In Silico/In Vitro Study of Hybrid D-modified Steroidal Alkylator Anticancer Activity Using Uridine Phosphorylase as Target Protein

GEORGE D. GEROMICHALOS[1], ELENA GEROMICHALOU[2], CHARALAMBOS CAMOUTSIS[3], MICHAEL KONTOS[4], PANAGIOTIS DALEZIS[1], ATHANASIOS PAPAGEORGIOU[1], ANASTASIOS A. GRIVAS[5], CHRISTOS TSIGRIS[4] and DIMITRIOS T. TRAFALIS[6]

[1]*Laboratory of Cell Culture, Molecular Modeling and Drug Design, Symeonidion Research Center, Theagenion Cancer Hospital, Thessaloniki, Greece;*
[2]*Department of Chemistry, University of Ioannina, Ioannina, Greece;*
[3]*Laboratory of Medicinal Chemistry, Department of Pharmacy, University of Patras, Patras, Greece;*
[4]*First Clinic of Surgery, Laikon University Hospital, Athens, Greece;*
[5]*Department of Medical Oncology-A, Metaxa Cancer Hospital, Piraeus, Greece;*
[6]*Department of Pharmacology, Medical School, University of Athens, Athens, Greece*

Abstract. *Background: In order to reduce toxicity and to enhance anticancer activity of nitrogen mustards, three hybrid steroidal esters were synthesized and tested in vitro against human pancreatic cancer cells expressing uridine phosphorylase (UPase). The inhibition potency against a target protein implicated in the chemotherapy of solid tumors, such as UPase, is of fundamental importance in the design and synthesis of new anticancer drugs. Materials and Methods: MTT colorimetric assay and molecular docking were employed for the in vitro and in silico drug evaluation, respectively. Results: A difference in cell sensitivity was found, which followed the known different UPase expression in the cell lines. Molecular docking studies on UPase protein, revealed the tested compounds to be bound to the binding cavity of the protein, with different affinity. Between the two D-modified compounds, the D-homo-aza (lactam)-hybrid compound (C2) was found to interact with the protein in a more efficient way. Conclusion: The molecular docking data were in accordance with the in vitro results, where the lactam steroid alkylator showed significantly higher cytostatic and cytotoxic activity than the non-D-modified compounds, which also correlated with the level of UPase expression in the pancreatic cancer cells.*

Correspondence to: George D. Geromichalos, Ph.D., Head, Laboratory of Cell Culture, Molecular Modeling and Drug Design, Symeonidion Research Center, Theagenion Cancer Hospital, 2, Al. Symeonidi Str., 54007, Thessaloniki, Greece. Tel: +30 2310898237, Fax: +30 2310845514, e-mail: geromchem@yahoo.gr

Key Words: Uridine phosphorylase, hybrid steroidal alkylators, *in silico* molecular docking, *in vitro* assay, mitrogen mustards, pancreatic cancer cells.

Hybrid compounds, which combine two active molecules in one, such as steroid alkylators containing steroidal hormones as biological vectors for cytotoxic agents, have been introduced into anticancer therapy based on, the potential for increased intracellular concentration of these agents due to their lipophilic steroid carrier, low systemic toxicity and the possibility of targeting steroid receptors and increasing specificity particularly against hormone-dependent neoplasms (1, 2).

Although the majority of alkylating or similar agents are inactive in the treatment of tumours of the gastrointestinal system, the homo-aza-(lactam) steroids, which contain a –NH–CO– group inside the A or D steroid nucleus, as biological carriers for carboxylic derivatives of *N,N-bis*(2-chloroethyl)aniline (nitrogen mustards), demonstrated high activity against colon cancer both *in vitro* and *in vivo*, as well as presenting very good activity against pancreatic cancer (3-5). They are also effective against leukaemia (6-9), melanoma (8, 10) and non-small cell lung carcinoma (NSCLC) *in vitro* and *in vivo* (11) and a variety of cancer cell lines (12).

Several hypotheses of the structure–activity relationships that lead to the anticancer effect of the modified lactam steroid alkylators, have been proposed, but as yet, precise molecular mechanism of lactamic steroid alkylator action is still unclear. However, other cellular moieties and pathways, beside the typical alkylating impact, may be implicated. Our

laboratory showed that these compounds highly interact with the enzymes that are involved in the metabolism of pyrimidines because of the structural similarity of the steroidal lactam ring with the common pyrimidine derivative uracil (3).

Uridine phosphorylases (UPases, EC 2.4.2.3) have been shown to be of medical and biological importance in the activation and catabolism of fluoropyrimidines, which are used in the chemotherapy of solid tumors (13). UPase can also catalyse the formation of nucleosides from uracil and ribose-1-phosphate and is the most important phosphorylase in the regulation of uridine homeostasis. UPase is a critical enzyme in the activation of 5-fluorouracil (5-FU) and its prodrug 5'-deoxy-5-fluorouridine (5'DFUR /capecitabine) *via* anabolism of 5-FU through the pyrimidine salvage pathway or the phosphorolysis of 5'DFUR into 5-FU. Additionally, UPase activity is elevated in various tumour tissues and is believed to contribute to drug selectivity and to confer 5-FU therapeutic advantage (14-16). However, the clinical effectiveness of these fluoropyrimidine antimetabolites is impeded by their toxicity to normal tissue (17-19). On the other hand, decreased levels of UPase provide a mechanism of acquired resistance to 5-FU in colorectal cancer (20). A high rate of UPase expression is also related to a higher incidence of tumour relapse, more advanced cancer stage, and worse survival in patients with head and neck or breast cancer (21, 22).

Pancreatic cancer is normally presented at an advanced stage and survival rates remain low despite the routine use of chemotherapy and radiotherapy and the development of targeted therapies. Novel more effective therapeutic agents and approaches are urgently needed (23). UPase is highly expressed in most pancreatic carcinomas and could potentially be useful in tumour targeting (24).

In order to reduce toxicity and to enhance the anticancer activity of nitrogen mustards, we have synthesized three new androstan D-modified steroidal alkylators, 3β-hydroxy-5α-androstan-[*p-[N,N-bis*(2-chloroethyl)amino]phenyl]acetate (compound **C1**), 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-[*p-[N,N-bis*(2-chloroethyl) amino]phenyl]acetate (compound **C2**) and 3β-hydroxy-17-oxo-17α-oxa-5α-androstan-[*p-[N,N-bis*(2-chloroethyl)amino] phenyl]acetate (compound **C3**) (Figure 1a) (25, 26). The anticancer activity of these three compounds against three human pancreatic cancer cell lines that differentially express the UPase enzyme is here described.

This study also aimed at the elucidation of the underlying mechanism of action of the three modified steroidal esters using molecular docking studies (based on previous crystallographic and molecular modeling studies) to identify the binding interactions of the compounds with amino acid residues of the UPase protein within the active site of the protein target.

Materials and Methods

Drug preparations. The three esters of *p-[N,N-bis*(2-chloroethyl)aminophenyl]acetic acid with, androstan (**C1**), D-homo-aza (lactam)-androstan (**C2**) and D-lactone androstan (**C3**) were synthesized as previously described (26, 27). Stock solutions of the tested compounds, were made immediately before use. **C1**, **C2** and **C3** were initially dissolved in a small volume of 10% dimethyl sulfoxide (DMSO).

Cell cultures. Human pancreatic adenocarcinoma cell lines Panc1, MiaPaCa2 and Hs766T (obtained from the American Type Culture Collection (ATCC); Manassas, VA, USA), which differentially express UPase (Hs766T>Panc1>MiaPaCa2) (10), were grown in a monolayer in RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin, streptomycin and 1 mM sodium pyruvate and maintained at 37˚C in air containing 5% CO_2.

In vitro testing. The cells were seeded out at a density of 50,000 cells /ml and after 24 h culture were treated with 10–100 μM of the tested compounds **C1**, **C2** and **C3** for 48 h. The viability of the cultured cells was estimated by MTT assay (28). MTT (Sigma, St. Louis, MO, USA) was dissolved in PBS at a concentration of 5 mg/ml, filter sterilized and stored at 4˚C. MTT (0.2 ml of stock solution per ml) was added to each culture and incubated for 3 h at 37˚C to allow metabolization. The formazan crystals produced were solubilized by acidic isopropanol (0.04 N HCl in absolute isopropanol in a ratio 1:3 v/v). Absorbance of the converted dye was measured at a wavelength of 540 nm on an Anthos Labtec AR 8001 microplate reader (Diagnostics Pasteur, Sanofi). The mean concentrations of each drug that generated 50% or total (100%) growth inhibition (GI_{50} and TGI, respectively) as well as the drug concentrations that produced cytotoxicity against 50% of the cultured cells (IC_{50}) were calculated by the linear regression method (29, 30). Using seven absorbance measurements (time 24 h [Ct24], control growth 72 h [Ct72], and test growth in the presence of drug at five concentration levels [Tt72×]), the percentage of growth was calculated at each drug concentration. The percentage growth inhibition was calculated according to National Cancer Institute (NCI) as: ([Tt72×][Ct24]/[Ct72][Ct24])×100 for concentrations for which Tt72× ≥Ct24, and ([Tt72×][Ct24]/[Ct24])×100 for concentrations for which Tt72×<Ct24. GI_{50} was calculated from ([Tt72×][Ct24]/[Ct72] [Ct24])×100=50, TGI from ([Tt72×][Ct24]/[Ct72][Ct24])×100=0 and IC_{50} from [(Tt72×)Ct24)/Ct24]×100=50. All the experiments were conducted in triplicate.

In silico computational methods (molecular modeling and docking calculations). All the compounds were screened virtually against a large Protein Drug Target Database comprising almost 1,000 target proteins. The search for protein targets of known crystal structure and biological significance was restricted to three major therapeutic categories: neoplastic, inflammation and bacterial infection diseases. All the molecules were built in 3D coordinates and their best, most stable (lower energy) conformation was detected by geometrical optimization of their structure in the gas phase, as implemented in the Spartan '08 Molecular Modeling program suite (Spartan '08 v.1.2.0, Wavefunction Inc., Irvine, CA, USA; www.wavefun.com). The structure of the molecules were initially optimised (*via* energy minimization) by conformational search using the Monte Carlo method with the MMFF94 molecular mechanics model, included in the Spartan '08 program suite. Geometry optimization (leading to the most stable conformer with the lowest

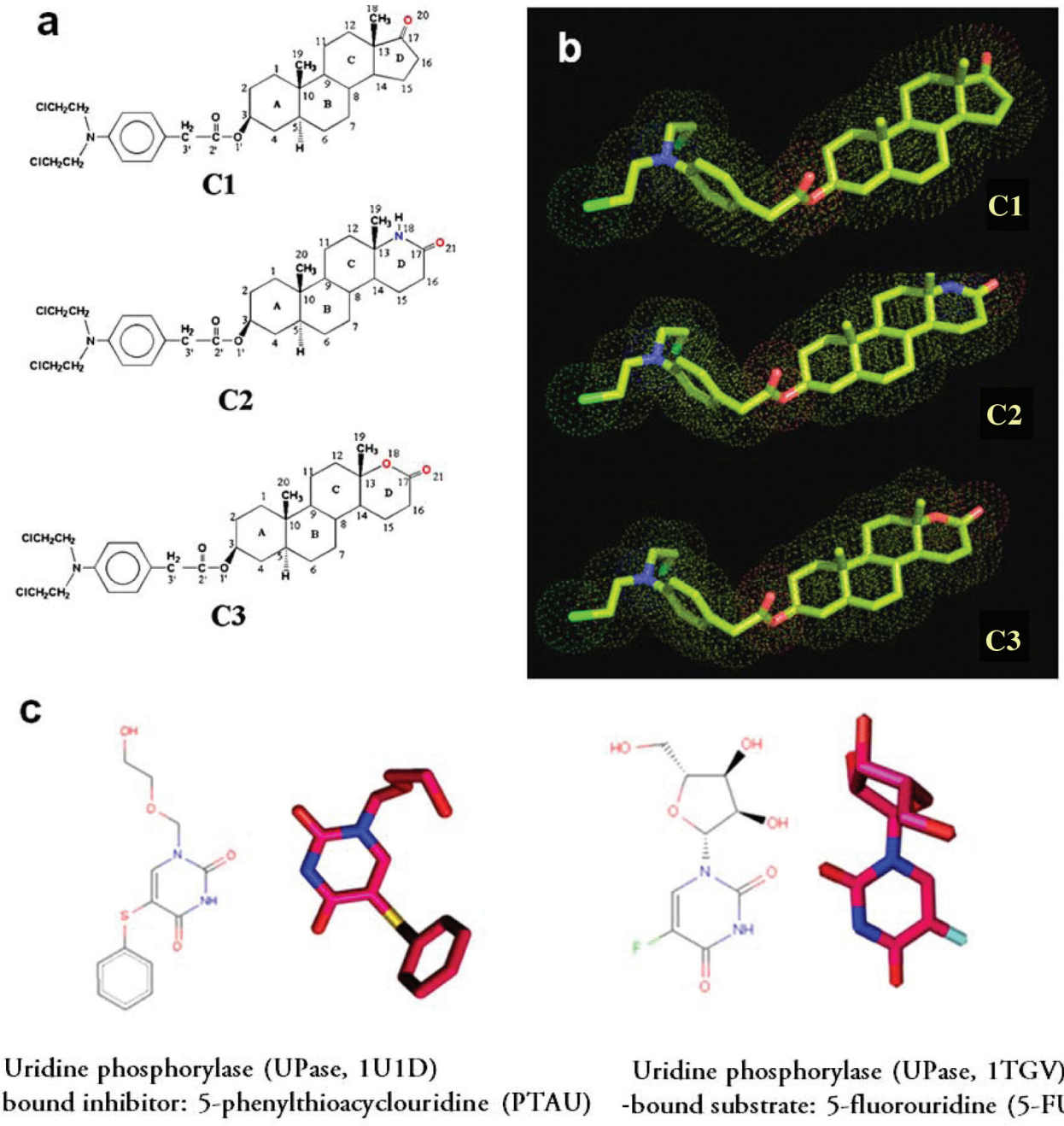

Figure 1. *Chemical (a) and molecular (b) structure of compounds **C1**, **C2**, and **C3**. In the 3-dimensional (3-D) models, molecules, omitting hydrogen atoms, are depicted as sticks colored by atom type; CL, N and O atoms are depicted in green, blue and red, respectively (a ray-traced representation surrounded by electron density surface as dots). Chemical and 3-D structures (c) of UPase inhibitor, 5-phenylthioacyclouridine (PTAU) (left) and substrate 5-fluorouridine (5-FU) (right) derived from the corresponding UPase crystal structures (Protein Data Bank, PDB entry codes: 1U1D and 1TGV, respectively).*

energy) was accomplished *via* quantum-chemical calculations by utilizing the *ab initio* Hartree–Fock method with a 6-31G* basis set (Figure 1b). The chemical structures of 5-phenylthioacyclouridine (PTAU or 1-[(2-hydroxyethoxy)methyl]-5-phenylthiouracil), a highly specific and potent inhibitor of UPase (31, 32), and the substrate used in the docking studies are shown in Figure 1c.

The molecular docking studies were carried out on two crystal structures of the UPase target protein (Protein Data Bank, PDB entry codes 1U1D and 1TGV), to investigate the effect of the synthesized compounds on this protein. Both X-ray structures of the UPase protein were obtained from the Brookhaven Protein Data Bank (operated by the Research Collaboratory for Structural

Table I. *In vitro cytostatic and cytotoxic effects of compounds **C1, C2, C3** on Panc1, MiaPaCa2 and Hs766T pancreatic cancer cells. Means of three independent experiments.*

Human pancreatic cancer cell line	Compound **C1**			Compound **C2**			Compound **C3**		
	GI_{50}	TGI	IC_{50}	GI_{50}	TGI	IC_{50}	GI_{50}	TGI	IC_{50}
MiaPaCa2	>100	>100	>100	25	45	77	86	>100	>100
Panc1	>100	>100	>100	14	32	61	59	>100	>100
Hs776T	>100	>100	>100	8	21	50	41	76	97
Mean	>100	>100	>100	15.7	32.7	62.7	62	>100	>100
P-value				<0.0001	<0.0001	<0.0001			

GI_{50} and TGI: mean drug concentrations generating 50% and total (100%) growth inhibition respectively; IC_{50}: drug concentration producing cytotoxicity against 50% of the cultured cells (according to NCI protocol). *P*-value refers to comparison between compound **C2** and the group of compounds **C1** and **C3**.

Bioinformatics, RCSB) (33-35). The molecular docking simulations were performed by BioMedCAChe 6.1 computer-aided chemistry software package, which is part of the CAChe package (CAChe WorkSystem Pro version 7.5.0.85, Fujitsu Co. Ltd., Tokyo, Japan) (36). The compound–protein complexes produced were ranked by the energy score, including their binding conformations. Best docked poses, with both lower binding energies and stronger interaction pattern, were derived from a number of solutions (docking results), usually with the higher ranking. Three-dimensional (3D) models of the above protein crystal structures were developed, after the deletion of the co-crystallized bound inhibitor. The docking procedure enables the flexibility of the ligand within the protein binding site by means of a 4-point chiral pharmacophoric comparison between the ligand and the site. The final output of the docking procedure is a set of solutions (docking results) ranked according to the corresponding scoring function values, each defined by the 3D coordinates of its atoms and expressed as a PDB file. In this study, the docking procedure with the aid of BioMedCAChe, was shown to accurately reproduce experimentally observed binding modes of the studied UPase inhibitor PTAU and its substrate 5FU, in terms of RMSD (root-mean squared deviation). The BioMedCAChe provided excellent results as low values of RMSD (best docked solutions of docking results 0.23-0.45 Å) were observed between the experimental and the best-scoring docked structures derived by superimposition of these structures (the accuracy of the docking results are good when the RMSD is mostly below 1.0 Å). The ability to accurately predict the binding conformation of the enzyme inhibitor and substrate, gave confidence that the BioMedCAChe would also exhibit a similar accuracy with the investigated molecules in the study. The PyMol molecular graphics system (DeLano Scientific, San Carlos, CA, USA, version 0.99), was used to visualize the molecules and the results of the docking and to construct the molecular models (37).

Statistical analysis. Statistical analysis was performed with SigmaPlot software v. 10.0 (SigmaPlot Software, Inc., Germany) using two-tailed Student's *t*-test. In all tests, a criterion of $p \leq 0.05$ was considered necessary for statistical significance. For all values derived from the corresponding dose–effect curves and drawn from triplicate determinations, coefficient of variation (CV) calculated to be lower than 5%.

Results

In vitro testing. The D-lactam steroid alkylator (**C2**) presented significant activity (compared to compounds **C1** and **C3**) against the 3 human pancreatic adenocarcinoma cell lines (Panc1, MiaPaCa2 and Hs766T) tested ($p<0.0001$, *t*-test). **C2**, in comparison to **C1** and **C3**, was the most active ($p<0.001$, paired *t*-test) whereas the respective D-lactone steroid ester (**C3**) showed modest activity and the unmodified androsterone alkylator (**C1**) was inactive (Table I). Furthermore, the anticancer effects of **C2** correlated with the expression of UPase in the pancreatic cancer cells. Thus, Hs776T cells that express the higher level of UPase were the most sensitive, Panc1 cells were less sensitive and MiaPaCa2 cells that present the lowest UPase expression demonstrated the least sensitivity to **C2** treatment (Figure 2).

In silico docking study of protein–ligand interactions. The computed binding energies (kcal/mol) of the tested compounds **C1**, **C2** and **C3** on the UPase protein are shown in Table II, and indicated that **C2** established better attachment than **C1** and **C3**, while C1 bound to protein better than **C3**.

The docking results of the **C1**, **C2** and **C3** molecules in complex with the crystal structure of the 1U1D UPase showed that in both subunits, in four out of the six chains of the hexameric structure of the UPase, the **C1**, **C2** and **C3** molecules were docked at the same place as PTAU was attached (Figure 3a). However, in addition to binding in the active site, in two docking poses for the compounds **C1** and **C2** and one for **C3**, the interaction of the **C1**, **C2** and **C3** molecules with separate binding pockets located near the center of the hexameric structure was identified (Figure 3a). The **C1**, **C2** and **C3** molecules were inserted in the binding pocket of the protein by their steroidal moieties, while the alkylating moieties were positioned at the opening of the binding pocket. Figure 3b illustrates the ligand-binding pocket of the protein in one chain of the hexameric structure

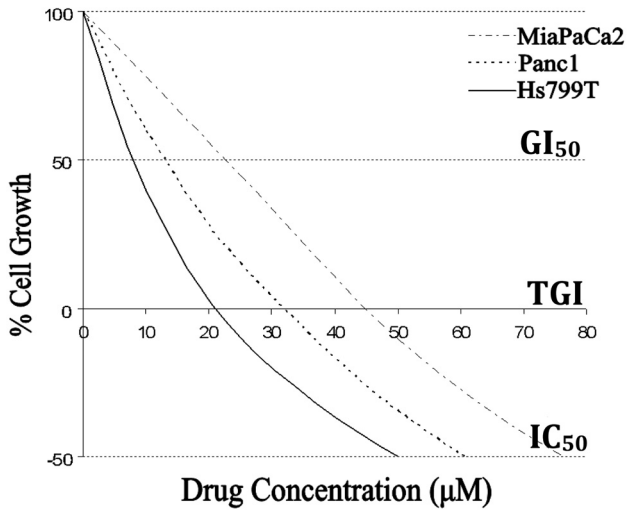

Figure 2. *Growth inhibition (sensitivity curves) of Panc1, MiaPaCa2 and Hs766T pancreatic cancer cells, produced by* ***C2*** *treatment.*

Table II. *Computed binding energies* (kcal/mol) for* ***C1***, ***C2***, ***C3****/UPase complexes.*

Uridine phosphorylase PDB entry code	Hybrid steroidal ester		
	C1	**C2**	**C3**
1TGV	–36.80	–37.96	–35.82
1U1D	–37.19	–38.12	–35.44

PDB: Protein Data Bank. *The lesser the binding energy of a complex, the more stable it is.

with the docked conformations of the **C1**, **C2**, **C3** and PTAU molecules. The stabilization of the compounds in the pocket was attributed to the formation of hydrogen bond, polar and hydrophobic contacts (the latter interactions for clarity are not shown in the figure).

The X-ray crystallography study revealed (Figure 3c, d) that the PTAU inhibitor anchored the UPase target protein *via* a network of hydrogen bond and hydrophobic binding interactions with the amino acid residues of the ligand-binding cavity of the protein. The C2 carbonyl O of the pyrimidine ring of PTAU interacted with the amino acid residue Gln166/NE2 (2.76 Å) and hydrogen bonded with water67/OH (3.48 Å). In addition, the C4 carbonyl O of the pyrimidine ring of PTAU contributed to the protein binding *via* polar and hydrogen bond interactions with the following amino acid residue and water molecules: Gln166/OE1 (3.45 Å), Arg168/NH1 (3.44 Å), Arg168/NH2 (2.81 Å) and water263/OH (2.74 Å). Additionally, the ethoxy oxygen atom of the (2-hydroxyethoxy)methyl group and the carbonyl oxygens of the

Table III. *Molecular docking interactions between* ***C1*** *and UPase (PDB entry code1U1D) elements (amino acid residues and water molecules). Binding contacts of best docked pose of the ligand with key residues in the active site of the model were determined.*

Amino acid residue of UPase or water molecule with its binding atom*	Interacting **C1** atom*	Distance (Å)	Type of interaction
Water159/OH	O20	1.90	H-bond
Water67/OH	O20	3.48	H-bond
Tyr195/OH	O20	2.70	H-bond
Thr94/OH	O20	3.31	H-bond
Water406/OH	O20	2.18	H-bond
Water120/OH	O20	2.14	H-bond
Water84/OH	O20	2.10	H-bond
Gly118/O	O20	3.13	H-bond
Glu127/OE2	O20	2.87	H-bond
Water269/OH	C2' carbonyl O	1.96	H-bond
Water148/OH	C2' carbonyl O	3.53	H-bond
Water397/OH	C2' carbonyl O	1.91	H-bond
Water54/OH	C2' carbonyl O	3.00	H-bond
Water59/OH	C2' carbonyl O	3.38	H-bond
Lys3/NHZ	N	2.64	H-bond
Water201/OH	N	1.80	H-bond
Ile220/CD1	C1	3.43	Hydrophobic
Ile220/CD1	C2	4.07	Hydrophobic
Glu196/C	C17	4.17	Hydrophobic
Glu196/CA	C17	3.41	Hydrophobic
Glu196/CA	C13	4.63	Hydrophobic
Pro229/CA	Aromatic C (*o*-position to N)	3.79	Hydrophobic
Pro229/C	Aromatic C (*o*-position to N)	2.30	Hydrophobic
Pro229/CB	Aromatic C (*o*-position to N)	2.60	Hydrophobic
Glu227/C	Cα of -N($CH_2CH_2Cl)_2$ group	1.29	Hydrophobic
Glu227/C	Cβ of -N($CH_2CH_2Cl)_2$ group	2.16	Hydrophobic
Ile228/CG1	Cα of -N($CH_2CH_2Cl)_2$ group	2.25	Hydrophobic
Ile228/CD1	Aromatic C (*o*-position to N)	2.20	Hydrophobic
Ile228/CB	Cα of -N($CH_2CH_2Cl)_2$ group	1.75	Hydrophobic
Ile228/CB	Cβ of -N($CH_2CH_2Cl)_2$ group	1.41	Hydrophobic
Ile228/CG2	Cα of -N($CH_2CH_2Cl)_2$ group	1.45	Hydrophobic
Ile228/CG2	Cβ of -N($CH_2CH_2Cl)_2$ group	1.72	Hydrophobic
Pro229/C	Cβ of -N($CH_2CH_2Cl)_2$ group	4.82	Hydrophobic

*Ligand, residue and water numbering according to PyMol v. 0.99c.

pyrimidine ring of PTAU accepted hydrogen bonds from the amino acid residues His11 (2.79 Å), Thr97 (3.12 Å) and Gln169 (2.68 Å) and water molecule water299/OH (3.00 Å). Furthermore, in the UPase/PTAU complex, it was established that hydrophobic interactions involved the residues Ile714 (3.76 Å), Val715 (3.86 and 3.79 Å), Thr589 (3.88 Å), Gln660 (3.85 Å), Phe656 (3.86, 3.77, 3.65, 3.68, 3.69 and 3.79 Å) and Gly590 (3.59, 3.80, 3.89 and 3.74 Å).

C1 compound was stabilized in the complex with the 1U1D UPase protein through a number of hydrogen bond and hydrophobic contacts as shown in Table III.

The **C2** molecule was found to be bound to the protein by many more interactions (polar, hydrogen bond or

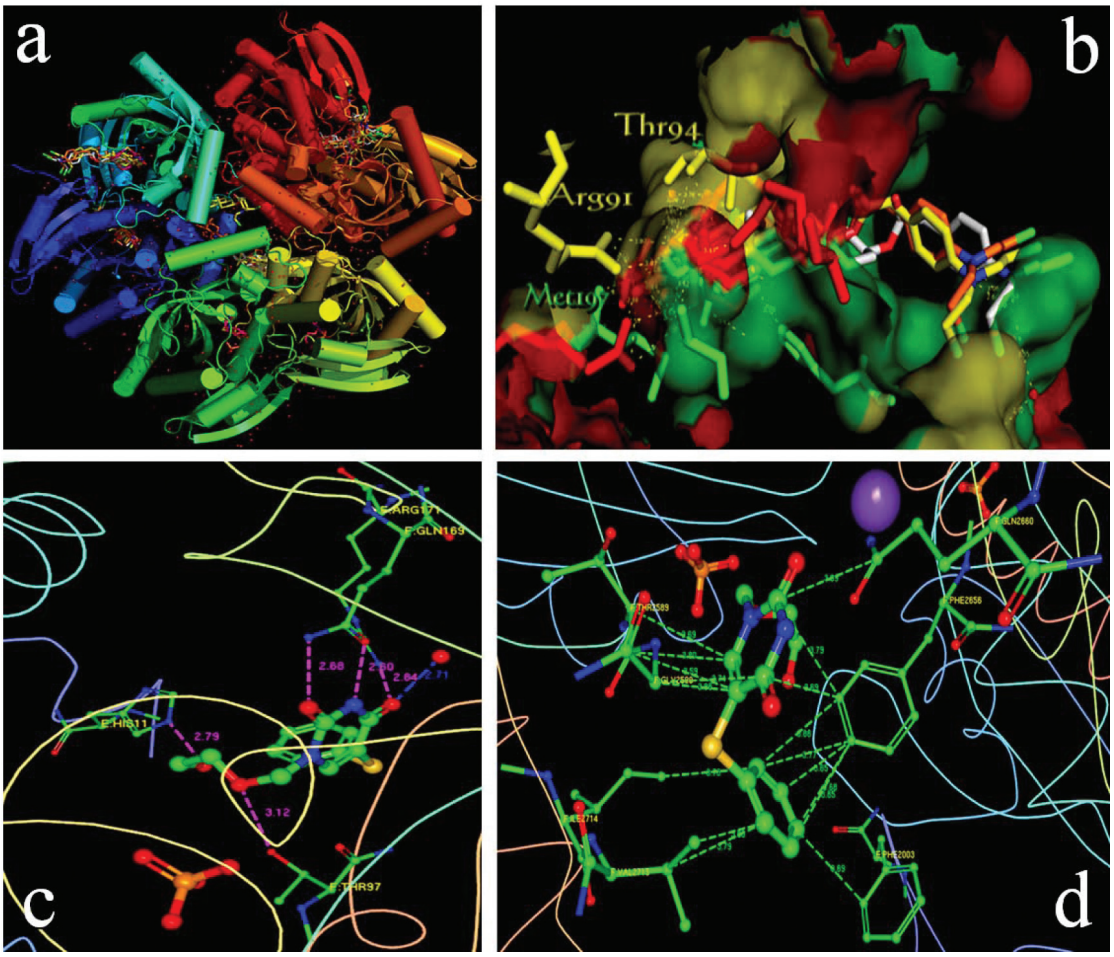

Figure 3. *UPase target protein (PDB entry code 1U1D) bound with 5-phenylthioacyclouridine (PTAU) and the docked compounds **C1**, **C2** and **C3**. Hexameric target protein-ligand complex (a) is depicted as ray-traced cylinder schematic coloured by spectrum and the ligand-binding cavity of the protein (b) in one chain is stick represented as a semitransparent surface coloured by secondary structure of the amino acid residues, while superimposed bound molecules are colored according to atom type (carbon, hotpink (PTAU), yellow (**C1**), orange (**C2**) and white (**C3**); CL, N and O, green, blue and red, respectively). Hydrogen atoms are omitted from all molecules for sake of clarity (the final structure (a, b) was ray-traced). PTAU docking orientations stabilized inside the ligand-binding cavity of the protein through hydrogen bond (mauve dashed lines) (c) and hydrophobic (green dashed lines) (d) binding interaction. Visualizations of the docking results were carried out via PyMol (a, b) and LigandExplorer software (c, d).*

hydrophobic) compared to **C1** and **C3**, with both its steroidal and alkylating moiety. The incorporation of the nitrogen atom in the D-ring of **C2** (N18) improved its binding capacity with the involvement of the amino acid residues, water molecules and phosphate group as shown in Table IV.

The **C3** compound was stabilized in the UPase/C3 complex more weakly than **C2** and again through both the alkylating and steroidal moieties of the molecule. The stabilization of the molecule in the alkylating pocket was attributed to a hydrogen bond network involving: O1' atom, **C2'** carbonyl oxygen and nitrogen atom of the **N**-chloroethyl group (Table V). The steroidal moiety of the **C3** molecule contributed to the binding through hydrogen bond and polar contacts of the D-ring C17 carbonyl oxygen (O21) atom and O18.

The stabilization of the compounds in the pocket was additionally attributed to hydrophobic contacts formed by the hydrophobic residues Ile220, Ile228, Leu121, Phe7, and Phe162. Hydrophobic interactions were also observed with the incorporation of Pro229, Thr95, Glu227, Glu196, Glu127, His8, Arg48, Lys3, Ser4 and His179 (Tables III-V).

Figure 4 illustrates the docked compounds **C1**, **C2** and **C3** superimposed with 5-FU within the 1TGV UPase binding site. As illustrated from the ligand-binding site architecture, the ligands occupied the same place as 5-FU in the crystal structure. All the compounds were shown to be anchored inside the ligand-binding pocket *via* polar, H-bond and van der Waals hydrophobic interactions with residues Ile220, Val221, Asn222, Ile228, Pro229, Phe7, His8, Thr9, Arg91,

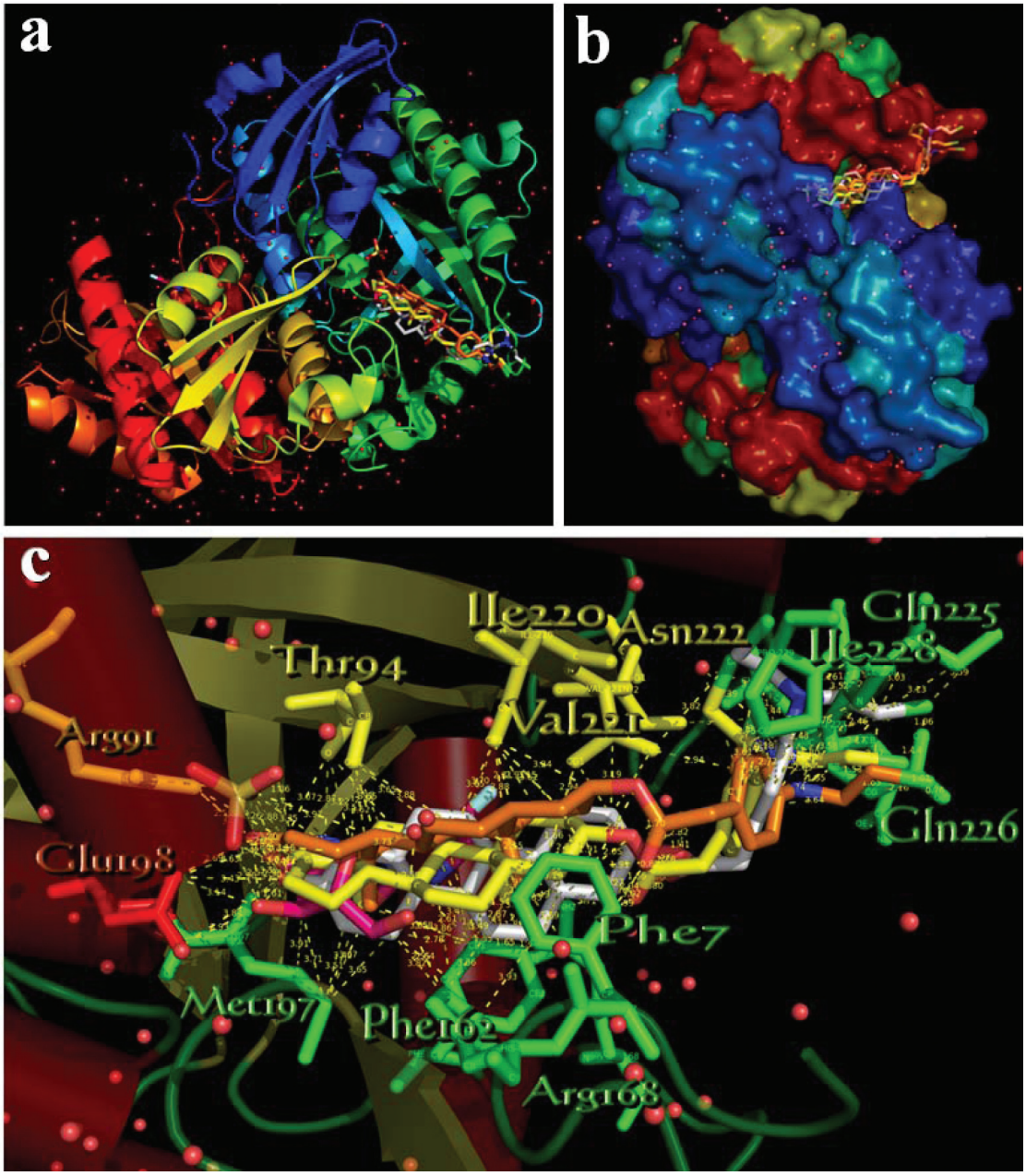

Figure 4. *UPase target protein (PDB entry code 1TGV) bound with 5-FU and the docked compounds **C1**, **C2** and **C3**. Protein–ligand complex is depicted as ribbon schematic coloured by spectrum (a) or semitransparent surface coloured by chain bow (b), while superimposed bound molecules are shown in stick representation and coloured according to atom type (carbon, hotpink (5-FU), yellow (**C1**), orange (**C2**) and white (**C3**); CL, N and O, green, blue and red, respectively). Polar, H-bond and hydrophobic binding interactions (c) (dashed yellow lines) are illustrated between amino acid residues of the ligand-binding site of UPase and 5-FU, **C1**, **C2** and **C3** ligands. Protein is depicted (c) as semitransparent ray-traced cylinder schematic coloured by secondary structure. Solvent water molecules are shown as red spheres. Hydrogen atoms are omitted from all molecules for sake of clarity (the final structure was ray-traced).*

Phe162, Arg168, Met197 and Glu198. The polar, H-bond and hydrophobic interactions between compounds **C1**, **C2**, **C3** and 5-FU and the amino acid residues of the ligand binding cavity of UPase (1TGV) are shown in Figure 4c. Binding interactions between the atoms of the steroidal and alkylating moieties of compounds **C1**, **C2** and **C3** and the atoms of the residues of the active site of UPase are shown in Table VI.

Beyond the UPase target protein, for each compound, after extended virtual screening with the aid of molecular modelling and docking techniques, more than 20 chief target

Table IV. *Molecular docking interactions between **C2** and UPase (PDB entry code1U1D) elements (amino acid residues and water molecules). Binding contacts of best docked pose of the ligand with key residues in the active site of the model were determined.*

Amino acid residue of UPase or water molecule with its binding atom*	Interacting **C2** atom*	Distance (Å)	Type of interaction
Water19/OH	N18	2.61	H-bond
PO_4/O1	N18	3.52	H-bond
Water406/OH	N18	2.27	H-bond
Water84/OH	N18	2.84	H-bond
Met197/N	O21	1.91	Polar
Glu198/OE2	O21	3.00	Polar
Water19/OH	O21	1.67	H-bond
Water43/OH	O21	2.49	H-bond
Water67/OH	O21	1.62	H-bond
Water84/OH	O21	2.55	H-bond
Water109/OH	O21	1.41	H-bond
PO_4/O4	O21	2.78	Polar
PO_4/O1	O21	3.31	Polar
Water446/OH	C2' carbonyl O	1.12	H-bond
Water248/OH	C2' carbonyl O	3.53	H-bond
Water111/OH	O1'	1.63	H-bond
Water394/OH	O1'	3.23	H-bond
Water398/OH	N	3.34	H-bond
Glu196/C	C17	3.87	Hydrophobic
His8/CE1	C1	3.01	Hydrophobic
His8/CD2	C11	3.94	Hydrophobic
Phe7/CD2	C3'	4.43	Hydrophobic
Phe7/CE2	C3	4.76	Hydrophobic
Pro229/CD	C2'	4.22	Hydrophobic
Phe162/CE1	C9	3.72	Hydrophobic
Phe162/CZ	C10	4.68	Hydrophobic
Thr95/CA	C19	4.26	Hydrophobic
Arg48/CZ	C11	3.57	Hydrophobic
Arg48/CZ	C12	3.50	Hydrophobic
Ser4/CB	Aromatic C (*o*-position to N)	4.29	Hydrophobic
Ser4/CB	Aromatic C (*o*-position to N)	3.46	Hydrophobic
Ser4/CB	Aromatic C (*m*-position to N)	4.28	Hydrophobic
Lys3/CD	Cα of -N($CH_2CH_2Cl)_2$ group	2.67	Hydrophobic
Lys3/CE	Cα of -N($CH_2CH_2Cl)_2$ group	4.08	Hydrophobic

*Ligand, residue and water numbering according to PyMol v. 0.99c.

Table V. *Molecular docking interactions between **C3** and UPase (PDB entry code1U1D) elements (amino acid residues and water molecules). Binding contacts of best docked pose of the ligand with key residues in the active site of the model were determined.*

Amino acid residue of UPase or water molecule with its binding atom*	Interacting **C3** atom*	Distance (Å)	Type of interaction
Water61/OH	O1'	3.04	H-bond
Water395/OH	O1'	1.40	H-bond
Water40/OH	C2' carbonyl O	1.82	H-bond
Water16/OH	C2' carbonyl O	2.30	H-bond
Water405/OH	C2' carbonyl O	2.94	H-bond
Water323/OH	N	2.33	H-bond
Water312/OH	N	2.76	H-bond
Water435/OH	N	3.31	H-bond
Water109/OH	O21	1.37	H-bond
Water254/OH	O21	1.79	H-bond
Water67/OH	O21	1.97	H-bond
Water201/OH	O21	1.82	H-bond
Met197/N	O21	3.03	Polar
Glu198/OE1	O21	2.66	Polar
Glu198/OE2	O21	3.10	Polar
PO_4/O3	O21	3.50	Polar
PO_4/O4	O21	3.11	Polar
Water201/OH	O18	2.57	H-bond
Water257/OH	O18	3.53	H-bond
PO4/O1	O18	2.82	Polar
Leu121/CD1	C4	2.19	Hydrophobic
Leu121/CD1	C5	2.04	Hydrophobic
Leu121/CD1	C20	3.03	Hydrophobic
Glu127/CD	C19	2.39	Hydrophobic
His179/CE1	Cα of -N($CH_2CH_2Cl)_2$ group	1.08	Hydrophobic

*Ligand, residue and water numbering according to PyMol v. 0.99c.

proteins were also found to be inhibited in a specific order of binding capacity. The produced compound–protein complexes were ranked by the energy score, including their binding conformations (Table VII).

Discussion

This preclinical research indicated that compound **C2** – a promising D-homo-aza-steroid alkylating ester – is a very active compound with a relatively low toxicity rate and a high therapeutic ratio against pancreatic cancer cells. Previously, the compound **C2** (NSC 290205) has shown *in vivo* cytostatic activity against murine pancreatic adenocarcinoma PAN02 in combination with adriamycin (38) was active in treating lung and breast xenografts, as well as a number of rodent tumors (25, 39) and leukaemia (40), and induced cytogenetic damage in human lymphocytes (41).

Prediction of the binding mode of a ligand within the active site of a protein of known structure is of paramount importance in rational drug design. The differences in activity of the studied compounds towards the tested cell lines, as shown in the *in vitro* experiments, could be partially explained by the molecular docking calculations which indicated better stabilization with **C2** than **C1** or **C3** inside the binding cavity of the protein. The lower predicted binding energy of compound **C2** (which included the lactamic group) compared to that of compounds **C1** and **C3** was in accordance with its extended contact network. The docking simulation studies showed that compounds **C1**, **C2** and **C3** entered into the binding pocket of UPase protein with their steroidal part and were positioned in the same

Table VI. *Molecular docking interactions between **C1, C2** and **C3** and UPase (PDB entry code1TGV) elements (amino acid residues and water molecules). Binding contacts of best docked pose of the ligands with key residues in the active site of the model were determined.*

Amino acid residue of UPase or water molecule with its binding atom*	Interacting **C1/C2/C3** atom*	Distance (Å)	Type of interaction
Thr94/O	O20 / C12 / O18	3.07 / 3.48 / 3.22	Polar / Polar / Polar
Thr94/C	– / D-ring N / –	– / 2.87 / –	– / Polar / –
Thr94/OG1	C12 / C9 / O18	3.61 / 3.88 / 3.53	Polar / Polar / Polar
	– / C13 / C12	– / 3.63 / 3.65	– / Polar / Polar
	– / C14 / –	– / 2.45 / –	– / Polar / –
Arg91/NH_2	O20 / O21 / –	3.29 / 2.52 / –	H-bond / H-bond / –
Arg168/NH_2	– / – / C4	– / – / 3.71	– / – / Polar
	– / – / C20	– / – / 1.87	– / – / Polar
	– / – / C2' carbonyl O	– / – / 3.70	– / – / H-bond
Phe7/CZ	C3 / C4 / C5	2.27 / 1.66 / 3.19	H-phobic / H-phobic / H-phobic
	C4 / C6 / –	1.32 / 2.15 / –	H-phobic / H-phobic / –
	C5 / C20 / –	1.83 / 2.71 / –	H-phobic / H-phobic / –
Phe7/CE1	O1' / C2' carbonyl O / –	2.05 / 1.91 / –	Polar / Polar / –
	– / O1'/ –	– / 2.91 / –	– / Polar / –
	C3 / C4 / C4	1.91 / 1.95 / 3.21	H-phobic / H-phobic / H-phobic
Phe7/CE2	C5 / – / –	1.93 / – / –	H-phobic / – / –
	C6 / – / –	1.54 / – / –	H-phobic / – / –
Phe7/CD1	C2' carbonyl O / C2' carbonyl O / –	2.99 / 2.04 / –	Polar / Polar / –
Phe162/CZ	C11 / C20 / C6	3.09 / 2.70 / 1.53	H-phobic / H-phobic / H-phobic
	C19 / – / C7	1.84 / – / 1.65	H-phobic / – / H-phobic
Phe162/CE1	C18 / C11 / C7	3.95 / 3.35 / 1.35	H-phobic / H-phobic / H-phobic
	C19 / C19 / C8	1.83 / 3.58 / 1.61	H-phobic / H-phobic / H-phobic
	C6 / C20 / C14, C15	3.49 / 2.87 /2.61, 2.78	H-phobic / H-phobic / H-phobic
Phe162/CE2	– / – / C5	– / – / 3.49	– / – / H-phobic
Phe162/CD1	– / – / C15	– / – / 2.55	– / – / H-phobic
Phe162/CD2	– / – / C5	– / – / 3.69	– / –/ H-phobic
	– / – / C6	– / – / 3.41	– / –/ H-phobic
Glu227/OE1	Cl / – / –	1.14 / – / –	Polar / – / –
Glu227/OE2	Cl / Cl / –	1.73 / 0.76 / –	Polar / Polar / –
Glu227/O	– / – / N	– / – / 2.26	– / – / Polar
	– / – / Cβ to Cl	– / – / 1.51	– / – / Polar
	– / – / Cα to Cl	– / – / 2.03	– / – / Polar
Glu227/N	– / – / Cl	– / – / 3.03	– / – / Polar
	– / – / Cβ to Cl	– / – / 1.65	– / – / Polar
	– / – / Cα to Cl	– / – / 1.65	– / – / Polar
Glu227/CA	– / Cβ to Cl / –	– / 3.01 / –	– / Polar / –
Glu227/CB	– / N (alkyl. moiety) / Cα to Cl	– / 1.35 / 2.17	– / Polar / H-phobic
	– / – / CAr	– / – / 2.05, 2.74	– / – / H-phobic
Glu227/CG	– / Cl / Cα to Cl	– / 2.16 / 2.57	– / Polar / H-phobic
	– / – / CAr	– / – / 3.64	– / – / H-phobic
Glu227/CD	– / Cl / –	– / 1.01 / –	– / Polar / –
Glu198/OE1	– / – / O21	– / – / 3.43	– / – / H-bond
Glu198/OE2	– / – / O21	– / – / 3.84	– / – / H-bond
Gln225/O	– / Cl / –	– / 2.71 / –	– / Polar / –
Gln226/O	– / Cl / N	– / 1.62 / 3.19	– / Polar / Polar
	– / Cα to Cl / Cβ to Cl, CAr	– / 1.99 / 3.44, 2.12	– / Polar / H-phobic
Asn222/O	– / Cl / –	– / 3.77 / –	– / Polar / –
His8/NE2	– / – / C7	– / – / 3.36	– / – / Polar
	– / – / C15	– / – / 3.82	– / – / Polar
His8/CE1	– / – / C6	– / – / 3.93	– / – / H-phobic
Ile220/CD1	– / C1 / C1	– / 2.91 / 3.62	– / H-phobic / H-phobic
	– / C3 / C11	– / 3.24 / 3.70	– / H-phobic / H-phobic
	– / C4 / –	– / 3.15 / –	– / H-phobic / –
	– / C5 / –	– / 2.31 / –	– / H-phobic / –
	– / C9 / –	– / 3.05 / –	– / H-phobic / –
	– / C10 / –	– / 2.88 / –	– / H-phobic / –

Table VI. *continued*

Table VI. *continued*

Amino acid residue of UPase or water molecule with its binding atom*	Interacting **C1/C2/C3** atom*	Distance (Å)	Type of interaction
Ile228/NH	– / N (alkyl. moiety) / –	– / 3.58 / –	– / H-bond / –
Ile228/CA	– / – / N	– / – / 2.76	– / – / Polar
Ile228/CD1	– / – / Cl	– / – / 3.59	– / – / Polar
Ile228/OE1	– / – / Cl	– / – / 1.06	– / – / Polar
Ile228/CB	– / – / Cα to Cl	– / – / 3.03	– / – / H-phobic
Ile228/CG1	– / – / Cα to Cl	– / – / 3.13	– / – / H-phobic
Ile228/N	– / – / Cα to Cl	– / – / 1.55	– / – / Polar
Val221/CB	– / – / C2	– / – / 3.19	– / – / H-phobic
Val221/CG1	– / – / C1	– / – / 2.99	– / – / H-phobic
	– / – / C2	– / – / 3.03	– / – / H-phobic
	– / – / C20	– / – / 3.69	– / – / H-phobic
Val221/CG2	Cα to Cl / Cα to Cl / C2	3.82 / 2.94 / 3.53	H-phobic / H-phobic / H-phobic
Met197/SD	C18 / – / C15, C16	3.42 / – / 3.61, 3.51	Polar / – / Polar
Met197/NH	O20 / – / –	3.32 / – / –	H-bond / – / –
Met197/CA	– / O21 / –	– / 2.69 / –	– / Polar / –
Met197/N	– / D-ring N / –	– / 4.09 / –	– / Polar / –
Pro229/CB	– / CAr / –	– / 2.75, 3.59 / –	– / H-phobic / –
Pro229/CG	– / CAr / CAr	– / 2.18, 1.46 / 2.72	– / H-phobic / H-phobic
Water19/O	– / Cl / –	– / 2.39 / –	– / Polar / –
Water119/O	– / Cα to Cl / –	– / 3.50 / –	– / Polar / –
Water119/OH	– / – / N	– / – / 3.55	– / – / H-bond
Water57/OH	– / – / O18	– / – / 3.73	– / – / H-bond

*Ligand, residue and water numbering according to PyMol v. 0.99c. H-phobic: Hydrophobic, CAr: aromatic C atom, Cα to Cl: C atom connected to Cl, Cβ to Cl: the second C atom away from Cl, N (alkyl. moiety): the N atom of the chloroethyl alkylating group.

Table VII. *Molecular docking of compounds **C1, C2, C3** with a panel of target proteins.*

Target protein	PDB entry code	Computed binding energy* (kcal/mol)		
		C1	**C2**	**C3**
Uridylate kinase	1UKZ	–	–38.86	–36.02
Thymidylate synthase	1BID	–30.08	–32.49	–31.59
Dihydrofolate reductase	1DHF	–37.30	–37.24	–37.71
FKBP, tacrolimus binding protein (FK506)	1TCO	–41.31	–37.87	–39.00
HIV protease	1HVR	–39.93	–41.32	–38.28
Glutamate mutase	1I9C	–	–39.39	–39.36
Protein farnesyl transferase	1FT2	–39.19	–39.47	–40.3
3-Alpha-hydroxysteroid dehydrogenase	1HDC	–36.5	–38.1	–38.88
PARP	2PAW	–	–37.99	–
Nitric oxide synthase	2NSE	–	–37.97	–36.19
Neuraminidase	2SIM	–37.94	–	–
Glutamate carboxypeptidase II	2C6C	–	–37.89	–
11-Hydroxysteroid dehydrogenase	1Y5M	–37.47	–35.79	–
Glycinamide ribonucleotide formyltransferase	1JKX	–37.45	–	–
Ornithine aminotransferase	2CAN	–	–37.29	–36.32
Peptidase	1FNO	–37.03	–	–
Phospholipase A2	5P2P	–36.96	–	–
Acetylcholinesterase	1VXR	–36.75	–	–
Dipeptidyl peptidase IV (DPP4)	2FJP	–36.72	–35.98	–37.69
Hyaluronate lyase	1OJN	–	–35.93	–
Flavohemoglobin	1E7W	–36.63	–38.45	–40.38
Peptide *N*-myristoyltransferase	1IIC	–	–36.38	–

PDB: Protein Data Bank. *The lesser the binding energy of a complex the more stable it is.

place as the specific and potent co-crystallized inhibitor PTAU and substrate 5-FU of the protein, mimicking their action. The enzyme–inhibitor complexes were stabilized mostly by van der Waals hydrophobic interactions in the hydrophobic steroidal region and by hydrogen bonds and polar interactions in the hydrophilic alkylating region.

The significance of the insertion of a lactamic group in homo-aza-steroidal esters, to increase antitumor activity has been pointed out in many studies (1-3, 6-9). In a series of hybrid steroidal esters of nitrosourea with an A-lactamic ring and nitrosourea esterified compounds at position 17, we have shown anticancer activity against PANO2 murine pancreatic adenocarcinoma (5), which was probably due to the lactam group (–NHCO–) modification. The presence of the characteristic group –NHCO– of the homo-aza-steroid molecule **C2** has proved important in binding with the target proteins, in order to lower acute toxicity and improve antitumor activity in cancer research (7, 8). Possibly the antineoplastic effects of these steroidal esters may be due to the multiple interactions of the –NHCO– group with similar groups or with specific structural domains which exist in DNA and proteins. It was suggested that the –NHCO– lactam group maybe transformed by a metabolic process or at least by an enzymatically catalyzed reaction to active species which strongly interact with similar groups existing in the DNA and proteins (–NH–CO– → $–N^{(–)}H + –C^{(+)}=O$) (7, 8). Molecular docking studies incorporating such active species active on DNA and various proteins are underway.

In conclusion, the *in silico* approach (molecular docking calculations) may provide a structural rationale of the receptor antagonism of the tested compounds, providing a molecular basis for understanding their inhibitory effect and suggesting that it could be attributed, at least in part, to the inhibition of the pathways involving UPase. On the other hand, taking into consideration the fact that cellular death and apoptosis are multifactor processes, a number of other pathways and target proteins could play a role in the observed *in vitro* inhibitory effect. More than 20 cancer-related target proteins were also inhibited by the studied compounds. The chief targets were proteins whose blockade might provide a mechanism to counteract cancer, inflammation and/or bacterial infection diseases.

References

1 Camoutsis C and Trafalis DT: An overview on the antileukemic potential of D-homo-aza- and respective 17 beta-acetamido-steroidal alkylating esters. Invest New Drugs *21(1)*: 47-54, 2003.

2 Trafalis DT, Geromichalos GD, Koukoulitsa C, Papageorgiou A, Karamanakos P and Camoutsis C: Lactandrate: a D-homo-aza-androsterone alkylator in the treatment of breast cancer. Breast Cancer Res Treat *97(1)*: 17-31, 2006.

3 Trafalis DT: Hybrid aza-steroid alkylators in the treatment of colon cancer. Cancer Lett *243(2)*: 202-210, 2006.

4 Trafalis DT, Geromichalos GD, Bountouroglou N, Koumbi D, Kontos M, Sougias D, Dalezis P, Karamanakos P, Papageorgiou A, Camoutsis C and Athanassiou AE: A preclinical survey on the efficacy of lactandrate in the treatment of colon carcinoma. J BUON *10(2)*: 227-234, 2005.

5 Papageorgiou A, Lialiaris T, Stergiou E, Stergiou I, Tsigris C, Kourti A, Geromichalos G, Stravoravdi P, Trafalis D, Athanassiou AE, Pitsas A and Camoutsis Ch: Preclinical studies of steroid-linked nitrosoureas in murine pancreatic adenocarcinoma PANO2. J BUON *13(2)*: 235-239, 2008.

6 Trafalis DT, Tsavdaridis D, Camoutsis C, Karayiani V, Mourelatos D, Dalezis P, Athanassiou A, Pangalis GA and Papageorgiou A: Preclinical studies on NSC290205 aza-steroid alkylator activity in combination with adriamycin against lymphoid leukaemia. Br J Haematol *128(3)*: 343-350, 2005.

7 Trafalis DT, Sambani C, Kapsimali V, Economidou J, Politis T and Catsoulacos P: Effects of homo-aza-steroids on acute non-lymphocytic leukaemia cell proliferation *in vitro*. Br J Haematol *91(4)*: 907-914, 1995.

8 Trafalis DT, Camoutsis C and Papageorgiou A: Research on the anti-tumour effect of steroid lactam alkylator (NSC-294859) in comparison with conventional chemotherapeutics in malignant melanoma. Melanoma Res *15(4)*: 273-281, 2005.

9 Kontos M, Nikolopoulou M, Trafalis DT, Geromichalos GD, Koukoulitsa C, Camoutsis JC, Bastounis E and Karamanakos P: The effect of an estrone D-lactam steroid ester derivative on breast cancer cells and its predicted binding interactions with the ligand-binding domain of estrogen receptor-alpha. Oncol Res *16(3)*: 129-142, 2005.

10 Trafalis DT, Camoutsis C, Karamanakos P, Arvanitis A, Tegou E, Ziras N and Athanassiou AE: Preclinical evaluation of the homo-aza-steroid ester 13beta-hydroxy-13alpha-amino-13,17-seco-5alpha-androstan-17-oic-13,17-lactam-*p-bis*(2-chloroethyl) aminophenoxy acetate for the treatment of malignant melanoma. J BUON *8(4)*: 333-339, 2003.

11 Trafalis DT, Camoutsis C, Dalezis P, Papageorgiou A, Kontos M, Karamanakos P, Giannakos G and Athanassiou AE: Antitumour effect of A- and D-lactam androgen nitrogen mustards on non-small cell lung carcinoma. J BUON *9(3)*: 275-282, 2004.

12 Camoutsis C, Sambani C, Trafalis DT and Peristeris P: On the formation of steroidal amidoesters of 4-[*N,N-bis*(2-chloroethyl) amino]benzoic acid and their cytotoxic activity. Eur J Med Chem *34(7-8)*: 645-649, 1999.

13 Cao D, Russell RL, Zhang D, Leffert JJ and Pizzorno G: Uridine phosphorylase (–/–) murine embryonic stem cells clarify the key role of this enzyme in the regulation of the pyrimidine salvage pathway and in the activation of fluoropyrimidines. Cancer Res *62*: 2313-2317, 2002.

14 Kawamura K, Takiguchi N, Wada A, Takenobu H, Kimura H, Soda H, Nagata M, Asano T and Tagawa M: Up-regulated expression of the uridine phosphorylase gene in human gastric tumors is correlated with a favorable prognosis. Anticancer Res *26(6C)*: 4647-4651, 2006.

15 Matsusaka S, Yamasaki H, Fukushima M and Wakabayashi I: Upregulation of enzymes metabolizing 5-fluorouracil in colorectal cancer. Chemotherapy *53(1)*: 36-41, 2007.

16 Yan R, Wan L, Pizzorno G and Cao D: Uridine phosphorylase in breast cancer: a new prognostic factor? Front Biosci *11*: 2759-2766, 2006.

17 Roosild TP, Castronovo S, Fabbiani M and Pizzorno G: Implications of the structure of human uridine phosphorylase 1 on the development of novel inhibitors for improving the therapeutic window of fluoropyrimidine chemotherapy. BMC Struct Biol *9*: 14, 2009.
18 Cao D and Pizzorno G: Uridine phosophorylase: an important enzyme in pyrimidine metabolism and fluoropyrimidine activation. Drugs Today *40(5)*: 431-443, 2004.
19 Ligo M, Nishikata K, Nakajima Y, Szinai I, Veres Z, Szabolcs A and Clercq E: Differential effects of 2,2'-anhydro-5-ethyluridine, a uridine phosphorylase inhibitor, on the antitumor activity of 5-fluorouridine and 5-fluoro-2'-deoxyuridine. Biochem Pharmacol *39*: 1247-1253, 1990.
20 Humeniuk R, Menon LG, Mishra PJ, Gorlick R, Sowers R, Rode W, Pizzorno G, Cheng YC, Kemeny N, Bertino JR and Banerjee D: Decreased levels of UMP kinase as a mechanism of fluoropyrimidine resistance. Mol Cancer Ther *8*: 1037-1044, 2009.
21 Miyashita H, Takebayashi Y, Eliason JF, Fujimori F, Nitta Y, Sato A, Morikawa H, Ohashi A, Motegi K, Fukumoto M, Mori S and Uchida T: Uridine phosphorylase is a potential prognostic factor in patients with oral squamous cell carcinoma. Cancer *94(11)*: 2959-2966, 2002.
22 Kanzaki A, Takebayashi Y, Bando H, Eliason JF, Watanabe Si S, Miyashita H, Fukumoto M, Toi M and Uchida T: Expression of uridine and thymidine phosphorylase genes in human breast carcinoma. Int J Cancer *97(5)*: 631-635, 2002.
23 Wong HH and Lemoine NR: Pancreatic cancer: molecular pathogenesis and new therapeutic targets. Nat Rev Gastroenterol Hepatol *6(7)*: 412-422, 2009.
24 Sahin F, Qiu W, Wilentz RE, Iacobuzio-Donahue CA, Grosmark A and Su GH: RPL38, FOSL1, and UPP1 are predominantly expressed in the pancreatic ductal epithelium. Pancreas *30(2)*: 158-167, 2005.
25 Catsoulacos P and Wampler GL: Activity of 3β-hydroxy-13α-amino-13,17-seco-5α-androstane-17-oic-13,17-lactam(*p-(bis*(2-chloroethyl)amino) phenyl)acetate (NSC 290205) in murine solid tumors. Oncology *39*: 109-112, 1982.
26 Catsoulacos P and Catsoulacos D: Hybrid anticancer compounds. Steroidal lactam esters of carboxylic derivatives of *N,N-bis* (2-chloroethyl) aniline. Anticancer Res 11(5): 1773-1777, 1991.
27 Catsoulacos P and Boutis L: Aza-steroids. Beckmann rearrangement of 3β-acetoxy-5α-androstan-17-one oxime acetate with boron fluoride. Alkylting agents. Chim Ther *8*: 215-217, 1973.
28 Finlay GJ, Wilson WR and Baguley BC: Comparison of *in vitro* activity of cytotoxic drugs towards human carcinoma and leukaemia cell lines. Eur J Cancer Clin Oncol *22*: 655-662, 1986.
29 Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH and Boyd MR: Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res *48*: 589-601, 1988.
30 Boyd MR and Paull KD: Some practical considerations and applications of the National Cancer Institute *in vitro* anticancer drug discovery screen. Drug Devel Res *34*: 91-109, 1995.
31 Martin DS, Stolti RL and Sawyer RC: Use of oral uridine as substitute for parenteral uridine rescue of 5-fluorouracil therapy, with and without the uridine phosphorylase inhibitor 5-benzylacyclouridine. Cancer Chemother Pharmacol *24*: 9-14, 1989.
32 al Safarjalani ON, Zhou XJ, Naguib FNM, Goudgaon NM, Schinazi RF and el Kouni MH: Modulation of the pharmacokinetics of endofenous plasma uridine by 5-(phenylthio)acyclouridine, a uridine phosphorylase inhibitor: implications for chemotherapy. Cancer Chemother Pharmacol *48*: 145-150, 2001.
33 Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN and Bourne PE: The Protein Data Bank. Nucleic Acids Res *28*: 235-242, 2000.
34 Berman HM, Henrick K and Nakamura H: Announcing the worldwide Protein Data Bank. Nature Struct Biol *10(12)*: 980, 2003.
35 Bernstein FC, Koetzle TF, Williams GJ, Meyer EE Jr, Brice MD, Rodgers JR, Kennard O, Shimanouchi T and Tasumi M: The Protein Data Bank: a computer-based archival file for macromolecular structures. J Mol Biol *112(3)*: 535-542, 1977.
36 Kogan TP, Dupre B, Bui H, McAbee K, Kassir JM, Scott IL, Hu X, Vanderslice P, Beck PJ and Dixon RAF: Novel synthetic inhibitors of selectin-mediated cell adhesion: synthesis of 1,6-*bis*[3-(3-carboxymethylphenyl)-4-(2-α-D-mannopyranosyloxy) phenyl]hexane (TBC1269). J Med Chem *41*: 1099-1111, 1998.
37 DeLano WL: The PyMOL Molecular Graphics System 0.99, DeLano Scientific, San Carlos, CA, USA, 2006.
38 Papageorgiou A, Andreadis Ch, Boutis A, Lialiaris Th and Mouratidou D: NSC 290205-based therapy in murine pancreatic adenocarcinoma PAN02 in combination with adriamycin ADR. Anticancer Res *26*: 243-248, 2006.
39 Catsoulacos P: Further studies on the anti-neoplastic activity of 3 β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam {*p*-[*bis*(2-chloroethyl)amino]-phenyl}acetate (NSC 290205). Cancer Lett *22(2)*: 199-202, 1984.
40 Wampler G and Catsoulacos P: Antileukemic effect of homo-aza-steroidal ester of [*p*-[*bis*(2-chloroethyl)amino]phenyl]acetic acid. Cancer Treat Rep *61(1)*: 37-41, 1977.
41 Mourelatos D, Petrou C, Boutis L, Papageorgiou A, Catsoulacos P and Dozi-Vassiliades J: Induction of cytogenetic damage by modified steroidal derivatives of *p-bis*(2-chloroethyl) aminophenylacetic acid in human lymphocytes. Mutat Res *190(3)*: 205-210, 1987.

Received September 28, 2010
Revised February 28, 2011
Accepted February 28, 2011