thymidylate synthase, (*see section 1.8.1.5.*) inhibits hTS activity and alters the cell growth, inducing a cytotoxic effect. A recently developed derivative of LR, [DGln<sup>4</sup>]LR, also showed the same inhibition profile but it is a more effective cytotoxic agent (*see section 2.1*).

In particular, peptides cytotoxicity was evaluated in human ovarian carcinoma cell line IGROV1, according to *section 2.1*. The cytotoxicity values were calculated by comparing cultures exposed to the drug to untreated (control) cultures. After treating for 72 hours, the cell growth inhibitory activity exhibited by the peptides was determined using a crystal violet dye assay; the dye extracted from the assay was proportional to the cellular biomass. The tested compounds were administered to cancer cells by exploiting a specific peptide delivery system that did not alter cell growth. Previous cytotoxicity studies, showed in *section 2.1*, against four cisplatin-sensitive and -resistant ovarian cancer cell lines (2008; A2780 cells and C13\*, A2780/CP (cDDP)-resistant counterpart sublines) demonstrated the general improved effectiveness of [DGln<sup>4</sup>] LR, compared to LR and 5-FU. Moreover, LR analogue exhibited noticeable growth-inhibitory activity against all four cell lines, when compared to untreated control cells. Also, cytotoxicity studies against IGROV1 cisplatin-sensitive ovarian cancer cell line demonstrated the same result. Figure 2.2.3.1. shows that the effectiveness of this peptide against IGROV1 was similar to the activity of LR and 5-FU, as previously observed in A2780/CP cisplatin resistant cells (*see section 2.1*). In particular, approximately 50% of the IGROV1 cisplatin resistant cells survived the treatment with [DGln<sup>4</sup>] LR. In particular, [DGln<sup>4</sup>] LR displayed superior activity against the cDDP-sensitive A2780 (*see section 2.1*).

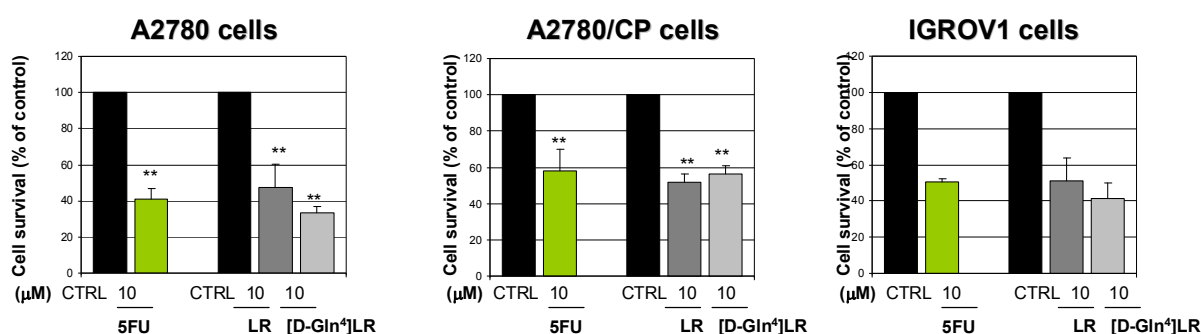


**Figure 2.2.3.1.** Inhibition of cell growth by the LR and [D-Gln<sup>4</sup>] LR, for cDDP-sensitive and -resistant cells. The peptides were transfected into A2780, A2780/CP and IGROV1 cells via a peptide delivery system. The effects of 10 μM peptides was compared to the same concentration of 5-FU. Cell survival percentages are the mean ± S:E:M: of at least three separate experiments performed in duplicate. Statistical significance was estimated by ANOVA test; a difference was considered to be significant at \* $P < 0.05$  or \*\* $P < 0.01$ .

In order to reach the aim of the present work, other colleagues of my research group developed a method based on the definition of a Protein Profile after the treatment with our peptide, and for identification of the metabolic pathways perturbed by the activity of the candidate drug purposing to hypothesize its mechanism of action.

A mass-spectrometry (MS) based semi-quantitative approach of the cellular proteome was the starting point to determine the proteins modulated by the treatment with the peptide; the global interpretation of data obtained in this study was driven by bioinformatics, through the integration of the interaction data from public domain repositories and pathway and molecular function annotation. In particular, 160 differentially expressed proteins are significantly modulated by LR treatment, and, among these, 64 proteins are connected by biological/physical interaction. After then, 4 main clusters of deregulated protein by LR treatment were generated: Cluster A, consisting mainly of ribosomal proteins, cluster D, which includes members of the proteasome complex (PSMB5, PSMC2, PSMC3, PSMD5, PSMD7), Cluster C that consists of ribonucleoproteins (SNRPA, SNRPA1, HNRNPC, SRSF7) and Cluster B, holding mostly down-regulated proteins of the original set, involved in pyrimidine ribonucleotide metabolic process, RNA and protein transport. Since our peptide targets the proteins involved in the folate pathway, which are low-abundance proteins at the cellular level, thus not detectable through mass spectrometry, the folate pathway-proteins and the proteins involved into the PMX mechanism of action, were connected to the deregulated proteins identified. Indeed, four folate/PMX-related proteins were identified as being highly connected via protein-protein interactions to the original set of LR peptide modulated proteins, being ATIC, DCK, MGMT and SHMT1. Therefore, among these proteins, which are identified as potentially involved in the action of LR, on the basis of the literature and

results of our proteomics and bioinformatics, several proteins were chosen to define a protein panel which could give information about the effects of peptides. The proteomic/bioinformatic approach showed that protein deregulations after treatment with LR can be mainly assigned to processes or functions, such as regulation of translational initiation, termination of RNA Pol-II transcription, transport, and proteasome. From this DPP approach, stress-related proteins TRAP1, HSP90AA1, and the eukaryotic translation initiation factor EIFS1 were selected as interesting proteins. In particular, TRAP1 is a mitochondrial chaperone, which has been described as an interesting target for ovarian cancer treatment while HSP90AA1 belongs to the same chaperone family and has an important role in protein homeostasis and cancer biochemistry. EIF2S1, eukaryotic translation initiation factor 2 subunit 1, is an important translation factor which catalyzes the first regulated step of protein synthesis initiation, coordinating the binding of the initiator tRNA to ribosomal subunits as ribosomal proteins were heavily modulated by LR treatment. The second group of proteins selected for validation refers to folate molecular partners, showing significant interactions with DEPs of our dataset, namely ATIC, GART and MGMT. WB assays in this case aimed to highlighting any biological effect which might arise from LR direct and indirect modulation of other folate pathway proteins, which could reasonably take place in addition to LR/[D-Gln<sup>4</sup>]LR hTS inhibition

Additionally, TS and DHFR were included as known targets for both our peptides and PMX, while MTHFR and SHMT1 levels were tested to monitor the folate pathway down-stream enzymes.

In order to validate the results obtained by the integrated proteomics and bioinformatics approach, we assessed previously selected protein expression levels by using the Western Blot assay.

Ours result showed that TS, DHFR, TRAP, HSP90, ATIC, GART proteins were similar regulated, while other selected proteins showed a different modulation in the three cell lines after the treatment with both peptides. However, while the treatment with both peptides induced a similar modulation of selected protein in the same cell line, it differed among the three cell lines.

In particular, as expected by previous observations (*see section 1.8.1.5.*), TS levels remain essentially unchanged or decrease after LR treatment, while DHFR levels appear to be always down-regulated. A similar pattern is observed after [D-Gln<sup>4</sup>]LR treatment.

Also, the expression levels of the stress-related chaperones TRAP-1 and HSP90AA1 decrease

in each of the three cell lines upon the above inhibitors treatment. Instead, the folate cycle proteins, SHMT1 and MTHFR were differently modulated by the peptide treatment. SHMT1 was found mainly up-regulated, excepted in A2780/CP cells treated with LR. The expression levels of MTHFR decrease in IGROV1 cells upon LR treatment, and increase in the other cell lines upon the treatment with the two peptides.

The expression of three enzymes directly linked to the folate cycle and indicated by the bioinformatic analysis as nodal proteins, significantly interacting with LR-modulated proteins (ATIC, MGMT and GART), were also assessed. While ATIC levels were always found decreased upon both peptides treatment in all the three cell lines, the GART levels are mainly increased after treatment with LR and its analog. Inhibitors effect on MGMT expression differs in the three cell lines, although in IGROV-1 and A2780/CP cell lines, inhibitors affect the protein more similarly.

At the same time, the biological effect of LR peptide was compared with pemetrexed (PMX)'s one. Results of cell growth inhibition assay showed that A2780, A2780/CP and IGROV1 appeared to be sensitive to the treatment with LR peptides, while only a low cytotoxic effect was observed after 48 hour from the treatment with 20  $\mu$ M Pemetrexed in all the cell lines tested. This mild response could be attributed to the rapid development of a mechanism of resistance. In fact the levels of TS and DHFR, which are known targets of PTX, were increased after 48 of treatment with pemetrexed in all cell lines tested. In particular, TS expression is a feature often observed in resistance to the common antifolate drugs. Furthermore, the levels of GART, another known target of PMX, decreased in all the cell lines tested.

On the contrary, the peptidic inhibitors have an opposite effect than PMX on the three folate-cycle proteins. Opposite effects between the peptidic inhibitors and the anti-folate drug PMX were clearly observed also for the stress-related proteins TRAP-1 and HSP90AA1. MGMT expression, instead, is clearly down-regulated, as a consequence of the treatment with PMX on each cell line considered. Finally, the treatment with PMX does not appear clearly affect the expression levels of the other selected proteins, considering the different results in the three cell lines tested.

#### 2.2.4. Conclusions

Our results showed that at least six out of the ten chosen proteins, TS, DHFR, TRAP, HSP90, ATIC, GART, have a similar modulation pattern for [DGln<sup>4</sup>] LR and LR. We hypothesized that this behavior could reflect also a similar mechanism of action of the two peptides.

In particular, our results show that TS and DHFR modulation pattern is the same for [DGln<sup>4</sup>] LR and LR, and both differ from that of PMX. Protein expression levels of SHMT1 and MTHFR are modulated, probably as a consequence of metabolic adaptation to thymidine depletion.

In conclusion, the adopted proteomic/bioinformatic approach yielded a protein set that included ten selected proteins, which was tested against a known antifolate drug and validated with a lead candidate analog (Figure 2.2.4.2.). Noticeably, [D-Gln<sup>4</sup>]LR protein profile fits almost completely with LR profile, suggesting that the two compounds could have a similar mechanism of action. Comparison with the protein profile modulated by the antifolate drug PMX supports the hypothesis that, although having the same main target (hTS), our peptides exert their action in the cell with a different mechanism. As a further development of this work, we plan to adopt the discovered protein panel to monitor the pharmacological behavior of other LR analogs in different OC cell lines and, reasonably, to extend this approach to primary cell culture to assess the candidate drugs efficacy.

Experimental procedures and more detailed information have been described in detail in the under submission version of the article reported below, in the *section 2.2.5*.

In the following panel (Figure 2.2.4.2.) the conceptual summary of the work has been reported.

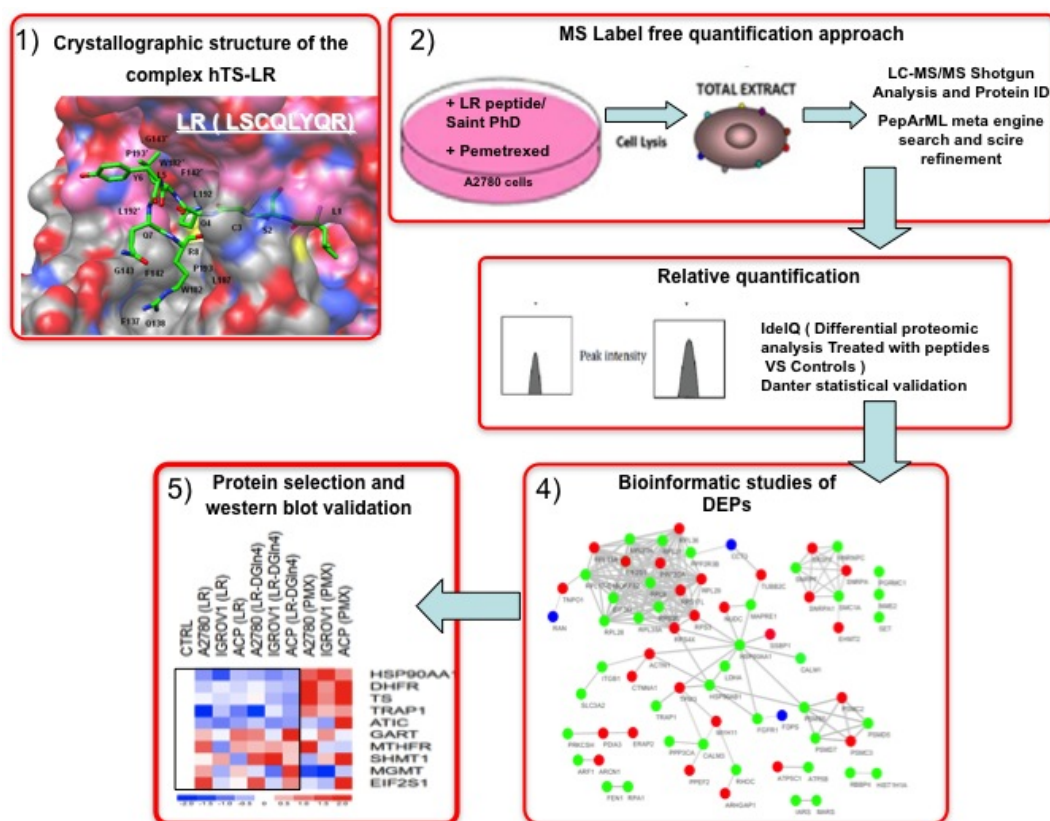


**Figure 2.2.4.1:** In this panel, the main results of the proteomic and bioinformatic integrated study for the definition of a pharmacodynamic profile of LR on OC cell lines were described.

Crystallographic structure of the complex hTS-LR, shown in Figure 1, was the starting point for a deeper investigation of LR peptide mechanism of action.

A mass-spectrometry (MS) based semi-quantitative approach of the cellular proteome, shown in Figure 2 and 3, determined the proteins modulated by the treatment with the peptide. Results were then integrated by a bioinformatics analysis, so that a panel of proteins differentially expressed (DEPs) after the treatment with the peptides emerged, as shown in Figure 4, which displays direct interactions among LR peptide modulated proteins. Up-regulated proteins are colored in red, while down-regulated proteins are colored in green. The three proteins with isoforms being both, up- and down-regulated, are depicted in blue.

In this work, I contributed confirming the modulation of the protein profiles (DEPs) emerged by the integration of data from proteomics and bioinformatics by western blot assay in three OC cell lines. Global results are collected in Figure 5, in which is shown a red-to-blue heatmap to display the numerical values corresponding to the expression of the ten selected proteins among A2780, IGROV1, and A2780/CP (ACP) OC cell lines after treatments with peptides LR, [DGln<sup>4</sup>]LR, and the reference drug, Pemetrexed (PMX).

Red colors are related to the proteins up-expression, while the blue colors to their down-expressions. Rows are standardized subtracting CTRL and divide by SD. The range of the standardized values displayed is  $\pm 2$ . Up- and down- expression fold changes colours and the correspondent values are indicated on the scale under the heatmap.

TS, DHFR, TRAP, HSP90, ATIC, GART proteins have a similar modulation pattern for [DGln<sup>4</sup>] LR and LR.

## 2.2.5

### **An Integrated Proteomic and Bioinformatic Approach to the Definition of the pharmacodynamic profile of a Peptide inhibiting ovarian cancer growth.**

*(submitted to Journal of Molecular and Cellular proteomics)*

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#### Abbreviations

AMBIC, Ammonium Bicarbonate; DEPs, Differentially Expressed Proteins; FA, Formic Acid; FBS, Fetal Bovine Serum; GFP, human Glu-1-Fibrinopeptide B; GO, Gene Ontology; HBS, HEPES Buffered Saline; FDR, False Discovery Rate; FMOC/tBu, Fluorenylmethyloxycarbonyl/*tert*-butyl; HSP, heat shock protein; hTS, human Thymidylate Synthase; KEGG: Kyoto Encyclopedia of Genes and Genomes; OC, Ovarian Cancer; PMX, Pemetrexed; RIPA, RadioImmunoPrecipitation Assay (buffer); RPMI, Roswell Park Memorial Institute (medium); TRAP1, TNF receptor-associated protein-1; TRIS-HCl,

Tris(hydroxymethyl)aminomethane hydrochloride; WB, Western Blot; XIC, eXtracted Ion Chromatogram.

## Summary

Ovarian cancer (OC) second-line therapy of relapsing forms is a daunting challenge, as current pharmacological approaches suffer from rapid resistance development. Recently, a new class of peptidic inhibitors of a key enzyme involved in the folic acid metabolism, Thymidylate Synthase, has been identified, binding the interface of the dimeric enzyme thus showing an innovative mechanism of inhibition which could overcome the limits of current drugs.

*In-vitro* behavior of LR, the lead octapeptide, and its cytotoxicity towards ovarian cancer cells have not been extensively studied yet; in this article, we suggest and validate a method aimed at defining a protein panel to be used in the early drug discovery process phase as a predictive and informative indicator of the peptide effect.

A profile of differentially expressed proteins of OC cell lines following treatment with LR octapeptide was drawn by preliminary blind semi-quantitative analysis of the cellular proteome, followed by bioinformatics-guided interpretation of the results and western blot validation of biologically relevant targets and nodal proteins of the folate pathway which display strict interactions with our original dataset of LR peptide modulated proteins (TS, DHFR, GART, SHMT1, MTHFR, MGMT, ATIC, TRAP1, HSP90AA1, EIF2S1).

Identified proteins were confirmed by western blot analysis and validated on a more active LR analog, [DGI<sup>n</sup>]<sup>4</sup>LR. A broader picture of the mechanism of action was also hypothesized by clustering the annotation features of the proteins modulated in cells treated with our lead candidate peptide.

The results confirm that this methodology provides an approach to the identification of a pharmacodynamic profile to monitor candidate drugs activity at the early phases of the drug development process. Different expression level changes observed consequently to LR treatment in comparison with an antifolate currently used in therapy, pemetrexed, are in agreement with the hypothesized different mechanism of action.

## Introduction

Ovarian Cancer (OC) is a major threat for women population; to date, the survival rates for advanced stage forms are very low, due to late diagnosis and to inadequate efficacy of standard first-line treatments, which currently include surgery and systemic chemotherapy. Platinum drugs, alone or in combination with a taxane, remain the mainstay of pharmacological treatment for high-grade serous cancers. However, initial treatment fails in

about 20% of patients and, among responding ones, the majority relapses within two years and progressively develops drug resistance (1). Several evidences demonstrate that inadequate drug concentration reached at the tumor site is one of the factors leading to the onset of untreatable drug-resistant ovarian cancer (2).

Thus, many efforts to improve survival of patients affected by OC are focused on the development of more effective systemic therapies and on the search for new therapeutic targets. Drug discovery is, however, a difficult and long process and, usually, mandatory steps include developing early phase drug leads and selecting them for the late pre-clinical phase/early clinical trials; one of the main issues in late drug development is lack of efficacy (3). While, in many cases, efficacy in pre-clinical models is easily observed, late preclinical and, often, early clinical drug development phases, suffer from a high failure rate, with consequent deep losses in efforts and costs. One way to diagnose poor drug efficacy at an early stage is to monitor the effects of the drug candidates in proper cell disease models, primary culture cells and, then, in animals. Observational parameters in pharmacokinetic studies are usually obtained by a classical approach, but often the specific effect of the candidate drug under study might not be clearly identified. In this context, a panel of markers of the drug lead activity might be a powerful tool in pushing the drug development process forward.

We applied this principle to a new class of peptidic drug candidates which showed a novel mechanism of action and efficacy in inhibiting ovarian cancer cell growth (4). The main validated molecular target of this inhibitor class is human Thymidylate Synthase (hTS), a homo-dimeric enzyme which plays a key role in DNA synthesis (5). Current drugs that target hTS (*e.g.*, 5-fluorouracil, pemetrexed and raltitrexed) suffer from toxicity and induce the development of resistance mechanisms, making the pharmacological treatment ineffective (6). Our group has designed a set of peptides to specifically target the hTS dimer interface. These compounds exhibited an innovative mechanism of inhibition, which relies on the stabilization of the di-inactive form of the enzyme (4). The peptide with the best activity profile, LR (LSCQLYQR), inhibits the intracellular enzyme in cisplatin-sensitive and -resistant OC cell lines without causing protein overexpression typical of the acquired resistance to pemetrexed and other substrate analogs, thus showing the potential of this innovative hTS inhibition strategy to overcome the limitations of OC chemotherapy (4).

Indeed, LR is an allosteric inhibitor of thymidylate synthase, it binds to the protein interface of this homodimeric enzyme and stabilizes its di-inactive form (2), as shown by X-ray crystallography (4). Pemetrexed and other substrate analogs, such as Raltatrexed and 5-FU bind at the protein active site (7), . This effect inhibits the intracellular hTS activity and alters the cell growth inducing a cytotoxic effect, with an inhibition rate of 50-60 % at 10  $\mu$ M after 72h on different ovarian cancer cells (8). cDDP sensible human ovarian carcinoma cells, A

recently developed derivative of LR, [DGln<sup>4</sup>]LR, obtained by a L to D configuration change at position 4, showed the same inhibition profile but is a more effective cytotoxic agent (70-75% inhibition of OC cells at 10  $\mu$ M) (8).

Highthroughput -omic techniques have been widely used in biomedical research in order to identify novel biomarkers for diseases, to decipher pathophysiological mechanisms, or to investigate the toxicological effect of chemical compounds during the drug discovery process. As the 'one drug one target' paradigm is more and more replaced by a poly-pharmacological conception of the effects of drugs, -omics technologies represent a powerful tool in deciphering the drug mode of action, especially in early drug discovery phases (9).

In ovarian cancer, a number of -omics studies have focused on the identification of biomarkers and classifiers in order to discriminate patients from healthy controls or patients showing fast relapse after platinum-based drug treatment (*e.g.*, 10,11). Only few studies focus on the discovery of drug activity markers at the cancer cell level or from primary tumors and, to our knowledge, no work has been published yet concerning the effects of an anticancer candidate drug during the early drug discovery phase. Label-free proteome quantification has found many applications in the study of tumor biology (12, 13), providing a wide picture of proteins related to a specific pathological state, biomarker signature, and an insight into the molecular mechanisms involved.

As a part of a wider investigational study on the biological effect and on the identification of a possible mechanism of action of the LR octapeptide, the present work aims at designing and validating a protein profile set which can serve as a biomarker of this drug candidate pharmacodynamic behavior, that could be used during the early drug development process. A mass-spectrometry (MS) based semi-quantitative blind approach was the starting point to determine, at a cellular proteome level, which molecular events are triggered directly or indirectly by this new class of hTS inhibitors; successive label-free quantitative proteomics highlighted a set of significantly differentially-expressed proteins which are modulated by our lead compound compared to a non-treated control group of epithelial OC cell lines.

The global interpretation of the data obtained in this study was driven by bioinformatic and consisted in integrating interaction data from public domain repositories in conjunction with pathway and molecular function annotation. The protein set was further expanded to include functional neighbors which show strong correlations with our original protein panel and folic acid molecular partners, as the LR peptide has been shown to interact with some important enzymes related to folate metabolism (4). This kind of approach has been successfully used for large dataset interpretation (14,15).

Differentially-expressed proteins which show a strong biological meaning and proteins emerging as highly connected to our protein dataset from bioinformatic analysis, including

folate pathway-related critical nodes, were validated through standard Western blot analysis on three different ovarian cancer cell lines.

The same set of proteins was monitored on OC cells treated with a more effective LR analog ([D-Gln<sup>4</sup>]LR) for validation purposes, and with a well-known anti-folate drug characterized by a different hTS inhibition mechanism, pemetrexed (PMX), with the aim of evaluating its proteomic behavior compared to our lead peptide. The use of folate-like inhibitors in OC has been recently reviewed by Walters and coworkers; the authors conclude that antifolates show a promising antitumor activity against platinum-resistant ovarian malignancies coupled with a favorable toxicity profile, although resistance development is still a concern (7). Our findings support the hypothesis that the identified peptides show a different protein profile modulation compared to a classical TS-targeting drug, which might reflect a different mechanism of action that does not include hTS overexpression and therefore represent a promising tool for the development of more potent and low drug resistance potential.

## **Experimental Procedures**

### *1. Cell Culture and Treatment*

A2780, its cisplatin-resistant counterpart A2780/CP and IGROV1 ovarian cancer cell lines were cultured in RPMI 1640 medium (Lonza), supplemented with 10% FBS and 200 mM L-Glutamine. Cells were incubated at 37°C, 5% CO<sub>2</sub> for at least 24 hours before the treatment with the peptides.

LR (LSCQLYQR, purity > 99%) and its [D-Gln<sup>4</sup>] analog (LSCqLYQR, purity > 99%) were obtained by canonical solid phase peptide synthesis on a Wang resin using Fmoc/t-Bu chemistry (16); crude peptides were further purified by standard preparative RP-HPLC.

Membrane barrier crossing of the peptides was promoted by the SAINT PhD delivery system (Synvolux Therapeutics, NL), according to the standard transfection protocol; a 0.8 mM stock solution of the peptide was prepared in HBS and stored at -80°C before use. For each treatment, delivery system-peptide complexes were prepared adding 20 µL/mL of SAINT PhD to a diluted peptide solution to reach a final LR/[D-Gln<sup>4</sup>]LR sub-cytotoxic dose of 5 µM. After the culture medium was aspirated from the cells, the SAINT-PhD/peptide complex was added to the wells and incubated (37°C; 5% CO<sub>2</sub>); complete RPMI was added after 4 hours. Cells were treated for 48 hours, then the culture medium was aspirated and cells were lysed for protein extraction. A delivery system solution at the same concentration was used as the control treatment.

For pemetrexed cell treatment, a 2 mM stock solution was obtained solubilizing the drug in DMSO and stored at -20°C before use. The drug was diluted to a 20 µM final concentration of PMX in complete RPMI medium and cells were incubated for 48 hours. Cells treated with 1% DMSO were used as control.

## 2. Proteomic Analysis

Cells were washed twice with ice-cold PBS and total proteins were extracted directly in the plate by scraping the surface in RIPA lysis buffer (50 mM TRIS-HCl, pH 7.4; 150 mM NaCl; 1% NP-40; 2 mM EDTA; 2 mM PMSF; 5 µg/mL leupeptin; 5 µg/mL pepstatin). Cell lysates were then precipitated with 4 volumes of cold acetone, left overnight at -20°C, resuspended with 30 µl 8 M urea solution and the protein concentrations, determined by a standard Bradford assay against bovine serum albumin, were normalized with deionized water to a final concentration of 1 µg/µl. 12 µg of protein extract per condition were reduced with DTT (10 mM, 1 µl in AMBIC 100 mM) at 56°C for 30 minutes and alkylated with iodoacetamide (55mM, 1 µl in AMBIC 100 mM) at room temperature in the dark for one hour; the alkylating agent excess was quenched with 10 µl DTT 10 mM, which was left reacting at room temperature for 10 minutes. After proper dilution of the urea concentration up to 2 M with AMBIC 10 mM, the resulting protein mixtures were digested with TPCK-modified sequencing grade trypsin (1:50 w/w enzyme to substrate final ratio) at 37°C overnight. Samples were then acidified with 1 µl 5% formic acid (FA) solution and dried in a vacuum evaporator. Peptides were resuspended in 30 µl of 1% FA/acetonitrile 98:2 solution spiked with Glu-1-Fibrinopeptide B (sequence: EGVNDNEEGFFSAR, Sigma Aldrich) as an internal standard, at a final concentration of 25 nM. Analyses were performed on an ESI-Q-TOF Accurate-Mass spectrometer (G6520AA, Agilent Technologies), controlled by MassHunter (v. B.02.00) and interfaced by a CHIP-cube to an Agilent 1200 nano-pump.

Chromatographic separation was performed on a high capacity loading chip, with a 75 µm I.D., 150 mm, 300 Å, C18 column, prior to a desalting step through a 500 nL trap column; injected sample (2µl, 0.8µg) was loaded on the trap column with a 4 µl/min 0.1% FA:ACN 98:2 phase; after 3min, the precolumn was switched in-line with the nanoflow pump (400 nl/min, phase A: water:ACN:FA 96.9:3:0.1, phase B: ACN:water:FA 94.5:5:0.1), equilibrated in 3% B. The peptides were eluted from the RP column through the following gradient: 3-45% B over a period of 75 minutes, 45–90% in 10', 90% B hold for 5', and back to 3% B in 8', for a total runtime of 110 minutes, including a 10' post-run reconditioning step. Each biological replicate was run three times; analytical controls (a mix of baker's yeast enolase and bovine serum albumin tryptic digests) were run twice a day to monitor chromatographic performances.

Centroided MS and MS<sup>2</sup> spectra were recorded from 350 to 1700 m/z and 50 to 1700 m/z, respectively, with a scan rate of 6 and 3 Hz. The eight most intense multi-charged ions were selected for MS<sup>2</sup> nitrogen-promoted collision induced dissociation; collision energy was

calculated according to the following expression:  $CE_{(v)} = 4 \left( \frac{m}{z} \right) - 3$ . A precursor active exclusion of 0.2 minutes was set; detector was operated at 2 GHz in extended dynamic range mode. Mass spectra were automatically recalibrated with two reference mass ions.

Raw data, converted from the vendor's data format into mzXML by msconvert from the Proteowizard toolbox (17), were searched against human IPI database (v. 3.82, 92104 entries; GFP internal standard sequence was appended) with PepArML, v. 1.1, a meta protein database search engine which, combining and interpreting the output of several types of peptide identification software, provides a significant increase in the number of peptide-to-spectrum matches and confidence, thanks to a machine-learning algorithm which discriminates true and false identifications (18). Search parameters were: 100 ppm precursor tolerance, 0.2 Da fragment mass error allowed, trypsin with semi-specific cleavage, carbamidomethyl as fixed modifier of cysteine residues, methionine oxidation, lysine and peptide N-terminus carbamylation as variable modifications; false discovery rate was estimated through an internal peparmml double decoy database search.

PepArML output underwent protein prophet validation for protein inference (19); only hits with at least two significantly scoring peptides or siblings per protein and a score > 0.9 were quantified. Ambiguous protein isoforms with the same score were all taken into account. No "one-hit-wonder" was included.

### 3. *Label-free relative quantification*

PepArML search results were cross-related to the corresponding MS1 profiles (mzXML format) by the quantitative software Ideal-Q, v. 1.024 (20); pooled search unique hits were manually validated and quantified on the *moverz*/RT 2D map. A non-endogenous peptide was used as an internal standard (GFP) for inter-run normalization. Peptide validated XIC area data were exported and elaborated through the DanteR tool (21); briefly, peak areas were Log<sub>2</sub> transformed, normalized (central tendency), then corresponding protein intensities were obtained from the peptides through an analysis of variance-based peptide roll-up of the most abundant 10 peptides per protein; entries with a p-value < 0.05 and a protein ratio < 0.5 or > 2 compared to the control group were considered as significantly down- or up-regulated by our lead candidate, respectively.

### 4. *Bioinformatic network analysis of differentially expressed proteins (DEPs)*

All significant DEPs were annotated with ENSEMBL Protein and Gene IDs using the ENSEMBL BioMart tool, as well as annotation from UniProtKB and NCBI gene databases. All the successive analyses are based on ENSEMBL IDs; official Gene Symbols are used in all graphical representations.

A network of direct protein-protein interactions between all members of the DEPs set was generated using data from three protein-protein interaction databases, namely BioGRID, IntAct, and Reactome. Interaction data from these three sources are consolidated in the omicsNET protein dependency graph framework, which takes into account direct protein-protein interactions and computationally calculated dependencies, based on shared pathway information, gene ontology information and common protein domains (22, 23 ).

In a second step, we extended the set of DEPs via an inter-neighbor expansion method, adding proteins to the initial set of DEPs having at least two interactions with functional neighbors, as determined by BioGRID, IntAct and Reactome interrogation; rationale for this expansion is to identify proteins of relevance due to their strong connectivity to members of the DEPs set which were, however, too diluted to be identified by mass spectrometry (24). The MCODE algorithm was further applied to the resulting network in order to identify highly connected functional complexes for further investigation (25).

#### *5. Bioinformatic analysis of folate pathway members*

In order to probe the effects of our lead candidate drug on the folate pathway members, we consolidated a set of folic acid-related proteins; this set was obtained from the Reactome pathway database (26), extracting all members of the “Metabolism of folate and pterines” pathways, as well as querying the gene ontology database for terms containing the substring “folate” or “folic acid”. In addition, we included protein targets of the drug pemetrexed as available in DrugBank (27). Both differential expression of these genes and proteins in the network neighborhood were investigated; the obtained list of folate molecular partners will be provided as Supplementary Information.

#### *6. Western Blot Analysis*

Cells were washed twice in ice-cold PBS, lysed in RIPA buffer (20 mM TRIS-HCl pH 7.5, 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, Complete Mini Protease inhibitor cocktail (Roche), and Phosphatase Inhibitor Cocktail 1 and 2 (Sigma)) and, then, centrifuged at 14000 rcf at 4°C for 30 minutes to remove debris. The protein concentration of each lysate was determined using the Bradford Protein Assay Reagent (Sigma Aldrich). Forty micrograms of the cell extracts were loaded on a polyacrylamide gel after denaturation according to the method of Laemmli. After SDS-PAGE, blotting was performed on PVDF membranes (Hybond-P, Amersham). The membranes were blocked in non-fat dry milk (2%) in TBS buffer containing 0.1% tween-20 at room temperature for one hour. Primary antibodies were incubated overnight in non-fat dry milk (2%) in PBS buffer containing 0.1% tween-20. Antibodies used were: anti-GART (clone 4D6-1D5, Abnova, dilution 1:1000), anti-ATIC (clone F38 P7 H9, Abnova, dilution 1:1000), anti-TS (clone TS106, Abnova, dilution 1:500), anti HSP90AA1 (clone 4F10, Abnova, dilution 1:5000), anti TRAP1 (clone TR-1A, Santa Cruz Biotech, dilution 1:2000), anti-EIF2S1 (clone [EIF2a], Abcam, dilution 1:500), anti-β-Actin (Santa Cruz Biotechnology, Inc., dilution 1:5000), anti-MTHFR (clone 1G12, Abnova, dilution 1:1000) and anti-SHMT1 (clone 4F9, Abnova, dilution 1:1000). After washing, membranes were incubated with a horseradish peroxidase-conjugated with a sheep anti-mouse secondary antibody (Amersham Biosciences, dilution 1:5000) or with a horseradish peroxidase-conjugated sheep anti-goat antibody (Sigma Aldrich, dilution 1:8000) for 60 min at room temperature. Antibody staining

was performed with a chemiluminescence detection system (ECL Plus, Amersham) and the signal detected by X-ray film (Amersham). The quantitative densitometric analysis of the western blots was performed using an Epson Photo Perfection 4180 scanner (Epson) and ImageJ densitometry software, v. 1.47 (National Institutes of Health, Bethesda, MD). Selected bands were quantified based on their relative intensities. The protein expression levels in samples treated with LR, [D-Gln<sup>4</sup>]LR and PMX were calculated by dividing each value obtained after treatment with LR, [D-Gln<sup>4</sup>]LR and PMX by the control value. Differences in Expression of proteins among the three cell lines after the treatment with the peptides were graphically represented with the use of heat maps generated by dChip Software ([www.hsph.harvard.edu/cli/complab/dchip/](http://www.hsph.harvard.edu/cli/complab/dchip/)). The protein levels were normalized against the housekeeping protein, actin, and the results were compared to the control (cell lines treated only with the delivery system), given an arbitrary value of 1.

## Results

### *1. Proteomics and Label-free Peptide Quantification*

HPLC-MS/MS shotgun analysis of trypsinized cell lysates was chosen for proteomic analysis; no pre-fractionation step was used to avoid introducing further variables. As in data-dependent analysis the ions are selected for MS/MS sequencing in a stochastic way merely based on their intensity, the dataset of identified proteins is inevitably biased towards the most abundant proteins; dynamic exclusion was applied to maximize proteome coverage.

PepArML allowed a significant increase of the peptides identified across six different protein database search engines, with around 800 proteins per technical replicate on average. The whole set of peptides identified across all groups (22000 peptides, 3300 proteins) was filtered excluding proteins with a protein prophet score < 0.9, corresponding to a 1% FDR (error) as estimated from the sensitivity to selectivity ROC curve, and processed by means of the IDEAL-Q software. After retention time alignment of all runs, based on the most intense and highest scoring identifications, peptides passing the SCI (Signal-to-noise-ratio, Charge, Isotope distribution) validation were quantified by extracted ion chromatogram integration. Applying this four-levels validation, peptide quantification was performed on the whole dataset of identified peptides.

After chromatogram areas processing for statistical evaluation of the interfered protein relative amounts, a total number of 160 differentially expressed proteins was determined to be modulated by LR treatment compared to the control group (76 were up-regulated, while 84 were down-regulated). The lists of identified and LR modulated proteins is provided as Supplementary Information. The cell lines selected for MS analysis were A2780 cell lines from human ovarian carcinoma.

## 2. Bioinformatic network analysis of DEPs

Based on data from BioGrid, IntAct and Reactome regarding the proteins correlated to LR peptide cellular effects, 171 direct protein-protein interactions were extracted. Protein to gene ID conversion of the initial set of differentially expressed proteins yielded 148 unique gene products; 64 could be connected this way, leading to the biological protein-protein interaction network represented in Figure 1.

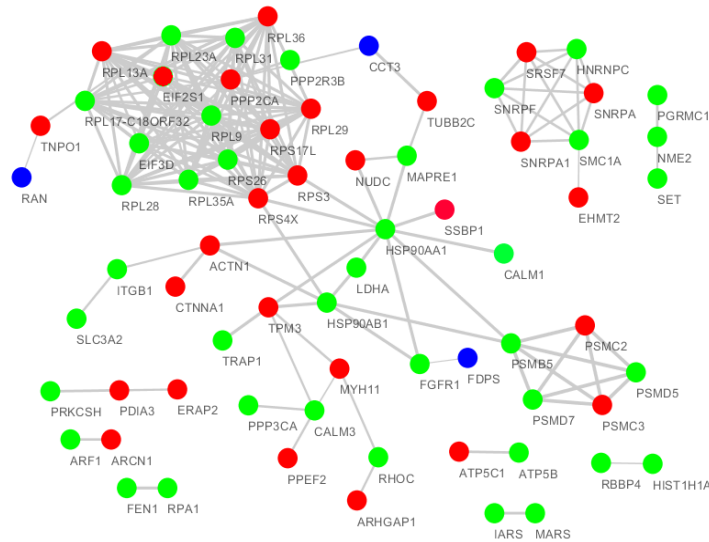


**Figure 1:** Direct interactions among LR peptide modulated proteins. Up-regulated proteins are colored in red, while down-regulated proteins are colored in green. The three proteins with isoforms being both, up- and down-regulated, are depicted in blue.

Three highly connected clusters are apparent: the first contains a number of ribosomal proteins (*e.g.*, RPL9, RPL29, RPL36) that show both up- and down-regulation. The second highly connected cluster of proteins contains members of the proteasome complex (PSMB5, PSMC2, PSMC3, PSMD5, PSMD7). The third cluster contains a set of ribonucleoproteins (SNRPA, SNRPA1, HNRNPC, SRSF7).

The inter-neighbor expanded network resulted in 6,013 edges linking in total 1,242 nodes. From the original set of 148 differentially regulated gene products, 124 showed significant interactions with at least two neighbors. Application of the MCODE algorithm on this biological network resulted in four strongly connected protein clusters, containing 23, 17, 7 and 5 proteins, respectively, represented in Figure 2.

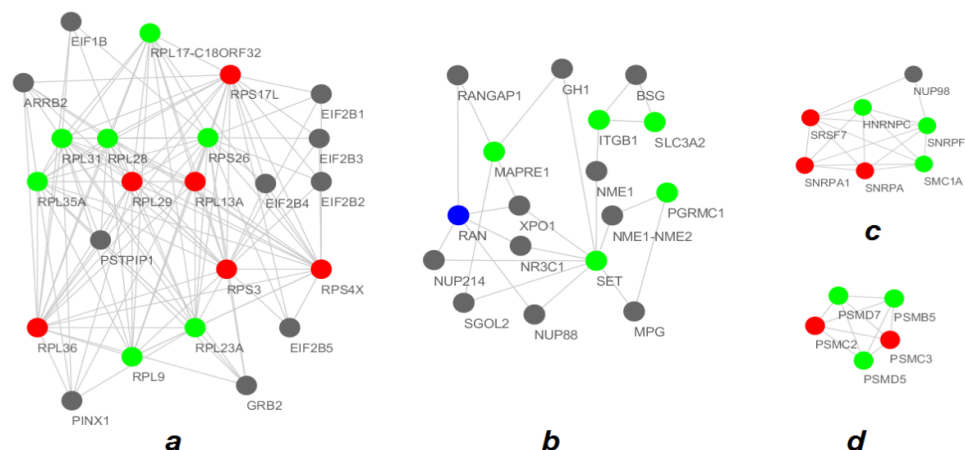


**Figure 2:** Set of four identified clusters of relevance correlating LR treatment DEPs to computationally expanded network neighbors.

On closer inspection, it becomes evident that cluster **a** overlaps strongly with the largest highly connected component of figure 1, consisting mainly of ribosomal proteins. Cluster **d** coincides completely with the second highly connected hub with members of the proteasome complex (PSMB5, PSMC2, PSMC3, PSMD5, PSMD7). Cluster **c** is related to the third cluster in figure 1 holding a set of ribonucleoproteins (SNRPA, SNRPA1, HNRNPC, SRSF7). Cluster **b** includes mostly down-regulated proteins of the original set involved in pyrimidine ribonucleotide metabolism, RNA and protein transport.

### 3. Bioinformatic analysis of folate pathway members

The bioinformatic analysis was conducted using the Reactome, GO and Drug bank databases. This study provided 46 proteins reported in the supplementary information. None of the 46 proteins extracted from the Reactome, GO and DrugBank databases was found to be differentially regulated. Then the correlation between folate-dependent proteins and members of the DEPs set was studied. OmicsNET, a software that allows the identification of biologically significant interactions among proteins, could not identify computationally inferred relationships with a score greater than 0.73 and therefore the conclusion was that no highly significant correlations between folate-associated proteins and members of the DEPs set were identified. The result of the extrapolation of significant interactions is represented in figure 3 as a graph holding 54 edges and 49 nodes (35 proteins from the original dataset and 14 folate-associated proteins). Four folate-related proteins, ATIC, DCK, MGMT and SHMT1, were found to be connected via protein-protein interactions with the original set of proteins modulated by the LR peptide.



Finally, hTS and DHFR (dihydrofolate reductase) were included as known targets for both our peptide analogs and PMX, while MTHFR and SHMT1 levels were tested to monitor these important folate pathway down-stream enzymes.

Table 1 summarizes the label-free quantification analysis output for directly modulated proteins and folate-related molecular partners among DEPs as determined by bioinformatic analysis.

Table 1. LR peptide directly modulated proteins and folate-related molecular partners among DEPs as determined by bioinformatic analysis.

| Accession ID                                             | Gene ID  | Unique peptides | Sequence coverage % | Protein Prophet score | LR/Ctrlratio |
|----------------------------------------------------------|----------|-----------------|---------------------|-----------------------|--------------|
| E9PM09_HUMAN                                             | CCT3     | 1*              | 21                  | 1                     | - ∞          |
| HNRPC_HUMAN                                              | HNRNPC   | 5               | 17.5                | 1                     | - ∞          |
| HS90A_HUMAN                                              | HSP90AA1 | 22              | 38.5                | 1                     | 0.31         |
| IF2A_HUMAN                                               | EIF2S1   | 1*              | 2.5                 | 0.9185                | + ∞          |
| MAOM_HUMAN                                               | ME2      | 1*              | 4                   | 0.996                 | + ∞          |
| NDK8_HUMAN                                               | NME2P1   | 2               | 13                  | 1                     | - ∞          |
| Q5SZX9_HUMAN                                             | CCT3     | 1*              | 13.5                | 1                     | 2.006        |
| SET_HUMAN                                                | SET      | 9               | 40                  | 1                     | - ∞          |
| SMC1A_HUMAN                                              | SMC1A    | 1*              | 2                   | 0.9833                | - ∞          |
| SNRPA_HUMAN                                              | SNRPA    | 1*              | 3.5                 | 0.9688                | + ∞          |
| TBB4B_HUMAN                                              | TUBB2C   | 16              | 71                  | 1                     | + ∞          |
| TRAP1_HUMAN                                              | TRAP1    | 5               | 6.9                 | 1                     | - ∞          |
| ∞ : the protein was quantified only in one group         |          |                 |                     |                       |              |
| * = has at least two sibling Peptide-to-Spectrum matches |          |                 |                     |                       |              |

### 5. Western-blot Validation.

Three ovarian cancer cell lines were selected for western blot validation of the selected protein panel. Noticeably, the immunoblot technique allowed the identification of low-abundance proteins, such as those of the folate pathway, overcoming the MS concentration bias limits. The compounds tested were LR and [D-Gln<sup>4</sup>]LR, that have been delivered to cDDP sensible human ovarian carcinoma cells, A2780, IGROV1, and to cDDP resistant human ovarian carcinoma A2780/CP (ACP) cells, which derived from the parent A2780 cell line. The experiments were repeated at least three times. The administered doses were sub-cytotoxic and below the IC<sub>50</sub> detected previously (8). Cell lines were grown for 48 hrs and the sample for WB analysis were collected. Immunoblot analysis were performed according to the section 6 of Experimental Procedures. Briefly the protein concentration of each cell lysate was determined using the Bradford Protein Assay Reagent and forty

micrograms of the cell extracts were used for the immunoblotting. To detect the protein of interest, primary antibodies were incubated overnight, followed by a further incubation with a horseradish peroxidase-conjugated secondary antibody (sheep anti-mouse or anti-goat antibody) in order to monitor the target protein at the end of the assay using a chemiluminescence detection system. The protein expression levels in samples treated with LR, [D-Gln<sup>4</sup>]LR and PMX, shown in Figure 4 and 5, were calculated by dividing each value obtained after treatment with LR, [D-Gln<sup>4</sup>]LR and PMX by the control value.

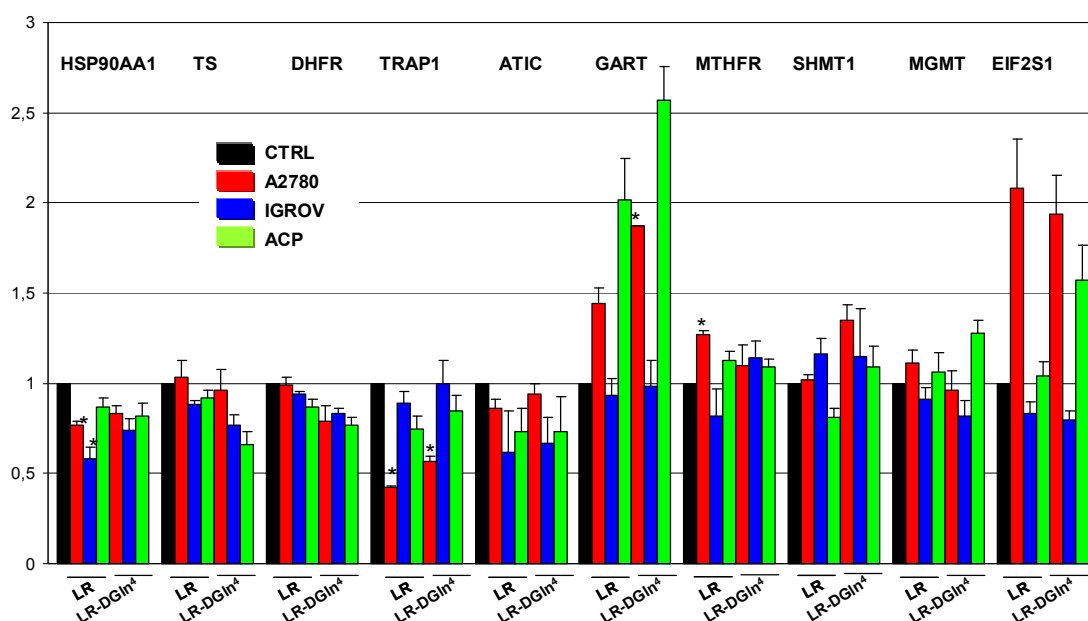


**Figure 4.** Western Blot analysis of selected protein levels in A2780, IGROV1 and ACP cells treated for 48h with 5  $\mu$ M LR and [D-Gln<sup>4</sup>]LR. Student's t test was performed to evaluate statistical significance of densitometry values of three replicates representative of three independent experiments. A p-value < 0.05 was considered to be significant for data acquired. The protein levels were normalized against the housekeeping protein  $\beta$ -actin, and the results were compared to the control (cell lines treated only with the delivery system), given an arbitrary value of 1.

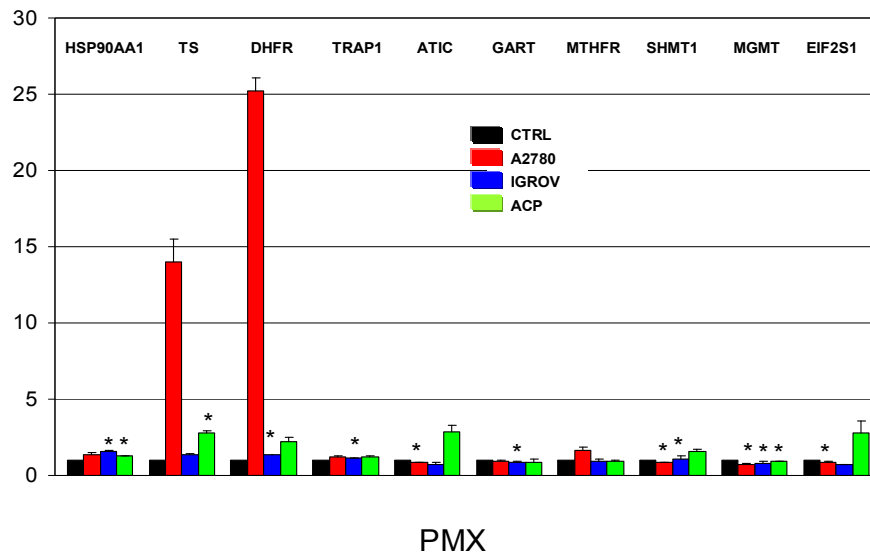


**Figure 5.** Western Blot analysis of selected protein levels in A2780, IGROV1 and ACP cells treated for 48h with 5  $\mu$ M LR and [D-Gln<sup>4</sup>]LR. Student's t test was performed to evaluate statistical of densitometry values of three replicates representative of three independent experiments. A p-value < 0.05 was considered to be significant for data acquired. The protein levels were normalized against the housekeeping protein  $\beta$ -actin, and the results were compared to the control (cell lines treated only with the delivery system), given an arbitrary value of 1.

In order to facilitate the interpretation of western blot's results, differences in expression of proteins among the three cell lines after the treatment with the peptides were graphically represented with the use of heat maps generated by dChip Software ([www.hsph.harvard.edu/cli/complab/dchip/](http://www.hsph.harvard.edu/cli/complab/dchip/)).

Figure 6 shows the heat map generated by the expression level analysis of each of the selected proteins upon after peptides and anti-folate pemetrexed treatment in A2780, ACP and IGROV1 cell lines. Red colors are related to the proteins up-expression, while the blue colors to their down-expressions. Rows are standardized subtracting CTRL and divide by SD. As expected by our previous observations (4), TS levels remain essentially unchanged or decrease after LR treatment, while DHFR levels appear to be always down-regulated. A similar pattern is observed after [D-Gln<sup>4</sup>]LR treatment. Interesting results were also obtained about two differentially expressed proteins as directly or indirectly altered by our anticancer peptide lead. In fact, the expression levels of the stress-related chaperones TRAP-1 and HSP90AA1 decrease in each of the three cell line upon the above inhibitors treatment.

The folate cycle proteins, which are strictly connected with both TS, the known target of LR, and DHFR, the second enzyme in the thymidylate cycle, were also examined. SHMT1 was found mainly up-regulated, excepted in A2780/CP cells treated with LR. The expression levels of MTHFR decrease in IGROV1 cells upon LR treatment, and increase in the other cell lines upon the treatment with the two peptides.

The expression of three enzymes directly linked to the folate cycle and indicated by the

bioinformatic analysis as nodal proteins, significantly interacting with LR-modulated proteins (ATIC, MGMT and GART), were also assessed. While ATIC levels were always found decreased upon both peptides treatment in all the three cell lines, the levels of GART are mainly increased after treatment with LR and its analog. The effect of the inhibitors on MGMT expression differs in the three cell lines, although in IGROV-1 and A2780/CP cell lines, the peptides affect the protein more similarly.

In the evaluation of the expression of selected proteins and targets of pemetrexed (PMX), a moderate to low cytotoxic (about 10-20%) effect was observed after the folate-like inhibitor treatment on the three cell lines (data not shown). The mild response after this drug administration could be attributed to the rapid development of resistance mechanisms (6, 32, 33), in fact the expression levels of PMX target protein, TS and DHFR, were rapidly up-regulated.

Up-regulation of key proteins in purine and pyrimidine synthesis, such as TS and DHFR, is a common phenomenon occurring in acquired resistance to substrate-like inhibitors (6, 32).

On the contrary, the peptidic inhibitors have an opposite effect with respect to PMX on the three folate-cycle proteins, as indicated in Figure 6. Previous studies, both in vitro and in ovarian cancer cell lines, have shown that LR (4) and its analog [D-Gln<sup>4</sup>]LR (8) are able to inhibit TS activity through a mechanism alternative to that of classical antifolates. TS and DHFR protein levels remain almost unchanged (TS) or down-regulated (DHFR) after peptidic inhibitors treatment. Interestingly, GART levels significantly increase after treatment with both peptides, especially in the case of [D-Gln<sup>4</sup>]LR.

Opposite effects between the peptidic inhibitors and the anti-folate drug PMX were clearly observed also for the stress-related proteins TRAP-1 and HSP90AA1. MGMT expression, instead, is clearly down-regulated as a consequence of the treatment with PMX on each cell line considered.

Finally, the treatment with PMX appeared to differently affect the expression levels of the other proteins (ATIC, MTHFR, SHMT1 and EIF2S1) on cells treated for the three cell lines tested.

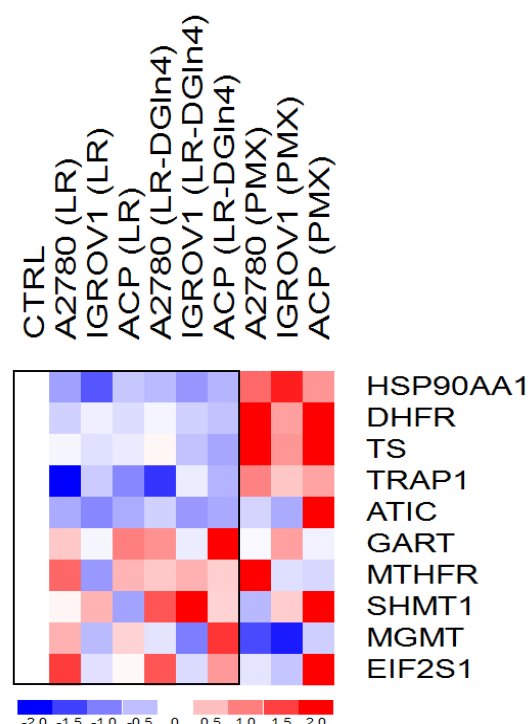


**Figure 6.** Representation of a red-to-blue heatmap to display the numerical values corresponding to the expression of the ten selected proteins among A2780, IGROV1 and ACP ovarian cancer cell lines after treatments with peptides LR, LR-DGln<sup>4</sup>, and the reference drug, Pemetrexed. Red colors are related to the proteins up-expression, while the blue colors to their down-expressions. Rows are standardized subtracting CTRL and divide by SD. The range of the standardized values displayed is  $\pm 2$ . Up- and down- expression fold changes colours and the correspondent values are indicated on the scale under the heat map.

## Discussion

General observations on the biological effect of the presented new class of inhibitors can be drawn from the evaluation of the molecular clusters obtained by bioinformatic analysis of DEPs modulated by our hTS interface-mimicking peptide LR. OC cell growth deregulation could be assigned to modulation of translational initiation, transport, termination of RNA Pol-II transcription, and protein catabolic events, as indicated by identified functional clusters **a**, **b**, **c** and **d** (Figure 2), respectively. Interestingly, proteasome related protein from cluster **d** play an important role in ovarian cancer inhibition (34); in fact, OC is characterized by high expression levels of proteasomal subunits, but they show elevated levels of poli-ubiquitinated proteins, as a symptom of catabolic stress: in this context, the proteasome is not able to counteract the elevated levels of misfolded proteins often present in many cancer forms. Consequently, proteasome seems an attractive target for anticancer therapies, and proteasome inhibitors are used in clinical treatments of many types of cancers (35). Based on our results, we can hypothesize that proteasomal subunits modulation represent a compensative response

of chaperones (HSP90 and TRAP1) down-regulation by LR, when the cells degrade misfolded proteins accumulated in the absence of chaperones that could refold them. The modulation of the expression of ribosomal associated proteins reveals a general metabolic reprogramming of treated cells, that could be the consequence of different processes:

- i. metabolic adaptation to thymidine depletion, that decreases DNA synthesis and, in turn, RNA and protein metabolism, leading cells to cell growth arrest aimed at counteracting stress induced by treatment;
- ii. specific regulation of the expression of ribosomal associated proteins set of proteins mediated by a transcription factor that could be, in turn, regulated by TS;
- iii. general translational blockage mediated by the translation initiation factor eIF2A<sub>1</sub>.

With regard to modulated specific proteins expression, bioinformatic DEPs analysis has led to identify a valuable protein panel, which might be used as early pharmacodynamic biomarker of this new class of anticancer peptidic inhibitors.

By Western blot analysis, at least six proteins (TS, DHFR, TRAP1, HSP90AA1, ATIC and GART) are similarly and constantly modulated in three ovarian cancer cell lines (A2780, A2780/CP and IGROV1) as a consequence of the treatment with the two peptides. Interestingly two proteins, ATIC and GART, are characterized by significant interactions with DEPs of our dataset, proving that proteomic/bioinformatic approach is consistent. In the above cell lines the two peptides have a similar effect on 6 proteins out of 10 selected. This observation suggests that the two peptides, LR and [D-Gln<sup>4</sup>]LR, act through a similar mechanism of action in the three cell lines studied. The six proteins: TRAP1, HSP90AA1, ATIC and GART together with TS and DHFR might warrant a deeper investigation since are directly modulated constantly by the peptides and represent the core set of the peptide profile. TRAP1 is a mitochondrial chaperone, and has recently been described as a potential novel target for ovarian cancer therapy; in fact, evidence exists that TRAP1 is overexpressed in an ovarian chemoresistant cancer cell line, PEO1 (28), and its expression is induced by estrogens in estrogen receptor-positive cancer cell lines (36). Although its role in OC has still to be fully investigated, in colorectal cancers TRAP1 is associated to chemoresistant and antiapoptotic phenotypes (37) because of its role in regulating mitochondrial transition pore opening together with HSP90AA1 and CyP-D (38).

TRAP1 is also localized on the outside of the endoplasmic reticulum, at the interface with mitochondria, where the proteasome complex is also located. In this context, TRAP1 forms a multi-protein complex with the 19S proteasome regulatory subunit PSMC4, which is modulated by LR treatment, and is responsible for the quality control and intracellular protein ubiquitination of mitochondria-destined proteins. This TRAP1/PSMC4 regulatory function is relevant in judging whether a damaged protein can be repaired and reach the final

mitochondrial destination or it needs to be degraded through the ubiquitin-proteasome system (37). Decreased TRAP1 expression leads to accumulation of ubiquitinated/misfolded proteins and to proteotoxic stress, a condition that renders cells more sensitive to apoptotic insults. Interestingly, TRAP1 has been described as a mitochondrial chaperone of HSP90AA1 (39). HSP90AA1, a molecular chaperone that promotes the maturation, structural maintenance and proper regulation of specific target proteins involved for instance in cell cycle control and signal transduction, is significantly down-regulated by LR and [D-Gln<sup>4</sup>]LR treatment. Many oncogenic proteins responsible for the transformation of cells to cancerous forms are client proteins of HSP90AA1. Targeting this chaperone with chemical inhibitors would degrade these oncogenic proteins and, thus, serve as a useful anticancer agent (40).

Interestingly, inhibition of both HSP90AA1 and TRAP1 with concomitant treatment with the anti-folate drug methotrexate, shows a synergistic effect interfering with the synthesis of purines (41). The two peptides show a similar behavior, downregulating HSP90AA1 and TRAP1 and therefore with the same effect as the classical antifolate. Furthermore, targeting simultaneously the multiple subcellular compartments of the HSP90AA1 in a model of glioblastoma, a highly lethal human malignancy, with a dual peptidomimetic inhibitor of mitochondrial and cytosolic HSP90AA1 and TRAP1 caused irreversible collapse of mitochondria, degradation of HSP90AA1 client proteins in the cytosol, and tumor cell killing by apoptosis and autophagy, with a positive effect on the treatment of the tumor (42). This mechanism of dual inhibition could explain one of the possible peptidic inhibitors-induced mechanisms of cell death due to the concomitant decrease in expression of these two chaperones.

As the selection of ions to be sequenced is stochastic and biased towards abundant peptides, members of the folate pathway, being relatively little abundant, could not be identified and quantitated in the MS investigation. However, in order to highlight a wider range of biological effect, which might arise from our peptides direct and indirect modulation, we monitored some of the proteins of the folate-associated genes, either having a high impact on our pharmacodynamic model or showing the largest number of dependencies to the species of our differentially expressed proteins dataset.

Among the other chosen folate pathway nodal proteins, ATIC, also known as AICAR transformylase, is an enzyme involved in purine biosynthesis. Two proteins of our dataset which are up-regulated by LR, ME2 and SNRPA, and the down-regulated NME2 were found to interact with this enzyme on a large-scale mapping of human protein-protein interactions by mass spectrometry (43). No functional evidence, however, has yet been found between these two proteins and ATIC. ME2 takes part to the pyruvate and carbon metabolism, while SNRPA is involved in RNA processing and has been evaluated as evaluated as a potential biomarker for resistance to platinum based primary chemotherapy in advance stage OC (44).

NME2, also known as nucleoside diphosphate kinase B, has a major role in the synthesis of nucleoside triphosphates other than ATP; interestingly, this protein has been described as a tumor metastasis suppressor, and its transcriptional regulation, as well as ATIC regulation, seems to be controlled by c-myc (45).

Immunoblot analysis showed a down-regulation of ATIC expression levels after LR and [D-Gln<sup>4</sup>]-LR treatment. Functional analyses revealed that ATIC activity is promoted by phosphorylation, abrogating partially the methotrexate inhibition of the transformylase activity, requiring higher doses of this PMX analog to produce a comparable inhibitory rate with respect to the native non phosphorylated protein (46). An exhaustive study of the involvement of ATIC in its down-stream targets modulation, as well as its possible direct/indirect involvement with the mechanism of action of the peptides, should take into account a fine post-translational modification analysis, in addition to shotgun mass spectrometry. It is interesting to notice that the antifolate PMX generates a significantly different protein expression profile. Indeed, the already mentioned low cytotoxic effect observed after PMX treatment is reflected on the expression level variations of two PMX target proteins namely TS and DHFR. After PMX treatment, TS and DHFR protein levels result increased, especially in A2780 cells, indicating a probable acquisition of drug resistance in this cell line after the treatment (32). On the contrary, our new class of peptidic anticancer agents shows a clear cytotoxicity on the same ovarian carcinoma cell line (8). PMX, in fact, inhibits TS with a different mechanism of action with respect to the hypothesized pharmacological model proposed by LR, and this probably reflects a consequently different effect on the levels of the proteins considered. Both peptides have, indeed, an opposite effect on the two folate-cycle proteins with respect to PMX. This class targets the hTS enzyme by binding at the protein interface of the di-inactive enzyme form, differently from the two classical active-site binding antifolates. This different binding mode prevent the induction of TS overexpression, maintaining unaltered the protein regulation at the mRNA level (4, 8).

Thus, direct inhibition of TS causes a downstream protein deregulation, further confirming the key role of TS in the entire folate cycle regulation. DHFR levels, in fact, decrease with both LR and [D-Gln<sup>4</sup>]-LR in the three cell lines considered.

On the contrary, GART tends to an increase after treatment with the peptides, a behavior that could be explained considering that the low level of tetrahydrofolate cofactor consumed and its consequent accumulation might promote substrate transformation activity and induce high level of GART activity, a mechanism already described in other organisms (44). Furthermore, it is likely that the two classes of inhibitors have different off-targets, which consequently might significantly influence the modulation of the proteins set identified in the present work. In conclusion, the adopted powerful proteomic/bioinformatic approach yielded a panel of

early drug discovery phase biomarkers composed of ten proteins, which was validated with the peptide lead candidate (LR). Noticeably, [D-Gln<sup>4</sup>]LR protein profile fitted almost completely with its lead one, confirming that the protein set we have identified could be of general value for compounds showing a similar inhibition mechanism with respect to LR.

New insights into LR mechanism of action of the new class of anticancer peptides have been provided, as direct inhibition of hTS causes a downstream down-regulation of the TS cycle proteins, further confirming the key role of TS in the folate cofactor regulation. The modulation of other important folate-related proteins, such as GART and SHMT1, suggests that the entire folate network is influenced at different levels after peptide treatment.

The differential proteomic signature between cells treated with Pemetrexed or with LR/PF34 could be interesting to investigate in terms of combinatorial action of the two drugs; in this view, the concept of “combinatorial lethality” is now emerging in tumor therapy. Rytelwski and collaborators recently show that it is possible to target multiple pathways with different drugs (for example, with the blockage of both BRCA2 and TS with specific inhibitors) to enhance cytotoxicity for tumors and to minimize the onset of drug resistance (47). Some effects of LR on indirect targets discovered through the proposed method as, for instance, deoxycytidine kinase or the proteasome-HSP90-TRAP1 functional cluster, require deeper investigation due to their complex role and molecular function.

As a further development of this work, we plan to examine the discovered protein panel using gene expression studies, to go deeper inside the metabolic connections among the mentioned proteins and to adopt this panel as a tool to monitor the cellular behavior of other LR analogs that will be designed showing improved efficacy against the mentioned OC cell line.

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## References

1. Stuart, G. C., Kitchener, H., Bacon, M., et al. (2011) 2010 Gynecologic Cancer InterGroup (GCIG) consensus statement on clinical trials in ovarian cancer: report from the Fourth Ovarian Cancer Consensus Conference. *Int. J. Gynecol. Cancer* 21, 750-755.
2. Ozols, R. F. (1990) Ovarian cancer. *Semin. Surg. Oncol.* 6, 328-338.
3. Gautier, L., Taboureau, O., Audouze, K. (2013) The effect of network biology on drug toxicology. *Expert Opin. Drug Metab. Toxicol.* in press, doi:10.1517/17425255.2013.820704.
4. Cardinale, D., Guaitoli, G., Tondi, D., Luciani, R., Henrich, S., Salo-Ahen, O. M. H., Ferrari, S., Marverti, G., Guerrieri, D., Ligabue, A., Frassinetti, C., Pozzi, C., Mangani, S., Fessas, D., Guerrini, R.,

- Ponterini, G., Wade, R. C., and Costi, M. P. (2011) Protein–protein interface-binding peptides inhibit the cancer therapy target human thymidylate synthase. *Proc. Natl. Acad. Sci. U. S. A.* 108, E542-E549.
5. Rahman, L., Voeller, D., Rahman, M., Lipkowitz, S., Allegra, C., Barrett, J. C., Kaye, F. J., Zajac-Kaye, M. (2004) Thymidylate synthase as an oncogene. *Cancer Cell* 5, 341-351.
6. Pullarkat, S. T., Stoecklacher, J., Ghaderi, V., Xiong, Y.-P., Ingles, S. A., Sherrod, A., Warren, R., Tsao-Wei, D., Groshen, S., Lenz, H.-J. (2001) Thymidylate synthase gene polymorphism determines response and toxicity of 5-FU chemotherapy. *Pharmacogenomics J.* 1, 65-70.
7. Walters, C. L., Arend, R. C., Armstrong, D. K., Naumann, R. W., Alvarez, R. D. (2013) Folate and folate receptor alpha antagonists mechanism of action in ovarian cancer. *Gynecol. Oncol.* in press, doi: 10.1016/j.ygyno.2013.07.080
8. Pelà, M., Saxena, P., Luciani, R., Santucci, M., Ferrari, S., Marverti, G., Marraccini, C., Martello, A., Pironi, S., Genovese, F., Salvadori, S., D'Arca, D., Ponterini, G., Costi, M. P., Guerrini, R. (2013) Optimization of peptides that target human thymidylate synthase to inhibit ovarian cancer cell growth. *J. Med. Chem.* Manuscript ID jm-2013-01156s.
9. Altman, R. B., Rubin, D. L., Klein, T. E. (2004) An “Omics” View of Drug Development. *Drug Dev. Res.* 62, 81-85.
10. Helleman, J., Smid, M., Jansen, M. P., van der Burg, M. E., Berns, E. M. (2010) Pathway analysis of gene lists associated with platinum-based chemotherapy resistance in ovarian cancer: the big picture. *Gynecol. Oncol.* 117, 170-176.
11. Pils, D., Tong, D., Hager, G., Obermayr, E., Aust, S., Heinze, G., Kohl, M., Schuster, E., Wolf, A., Shouli, J., Braicu, I., Vergote, I., Van Gorp, T., Mahner, S., Concin, N., Speiser, P., Zeillinger, R. (2013) A combined blood based gene expression and plasma protein abundance signature for diagnosis of epithelial ovarian cancer--a study of the OVCAD consortium. *BMC Cancer* 13, 178.
12. Wiśniewski, J. R., Ostasiewicz, P., Duś, K., Zielińska, D. F., Gnäd, F., Mann, M. (2012) Extensive quantitative remodeling of the proteome between normal colon tissue and adenocarcinoma. *Mol. Syst. Biol.* 8, 611.
13. Rezaul, K., Thumar, J. K., Lundgren, D. H., Eng, J. K., Claffey, K. P., Wilson, L., Han, D. K. (2010) Differential Protein Expression Profiles in Estrogen Receptor-Positive and -Negative Breast Cancer Tissues Using Label-Free Quantitative Proteomics. *Genes Cancer* 1, 251-271.
14. Perco, P., Mühlberger, I., Mayer, G., Oberbauer, R., Lukas, A., Mayer, B. (2010) Linking transcriptomic and proteomic data on the level of protein interaction networks. *Electrophoresis* 31, 1780-1789.
15. Wang, X., Zhang, A., Sun, H., Wu, G., Suna, W., Yana, G. (2012) Network generation enhances interpretation of proteomics data sets by a combination of two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. *Analyst* 137, 4703-4711.
16. Benoiton, N. L. (2005) *Chemistry of Peptide Synthesis.*, pp. 125-154, Taylor&Francis, London, UK.
17. Chambers, M. C. et al. (2012) A cross-platform toolkit for mass spectrometry and proteomics. *Nat. Biotechnol.* 30, 918-920.

18. Edwards, N., Wu, X., Tseng, C.-W. (2009) An Unsupervised, Model-Free, Machine-Learning Combiner for Peptide Identifications from Tandem Mass Spectra. *Clin. Proteomics* 5, 23-36.
19. Nesvizhskii, A. I., Keller, A., Kolker, E., Aebersold, R. (2003) A Statistical Model for Identifying Proteins by Tandem Mass Spectrometry. *Anal. Chem.* 75, 4646-4658.
20. Tsou, C.-C., Tsai, C.-F., Tsui, Y.-H., Sudhir, P.-R., Wang, Y.-T., Chen, Y.-J., Chen, J.-Y., Sung, T.-Y., Hsu, W.-L. (2010) IDEAL-Q, an Automated Tool for Label-free Quantitation Analysis Using an Efficient Peptide Alignment Approach and Spectral Data Validation. *Mol. Cell. Proteomics* 9, 131-144
21. Taverner, T., Karpievitch, Y. V., Polpitiya, A. D., Brown, J. N., Dabney, A. R., Anderson, G. A., Smith, R. D. (2012) DanteR: an extensible R-based tool for quantitative analysis of -omics data. *Bioinformatics* 28, 2404-2406.
22. Bernthaler, A., Mühlberger, I., Fechete, R., Perco, P., Lukas, A., Mayer, B. (2009) A dependency graph approach for the analysis of differential gene expression profiles. *Mol. Biosyst.* 5, 1720-1731.
23. Heinzel, A., Fechete, R., Söllner, J., Perco, P., Heinze, G., Oberbauer, R., Mayer, G., Lukas, A., & Mayer, B. (2012) Data Graphs for Linking Clinical Phenotype and Molecular Feature Space. *IJSBBT* 1, 11-25.
24. Fechete, R., Heinzel, A., Söllner, J., Perco, P., Lukas, A., Mayer, B. (2013) Using Information Content for Expanding Human Protein Coding Gene Interaction Networks. *J. Comput. Sci. Syst. Biol.* 6, 73-82.
25. Bader, G. D., Hogue, C. W. V. (2003) An automated method for finding molecular complexes in large protein interaction networks. *BMC Bioinf.* 4, 2.
26. Croft, D., O’Kelly, G., Wu, G., Haw, R., Gillespie, M., Matthews, L., Caudy, M., Garapati, P., Gopinath, G., Jassal, B., Jupe, S., Kalatskaya, I., Mahajan, S., May, B., Ndegwa, N., Schmidt, E., Shamovsky, V., Yung, C., Birney, E., Hermjakob, H., D’Eustachio, P., Stein, L. (2011) Reactome: a database of reactions, pathways and biological processes. *Nucleic Acids Res.* 39, D691-D697.
27. Knox, C., Law, V., Jewison, T., Liu, P., Ly, S., Frolkis, A., Pon, A., Banco, K., Mak, C., Neveu, V., Djoumbou, Y., Eisner, R., Guo, A. C., Wishart, D. S. (2011) DrugBank 3.0: a comprehensive resource for ‘Omics’ research on drugs. *Nucl. Acids Res.* 39, D1035-D1041.
28. Landriscina, M., Amoroso, M. R., Piscazzi, A., Esposito, F. (2010) Heat shock proteins, cell survival and drug resistance: the mitochondrial chaperone TRAP1, a potential novel target for ovarian cancer therapy. *Gynecol. Oncol.* 117, 177-182.
29. Taipale, M., Jarosz, D. F., Lindquist, S. (2010) HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nat. Rev. Mol. Cell Biol.* 11, 515-528.
30. Mahalingam, D., Swords, R., Carew, J. S., Nawrocki, S. T., Bhalla, K., Giles, F. J. (2009) Targeting HSP90 for cancer therapy. *Br. J. Cancer.* 100, 1523-1529.
31. de Haro, C., Méndez, R., Santoyo, J. (1996) The eIF-2alpha kinases and the control of protein synthesis. *FASEB J.* 10, 1378-1387.
32. Skacel, N., Menon, L. G., Mishra, P. J., Peters, R., Banerjee, D., Bertino, J. R., Abali, E. E. (2005) Identification of Amino Acids Required for the Functional Up-regulation of Human Dihydrofolate Reductase Protein in Response to Antifolate Treatment. *J. Biol. Chem.* 280, 22721-22731
33. Lu, X., Errington, J., Curtin, N. J., Lunec, J., Newell, D. R. (2001) The Impact of p53 Status on Cellular Sensitivity to Antifolate Drugs. *Clin. Cancer Res.* 7, 2114-2123.

34. Bazzaro, M., Lee, M. K., Zoso, A., Stirling, W. L. H., Santillan, A., Shih, I.-M., Roden, R. B. S. (2006) Ubiquitin-Proteasome System Stress Sensitizes Ovarian Cancer to Proteasome Inhibitor-Induced Apoptosis. *Cancer Res.* 66, 3754-3763.
35. Richardson, P. G., Mitsiades, C., Hideshima, T., Anderson, K. C. (2006) Bortezomib: proteasome inhibition as an effective anticancer therapy. *Annu. Rev. Med.* 57, 33-47.
36. Walker, G., MacLeod, K., Williams, A. R., Cameron, D. A., Smyth, J. F., Langdon, S. P. (2007) Estrogen-regulated gene expression predicts response to endocrine therapy in patients with ovarian cancer. *Gynecol. Oncol.* 106, 461-468.
37. Matassa, D. S., Amoroso, M. R., Maddalena, F., Landriscina, M., Esposito, F. (2012) New insights into TRAP1 pathway. *Am. J. Cancer Res.* 2, 235-248.
38. Kang, B. H., Plescia, J., Dohi, T., Rosa, J., Doxsey, S. J., Altieri, D. C. (2007) Regulation of tumor cell mitochondrial homeostasis by an organelle-specific Hsp90 chaperone network. *Cell* 131, 257-270.
39. Altieri, D. C., Stein, G. S., Lian, J. B., Languino, L. R. (2012) TRAP-1, the mitochondrial Hsp90. *Biochim. Biophys. Acta* 1823, 767-773.
40. Mahalingam, D., Swords, R., Carew, J. S., Nawrocki, S. T., Bhalla K., and Giles F. J. (2009) Targeting HSP90 for cancer therapy. *Br. J. Cancer* 100, 1523 – 1529.
41. French, J. B., Zhao, H., An, S., Niessen, S., Deng, Y., Cravatt, B. F., Benkovic, S. J. (2013) Hsp70/Hsp90 chaperone machinery is involved in the assembly of the purinosome. *Proc. Natl. Acad. Sci. U. S. A.* 110, 2528-2533.
42. Siegelin, M. D., Plescia, J., Raskett, C.M., Gilbert, C. A., Ross, A. H., Altieri, D. C. (2010) Global targeting of subcellular heat shock protein-90 networks for therapy of glioblastoma. *Mol. Cancer Ther.* 9, 1638-1646.
43. Ewing R. M., Chu, P., Elisma, F., Li H., et al., (2007) Large-scale mapping of human protein-protein interactions by mass spectrometry. *Mol. Syst. Biol.* 3:89; doi:10.1038/msb4100134.
44. Dressman, H. K., Berchuck, A., Chan, G., Zhai, J., Bild, A., Sayer, R., Cragun, J., Clarke, J., Whitaker, R. S., Li, L., Gray, J., Marks, J., Ginsburg, G. S., Potti, A., West, M., Nevins, J. R., Lancaster, J. M. (2007) An Integrated Genomic-Based Approach to Individualized Treatment of Patients With Advanced-Stage Ovarian Cancer. *J. Clin. Oncol.* 25, 517-525.
45. Postel, E. H., Berberich, S. J., Flint, S. J., Ferrone, C. A. (1993) Human c-myc transcription factor PuF identified as nm23-H2 nucleoside diphosphate kinase, a candidate suppressor of tumor metastasis. *Science* 261, 478-480.
46. Boccalatte, F. E., Voena, C., Riganti, C., Bosia, A., D'Amico, L., Riera, L., Cheng, M., Ruggeri, B., Jensen, O. N., Goss, V. L., Lee, K., Nardone, J., Rush, J., Polakiewicz, R. D., Comb, M. J., Chiarle, R., Inghirami, G. (2009) The enzymatic activity of 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase is enhanced by NPM-ALK: new insights in ALK-mediated pathogenesis and the treatment of ALCL. *Blood* 113, 2776-2790.
47. Rytelwicz, M., Ferguson, P. J., Maleki, V. S., Figueredo R., Vincent, M, Koropatnick, J. (2013) Inhibition of BRCA2 and Thymidylate Synthase Creates Multidrug Sensitive Tumor Cells via the Induction of Combined “Complementary Lethality“. *Mol Ther Nucleic Acids*, 2:e78.doi:10.1038/mtma.2013.

### SECTION 3.

In this section I present the work performed in order to evaluate the FR expression in EOC cell lines (*see section 1.11.3.*).

In particular, to evaluate the efficiency of the FR $\alpha$ -targeted strategy for selectively delivering our peptides within ovarian cancer cells, a first-generation pteroyl-nonapeptide conjugated (PNPC) has been synthesised by the collaborator team of Ferrara University (Dr. Remo Guerrini and colleagues) (*see section 1.12.3.*). In this context, I performed the characterization of a panel of OC cell lines (A2780, A2780/CP, 2008, C13\*, IGROV1, TOV112D, COV504, OAW28) for FR $\alpha$  expression (*see section 1.12.3.1.*), FA binding and the preliminary evaluation of the biological activity of the PNPC on ovarian cancer cells IGROV1, TOV112D, COV504 and OAW28 (*see section 1.12.3.2.*).

The results obtained from this study have been recently described in the manuscripts *Targeting cancer-cell folate receptors with a pteroyl-nonapeptide conjugate*, in preparation.

### 3.1. Introduction to manuscripts: Targeting cancer-cell folate receptors with a pteroyl-nonapeptide conjugate

#### 3.1.1. Background

In the following manuscripts, currently being prepared for publication, an alternative strategy that exploits the  $\alpha$  isoform of FR (FOLR1) has been explored for the treatment of ovarian cancer cells. FR $\alpha$ , as reported in *section 1.8.2.2.*, represents both a biomarker and a potential target of therapeutic approaches. In fact, it is expressed in more than 90% of non-mucinous ovarian carcinomas (OCs), while is almost absent in normal cells. Moreover, FR $\alpha$  is able to specifically internalize folic acid-drug conjugates to cancer cells via receptor-mediated endocytosis. Within these premises, targeted drug delivery via FR promises to expand the therapeutic windows of drugs by increasing delivery to the target malignant tissue as well as the target/non-target-tissue ratio. It is currently being explored for the latest generation of drug delivery approaches (*see section 1.8.3.2.*).

In the present work, a first-generation pteroyl-nonapeptide conjugate (PNPC, or FA-LR) derived by the conjugation of a folic acid residue to an octapeptide known to inhibit hTS, has been designed to enter cancer cells by FOLR1-mediated endocytosis.

#### 3.1.2. Aims

The objective of this study was to verify whether the conjugation of a known hTS inhibitor with a folic acid residue could represent an effective strategy to specifically deliver the drug to cancer cells.

#### 3.1.3. Results

To assess the FR $\alpha$  targeting strategy for internalizing our peptides into ovarian cancer-cell models, a PNPC of first generation, shown in Figure 3.1.3., has been synthesised by our collaborators from Ferrara University (Dr. Remo Guerrini and colleagues); FA-LR inhibition studies against hTS showed 50% of inhibition at 100  $\mu$ M (data not shown).

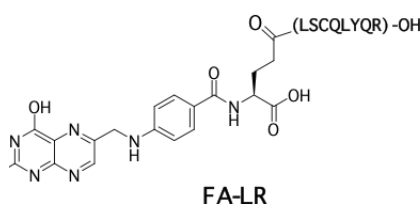


**Figure 3.1.3.1.** Chemical structure of Pteroyl-nonapeptide conjugate (PNPC) of first generation. The synthesis of this compound has been performed by our collaborators of Ferrara University.

During my PhD work, I identified the most suitable cellular models for testing our newly designed FA-conjugated from a panel of eight human OC cell lines, using both quantitative and semi-quantitative assays in order to evaluate the expression levels of FR $\alpha$ . Indeed, since targeted drug delivery efficiency via FR $\alpha$  depends on the expression of this surface protein, its levels were investigated in the following ovarian cancer cell lines: OAW28, COV504, IGROV1, TOV112D, 2008, C13\*, A2780 and A2780/CP. In particular, radiometric methods were used to measure the competition between FA-LR and [ $^3$ H] folic acid for the binding with FR $\alpha$ ; flow cytometry was used to assess the FR $\alpha$  cell surface expression; the expression levels of total FR $\alpha$  mRNA were measured with quantitative PCR (Power SYBR® Green strategy), while the total protein was assessed via Western Blot assay [1-3].

IGROV1 and OAW28 cell lines exhibited the highest levels of FR $\alpha$  mRNA (relative mRNA expression,  $y > 10$  in Figure 3.1.3.2. a), A2780 and A2780/CP cells showed intermediate ( $5 < y < 10$ ), while TOV112D and COV504 cells exhibited very low levels of FR $\alpha$  mRNA ( $y < 5$ , Figure 3.1.3.2. a). A similar result was obtained with the WB analysis of total FR $\alpha$  protein levels (Figure 3.1.3.2. b). Indeed, FR $\alpha$  bands were detected only with OAW28 and IGROV1 cells, the latter showing the highest FR $\alpha$  cellular content.

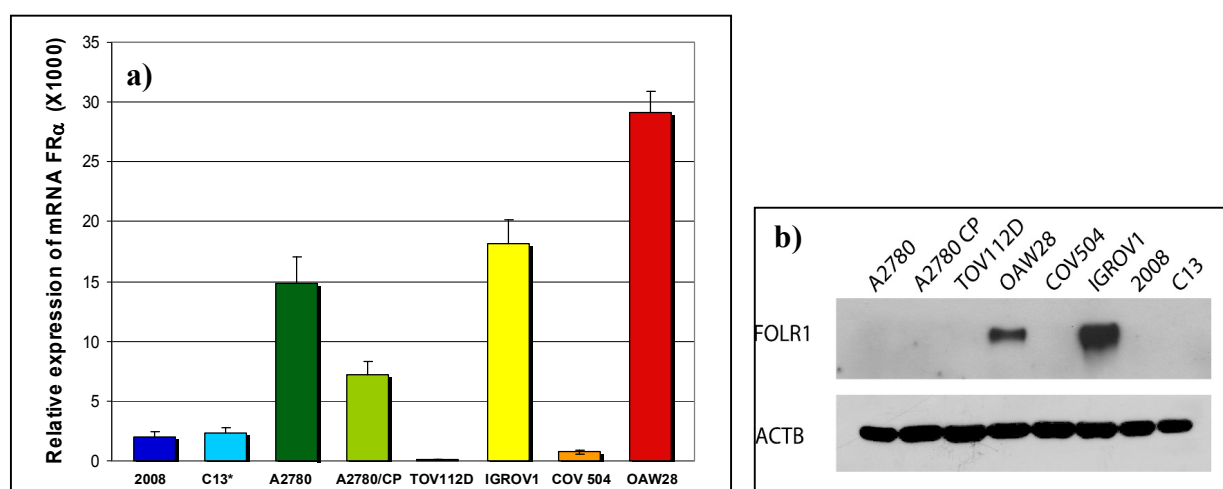


**Figure 3.1.3.2.** Expression levels of FR $\alpha$  mRNA and total protein in eight ovarian cancer cell lines: a) relative levels of FR $\alpha$  mRNA. y axis: levels of FR $\alpha$  mRNA relative to GAPDH mRNA; b) Example of western blot analysis of the 38 KDa FR $\alpha$  protein in cell line extracts. ACTB: anti-human  $\beta$ -actin.

Flow cytometry was used to assess the expression levels of FR $\alpha$  protein on the cell surface (Figure 3.1.3.3. a), confirming that OAW28 and IGROV1 cells express the largest amounts of FR $\alpha$ . Radioligand binding assays, that exploited the binding between the [ $^3$ H] folic acid and FR $\alpha$  [4], has also been performed (Figure 3.1.3.3. b) to assess both the expression levels of FR $\alpha$  on the cell surface and the levels of functional FR $\alpha$ , a crucial property for FR-targeted

therapies [5]. These assays confirmed the previous results. IGROV1 cells, indeed, exhibited the highest amount of functional FR $\alpha$  on their surface: more than 90 fmol of [ $^3$ H]folic acid bound per mg of protein, versus about 40 fmol/mg for OAW28 cells; values lower than 30 fmol/mg were measured for the other cell lines.

The combination of the results from both qualitative and quantitative assays enlightened that the highest levels of FR $\alpha$  are expressed by IGROV1 and OAW28, while the lowest by COV504 and TOV112D cell lines. For this reason, we decided to select IGROV1 and TOV112D for our further experiments with FA-LR as positive and negative controls, respectively.

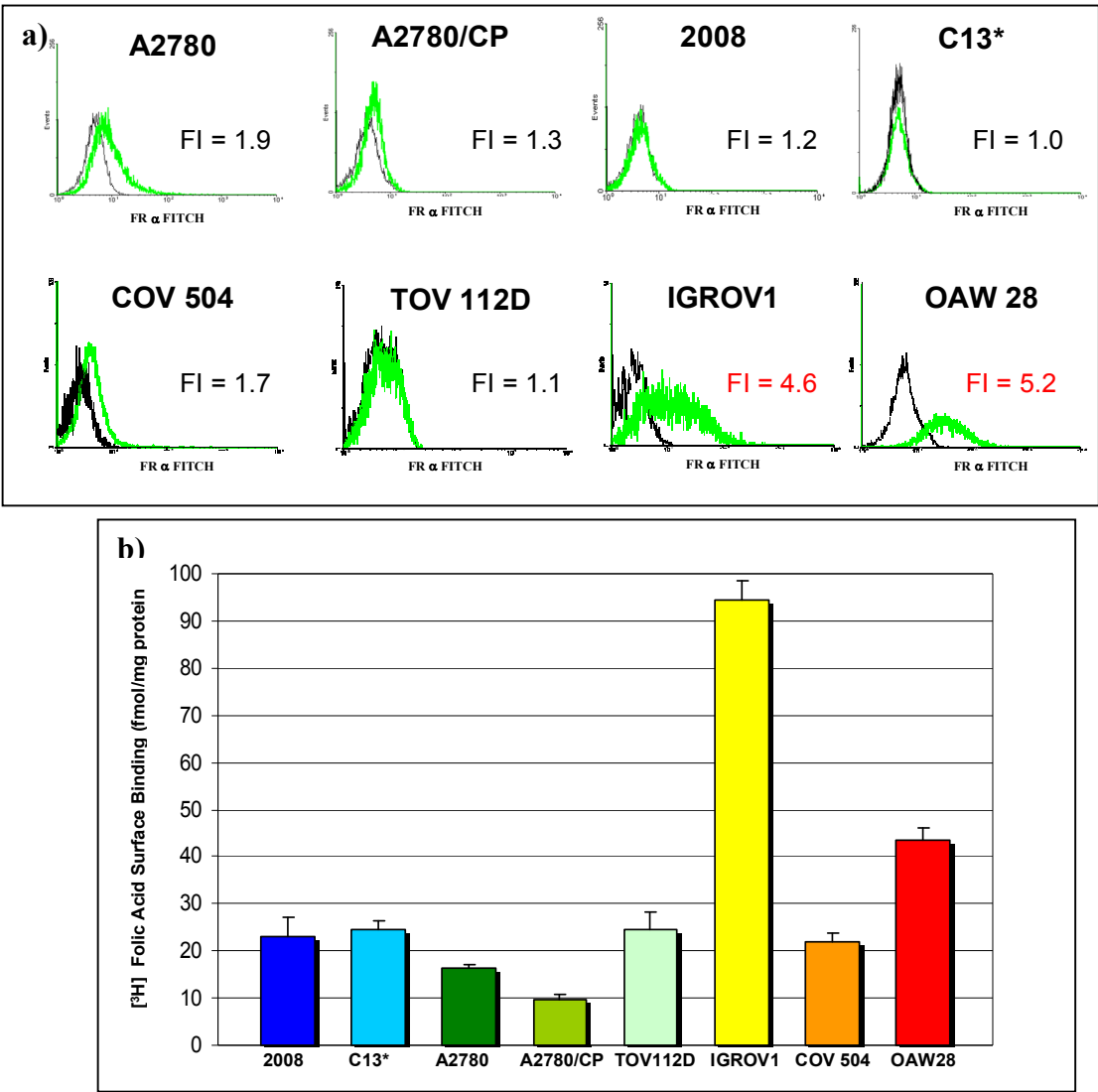


**Figure 3.1.3.3.** Expression of FR $\alpha$  in ovarian cancer cell lines: a) Flow cytometric assessment of FR $\alpha$  cell surface expression. Black: cells labeled with secondary antibody alone; green: cells labeled with anti-FR $\alpha$  Mov18 antibody. FI: ratio of the mean fluorescence intensities in the presence and absence of the primary antibody. b) Substrate specificity of the [ $^3$ H]folic acid surface binding by the indicated human ovarian cancer cell lines.

To evaluate the levels of functional FR $\alpha$ , a crucial property for FR-targeted therapies [5], radioligand binding assays that measure the competition between FA-LR and [ $^3\text{H}$ ] folic acid for the binding with FR, have been performed in ovarian cancer cell lines. Results indicate that the binding of [ $^3\text{H}$ ]folic acid to FR $\alpha$  was greatly inhibited in presence of unlabeled FA in all cell lines (Figure 3.1.3.4. a). Quite similar [ $^3\text{H}$ ]folic-acid binding-inhibitory effects were observed in presence of our FA-LR conjugate. The inhibition was more evident in FR $\alpha$ -highly-expressing cells, i.e., IGROV1 and OAW28 (80% and 70%, respectively). As an expected consequence of the competitive binding to FR $\alpha$ , [ $^3\text{H}$ ]folic acid uptake was reduced in the eight cell lines in presence of both unlabeled FA and FA-LR conjugate (Figure 3.1.3.4. b), a behavior consistent with our previous observations on FA and folate analogs [6]. We observed that, while in most cell lines competition with the FA-LR conjugate caused a decrease of [ $^3\text{H}$ ]folic acid uptake of 50% or less, this decrease was about 80% (from  $583.3 \pm 42$  to  $92.2 \pm 11$  fmol/mg of protein) and 75% (from  $271.5 \pm 27$  to  $56.6 \pm 8$  fmol/mg of protein) with IGROV1 and OAW28 cells, respectively (Figure 3.1.3.4. b). Then, we used these two FR $\alpha$ -highly-expressing cell lines to better study the ability of FA-LR to directly compete with folic acid for binding to cell-surface FR $\alpha$ . The dose-response plots, in Figure 3.1.3.3. c, indicate that FA-LR efficiently competes with [ $^3\text{H}$ ] folic acid uptake already at 0.5  $\mu\text{M}$ , and behaves very similarly to unlabeled FA with both cell lines.

Observation and quantitation of the internalized FA-LR conjugate were obtained by label-free spectrofluorometry, performed with the supervision of Prof. Glauco Ponterini (University of Modena and Reggio Emilia). The pterate moiety of FA exhibits a characteristic fluorescence emission [7] that can be enhanced by UV-induced oxidative photocleavage of the bond between the fluorophore and the quenching p-aminobenzoate unit [8]. Emission and excitation measurements on IGROV1 cell lysates provided pterate signals that increased upon repeated exposures to the excitation lamp up to final, stable values (Figure 3.1.3.4. d). The experiments yielded average emission intensities of  $834 \pm 32$  from the FA-LR conjugate treated cells and  $747 \pm 36$  kcounts from the untreated cells; the excitation average signals were  $1120 \pm 25$  and  $994 \pm 80$  kcounts for the treated and untreated samples, respectively. The differences between positive and control signals, proportional to the uptaken FA-LR concentrations, were therefore  $87 \pm 48$  and  $126 \pm 84$  kcounts, respectively. Using the number of cells (around  $6 \times 10^6$ ) and an average cell volume of  $1.86 \times 10^{-12}$  L [9] we estimated the corresponding intracellular concentrations to be  $12.1 \pm 6.6$  and  $14.6 \pm 9.7$   $\mu\text{M}$  from FA-LR emission and excitation signals, respectively. Thus, in these conditions, we estimate a three-fold increase of FA-LR in IGROV1 cells relative to the external incubation concentration.

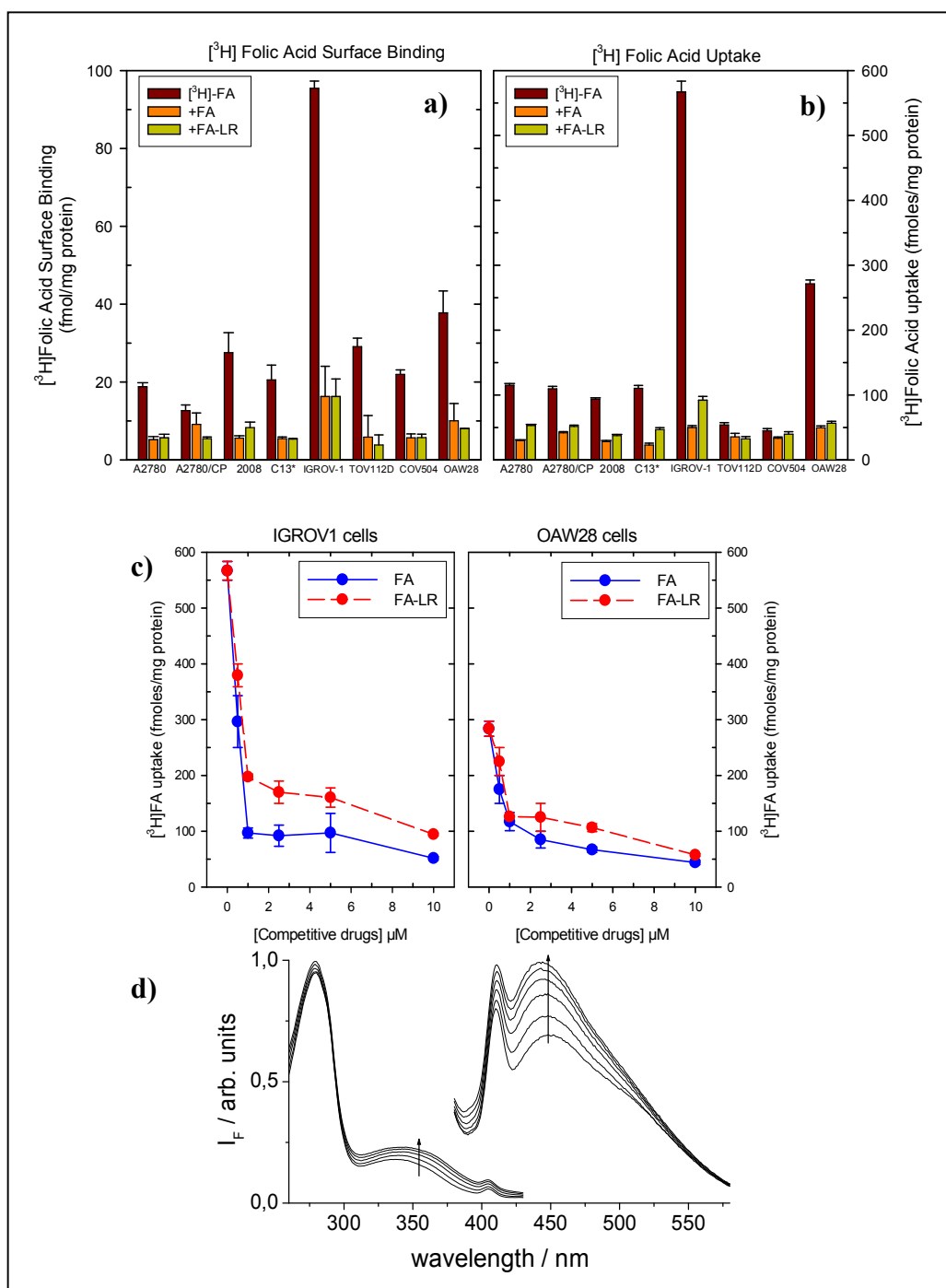


**Figure 3.1.3.4.** Substrate specificity of the  $[^3\text{H}]$ folic acid surface binding and uptake by the indicated human ovarian cancer cell lines. a) surface binding; b) uptake; c) relative folate receptor affinity of the FA-LR conjugate. Each data point represents the mean  $\pm$  S.E.M of three separate determinations. d) Pterate excitation ( $\lambda_{\text{em}} = 450 \text{ nm}$ ) and emission ( $\lambda_{\text{exc}} = 360 \text{ nm}$ ) fluorescence spectra of a lysate of IGROV1 cells treated with FA-LR (see text). The arrows indicate the spectral evolution upon subsequent emission-excitation acquisition cycles. Data are normalized to the maxima of the final spectra for ease of presentation.

These findings suggest that the pterate-peptide conjugate can be developed as a tool for specifically targeting FR $\alpha$ -expressing cancer. Next, a preliminary evaluation of the biological

effect of our new conjugates has been performed in ovarian cancer cell lines. FA-LR and its derivative FA-[D-Gln<sup>4</sup>]LR, shown in Figure 3.1.3.5. were tested at the concentrations of 5 and 10  $\mu$ M.

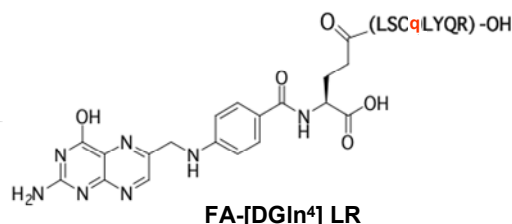


**Figure 3.1.3.5.** Chemical structure of first generation PNPC derivate. The synthesis of this compound has been performed by our collaborators of Ferrara University.

In all the cell lines considered, both the conjugated peptides did not significantly affect the cellular proliferation (Figure 3.1.3.6.). In cells expressing low levels of FR $\alpha$  (TOV112D and COV504) this behavior could be explained by the absence of the specific target recognized by the folic acid portion of the conjugate: in these cells, therefore, the peptide conjugates should not be able to cross the cell membrane. In cells over-expressing FR $\alpha$  (IGROV1 and OAW28), instead, the peptide conjugates bind the receptor (as demonstrated by the radioligand binding assays) and are internalized within the endosomes but the absence of a cleavable bond prevents them to exit the vesicles to exert their inhibitory activity.

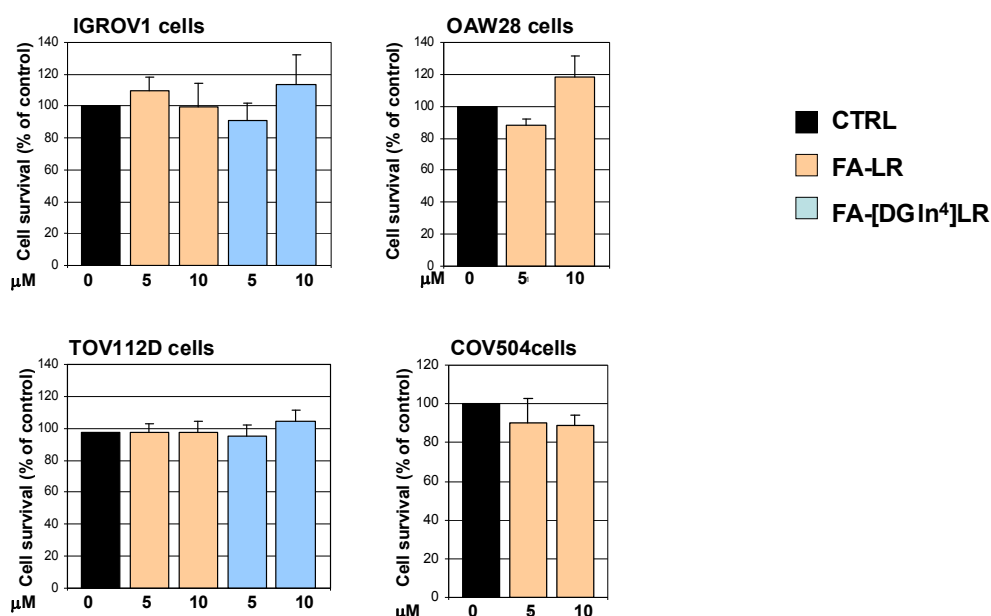


**Figure 3.1.3.6.** Inhibition of cell growth after treatment with FA-LR and FA-[D-Gln<sup>4</sup>]LR (5-10  $\mu$ M in fresh FFRPMI). Percentage of cell survival after treatment with FA-LR is represented with dark grey bars, while percentage of cell survival after treatment with FA-[D-Gln<sup>4</sup>]LR is represented by light grey bars. The concentration FA-peptides (grey) is given on the x-axis in  $\mu$ M.

#### 3.1.4. Conclusions and future perspectives

To conclude, we recall that ovarian cancer is a severe cancer type for which adequate therapy is not yet available. FR $\alpha$ , which is expressed in epithelial ovarian cancer cells but is almost absent in normal epitheliums, offers a major therapeutic advancement opportunity. In our effort to design new drugs directed to hTS, we have combined hTS inhibition with the concept of targeted delivery by conjugating the LR peptide with FA. Our competition binding experiments have demonstrated that FA-LR targets FR $\alpha$  with an affinity comparable with that of FA. The competing effect of FA-LR on FA uptake is more pronounced with cells, like IGROV1 and OAW28, that highly express FR $\alpha$ ; FA-LR is thus internalized via FR $\alpha$  and may reach concentrations of several micromoles/litre, being therefore able to reach and bind its intracellular target. FA-LR internalization cannot occur via RFC, which is the preferred path for antifolate drugs, such as raltitrexed, pemetrexed and methotrexate [10]. Because FR $\alpha$  is undetected in normal cells [11], our approach may avoid toxicity onset. Thus this folate-peptide conjugate has the potential to be developed into a new tool for cancer chemotherapy. To quantify the intracellular concentration of FA-LR conjugate we set up a new, label-free spectrofluorometric method. As it relies on pterate fluorescence, this method could be extended to a wide range of FA-drug conjugates and, more importantly, it could be directly applied to studies of FA-drug conjugate pharmacokinetics in clinical samples. These findings suggest that the pterate-peptide conjugate can be developed as a tool for specifically targeting FR $\alpha$ -expressing cancer.

Studies on the stability and intracellular concentration of the peptide conjugate FA-LR via LC-MS/MS are currently under evaluation. Stability assays with mass spectrometry, as well as the fluorescence microscopy assessment of the intracellular distribution of a pteroyl-nonapeptide conjugated with a fluorescent probe (BODIPY), will complete our information on both internalization and intracellular localization of the compound. A new folic acid conjugate bearing a cleavable bond is currently being synthesized: this compound, unlike FA-LR, could not only be able to cross the cell membrane via FR $\alpha$ , but could also be released in the cytoplasm to exert its inhibitory activity on hTS and/or on other, not yet identified, targets. Indeed, in addition to lead optimization, future work will focus on cellular pharmacokinetic properties and biological effect of the new conjugates in cancer cells.

The compound itself can inhibit the hTS protein showing a IC<sub>50</sub> of 4  $\mu$ Molar. Indeed, FA-LR has a cytotoxicity similar to LR when the two compounds are delivered with the same delivery system, Saint-PhD delivery, on 2008, C13\*, A2780 and A2780/CP cell lines, after 72 hours (Figure 3.1.3.7.).

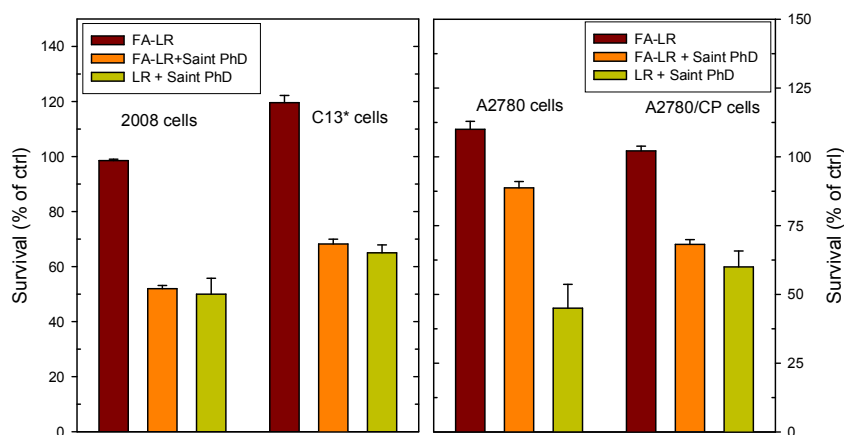


**Figure 3.1.3.7.** Cell growth inhibition of LR peptide and of bioconjugate FA-LR in the presence or absence of peptide delivery system. Cells have been allowed to attach overnight and then transfected with the peptides delivered by means of Saint-PhD, peptide delivery system. After 72 hours, cells have been stained with a crystal violet solution and the percentage of cytotoxicity was calculated by comparing the absorbance of cultures exposed to the drug to untreated controls.

### 3.1.5. Experimental procedures

**Cell lines.** The human ovarian cancer cell lines OAW28, COV504, IGROV1, TOV112D, 2008, C13\*, A2780 and A2780/CP were grown as monolayers in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and 50  $\mu$ g/ml gentamycin sulfate. All cell media and serum were purchased from Lonza (Verviers, Belgium). Cultures were equilibrated with humidified 5% CO<sub>2</sub> in air at 37 °C.

**Real Time PCR of FR $\alpha$  mRNA.** Cells were harvested by scraping and total RNA was isolated using the InnuSOLV RNA Reagent (Analytik Jena, Germany). Reverse transcription was performed with 1  $\mu$ g of total RNA using MMLV reverse transcriptase and oligo(dT) (Promega, Milan, Italy). The cDNA strand generated was used for PCR amplification in a total volume of 20  $\mu$ l. qPCR was performed with 40 ng of cDNA using the Power SYBR® Green PCR Master Mix (Applied Biosystems). Samples were amplified by 40 cycles at 95°C for 10 min, 94°C for 15 sec, 60°C for 1 min in Applied Biosystems 7900HT Fast Real-Time PCR System. We performed dissociation curve analysis and agarose gel electrophoresis to confirm the amplification [12]. The amount of RNA expressed was normalized with GAPDH

and detected by  $2^{-\Delta Ct}$  method [13]. FOLR1 (target) primers [NCBI,CoreNucleotide: NM\_016725.2]: forward: 5' GTGAGCAATGGTGGGAAGAT 3', reverse: 5' GTGGGTGTGGGGAAGTAGAA 3'; GAPDH (reference) primers [NCBI, CoreNucleotide: NM\_002046.3]: forward: 5' CAAGGTCATCCATGACAACTTTG 3',reverse: 5' GGGCCATCCACAGTCTTCTG.

**Western Blot Analysis.** Protein concentrations in cellular extracts were determined using the Bradford Protein Assay Reagent (Sigma Aldrich). For the assessment of TS levels, 40  $\mu$ g of each protein sample were resolved by SDS-PAGE according to the method of Laemmli [14]. For the assessment of FR $\alpha$  levels, non-reducing and non-denaturing conditions (no SDS) were used. Gels were blotted onto PVDF membranes (Hybond-P, Amersham). Antibody staining was performed with a chemiluminescence detection system (ECL Plus, Amersham), using a 1:500 dilution of the anti-human mouse Mov18 monoclonal primary antibody (Enzo Lifesciences) and 1:2000 dilution of anti-human  $\beta$ -actin mouse antibody (Santa Cruz Biotechnology, Inc.) in TBS-T with 5% dry milk for normalization, in conjunction with a 1:5000 dilution of a horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham).

**Flow cytometric analysis of FR $\alpha$  cell surface expression.** Cells were pre-treated with folate-free RPMI-1640 medium (Lonza) for 24 h (37°C, 5% CO<sub>2</sub>) before analysis. Mov18 primary antibody (10  $\mu$ g/mL, Enzo Lifesciences) was added to tumor cells ( $3 \times 10^5$ ) in 100 $\mu$ L PBS+1% Bovine Serum Albumin, and the mixture was incubated for 1h at room temperature. Antibody binding was detected by a FITC-conjugated secondary antibody (1:200, Dako) for 30 minutes, 4°C in dark. For each sample, at least 10000 cells were acquired with an Epics-XL Flow Cytometer (Beckman Coulter) and data were subsequently analyzed using the WinMDi software [15].

**Radioligand binding assays.** Cells were seeded in 6-well plates, and were allowed to grow for two more days to reach near-confluence. 24 h before measurement, the cell culture medium was replaced with folate-free (FF) medium, to allow the recycle of receptors to the cell surface. Cells were then washed twice with ice-cold acid buffer (acetate pH 3.5) to remove endogenous folates bound to FR at the cell surface, followed by two washes with ice-cold PBS (pH 7.4). To assess FR $\alpha$  protein at the cell surface, cells were incubated for 10 min with 5 nM [<sup>3</sup>H]folic acid (specific activity 0.5 Ci/mmol) in ice-cold PBS in the presence or absence of 5  $\mu$ M nonlabeled folic acid or FA-LR (1000-fold in excess of [<sup>3</sup>H]folic acid), and incubated for 10 min at 4°C. Uptake studies were conducted with 30 nM [<sup>3</sup>H] folic acid at 37°C in the presence and absence of 10  $\mu$ M unlabeled FA or FA-LR conjugate. After three

ice-cold PBS washes, cells were lysed in 0.7 ml of 0.1 N NaOH and 0.1% Triton X-100, and 50  $\mu$ l of cell lysate were used for protein determination by Lowry method, whereas 0.55 ml were analyzed for radioactivity using a scintillation counter (model Tri-Carb 2100, Packard Inc., USA).

**Spectrofluorometry.** Uptake of FA-LR was spectrofluorometrically measured following incubation of ca.  $6 \times 10^6$  IGROV1 cells with PBS in the presence and absence of 5  $\mu$ M FA-LR at 37 °C for 20 minutes, three homogeneous samples each. Cell extracts had a final volume of 700  $\mu$ l. 2  $\mu$ l of 1 M NaOH were added to each solution to a pH around 11, in order to convert all pterins to pteroate fluorophores. Emission ( $\lambda_{\text{exc}} = 360$  nm) and excitation ( $\lambda_{\text{em}} = 470$  nm) spectra were measured on a Horiba FluoroMax2 spectrofluorometer in 4x10 mm<sup>2</sup> quartz cuvettes at room temperature (25-28 °C). Because irradiation caused an increase in the pteroate fluorescence intensity, likely associated with photocleavage of the pteroyl/p-aminobenzoate bond [7], emission/excitation spectral measurements were repeated until the spectra did not change in two subsequent measurement cycles. Pteroate emission was read at 445 nm, excitation at 360 nm. In order to obtain the calibration factors needed to convert these signals into pteroate concentrations, the same measurements were carried out on the three control samples following additions of known, increasing amounts of the 5  $\mu$ M FA-LR supernatant.

**Cellular proliferation inhibition assay of FA-LR and FA-[D-Gln4]LR bioconjugates.**

Cells were seeded in 24-well plates (BD Falcon, Lincoln Park, NJ, USA) with RPMI complete medium to form adherent monolayers (60-80% confluent). 24 hours before the treatment with the peptide conjugates, cells were pre-treated with folate-free RPMI 1640 medium (FFRPMI, 37°C, 5% CO<sub>2</sub>) to induce a FOLR1 over-expression on their cell surface. Cells were, then, treated with FA-LR and FA-[D-Gln4]LR peptides conjugated (5-10  $\mu$ M in fresh FFRPMI), incubated at 37°C, 5% CO<sub>2</sub> for 1 hour and treated again with the same concentration. After a total 2 hours exposure, the peptide conjugated-containing media were replaced and the cells were allowed to further incubate for 70 hours in fresh RPMI complete medium. To verify whether a possible cytotoxic effect of the peptides was determined by the targeting specificity of the conjugates to FR $\alpha$ , an excess of folic acid was also used for the treatment of some wells to saturate the receptors. Briefly, thirty minutes before the addition of the peptide conjugates, spent medium was aspirated from some wells and replaced with FFRPMI supplemented with 100  $\mu$ M folic acid. After 30 minutes, cells were washed with fresh FFRPMI and treated with the peptide conjugates as described above [16,17].

In addition, treatment with LR peptide or the bioconjugate FA-LR was performed according to the standard transfection protocol of SAINT PhD delivery system (Synvolux Therapeutics, NL) (*see section 2.1.5*). The culture medium was aspirated from the cells, the SAINT-PhD/peptide complex was added to the wells and incubated 4 hours (37°C; 5% CO<sub>2</sub>). After this time, complete RPMI was added to reach the appropriate volume. The cells were allowed to further incubate for 68 hours in fresh RPMI complete medium.

The cytotoxicity of each compound was assessed by the crystal violet dye assay [18] (*see section 2.1.5*). The culture medium was removed and the cell monolayer was fixed with methanol prior to staining with 0.2% crystal violet solution in 20% methanol for 1 hour. After washing several times with distilled water to remove the dye excess, the cells were let to dry. The incorporated dye was solubilised in acidic isopropanol (1N HCl:2-propanol 1:10). Dye was determined spectrophotometrically at 540 nm. The extracted dye was proportional to cell number. Percentage of cytotoxicity was calculated by comparing the absorbance of exposed to non-exposed (control) cultures. All results were expressed as the percentage of absorbance (minus background) relative to the untreated control cells.

### 3.1.6. References

- [1] Zhao R., et al., *J. Biol. Chem.*, 2009, 284, 4267–4274;
- [2] Holm J., et al., *Biosci. Rep.*, **1997**, 17, 415–427;
- [3] Parker N., et al., *Anal. Biochem.*, **2005**, 338, 284–293;
- [4] Mauritz R., et al., *Cancer Chem. Pharmacol.*, **2008**, 62, 937–948;
- [5] Reddy J. A., et al., *Blood*, **1999**, 93(11), 3940–3948;
- [6] Kansara, V., et al., *Int. J. Pharm.*, **2008**, 355, 210–219;
- [7] Cabrerizo, et al., *Photochemistry and Photobiology*, **2005**, 81, 1234–1240;
- [8] Hirakawa K., et al., *Archives of Biochemistry and Biophysics*, **2003**, 410, 261–268;
- [9] Ligabue A., et al., *PLoS ONE*, **2012**, 7(10): e47318. doi:10.1371/journal.pone.0047318;
- [10] Leamon C. P. and Reddy J. A., *Adv. Drug Deliv. Rev.* 56(8), **2004**, 1127–1141;
- [11] Jackman, A. L., et al., *Adv. Drug Deliv. Rev.*, **2004** 56, 1111–1125;
- [12] Arocho A, et al., *Diagn. Mol. Pathol.*, **2006**, 15, 56–61;
- [13] Corona G, et al., *Int J Cancer*, **1998**, 75, 125–133;
- [14] Laemmli U. K., *Nature*, **1986**, 227, 680–685;
- [15] Figini M., et al., *Gene Therapy*, **2003**, 10, 1018–1025;
- [16] Leamon C. P., et al., *Bioconjugate Chem.*, **2006**, 17, 122–1232;
- [17] Leamon C. P., et al., *Cancer Res.*, **2008**, 68, 9839–984;
- [18] Kueng W., et al., *Anal. Biochem.*, **1989**, 182:16–9.

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