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ORIGINAL ARTICLE

Antitumour effect of a- and d- lactam androgen nitrogen mustards on non-small cell lung carcinoma

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Summary

Purpose: *We tested 3 alkylating esters of D-lactam androsterone, 3 alkylating esters of A-lactam testosterone and the alkylating nitrogen mustard components of these esters, for antineoplastic activity on non-small cell lung carcinoma (NSCLC) in vitro and in vivo.*

Materials and methods: *Cytostatic and cytotoxic activity was evaluated in vitro against 10 human NSCLC cell lines. The in vitro testing was performed with the MTT metabolic-colorimetric assay and the mean concentrations of each drug that generated 50% or total (100%) growth inhibition (GI50 and TGI, respectively) as well as the drug concentrations that produced cytotoxicity against 50% of the cultured cells (IC50) were calculated. Furthermore, the in vivo antitumour effect was determined against the relatively chemo-resistant Lewis lung carcinoma (LLC) on mice. The acute toxicity of the tested compounds was appointed in C57BL mice and the antitumor effect on LLC was assessed from the percent increase in median lifespan of the treated animals over the untreated (control) (T/C%).*

Results: *The lactam steroidal esters presented lower toxicity and increased antineoplastic activity in vitro and in vivo compared to their respective alkylating components. An A-lactam testosterone ester namely: 17β-hydroxy-3-aza-A-homo-4α-androsten-4-one-p-N,N-bis (2chloroethyl) amino phenoxy acetate (ALT-CAPOA) performed significantly higher anticancer activity in vitro and in vivo. This compound generated 37.5% 90-day disease free survivors (cures) against LLC.*

Conclusion: *These results indicate a high antitumor potential of lactam steroid alkylating esters depended on the alkylating moiety as well as on the modified steroidal carrier. Preclinical research supports that ALT-CAPOA generates well-tolerated toxicity as well as superior antitumor activity against NSCLC. These significant results call for further clinical development.*

Key words: human cell lines, lactam steroid alkylator, Lewis lung carcinoma, nitrogen mustards, non-small cell lung carcinoma,

Introduction

In the majority of the cases, lung cancer is diagnosed at advanced stages when treatment options are limited and mainly palliative. At the time of diagnosis, most patients are older than 65 years and have stage III or IV disease. More than 80% of the patients have NSCLC and the rest have small cell lung cancer [1]. Most of the patients will die of the disease after aggressive modern treatments. Therefore, significant improvement in therapeutic methods must be implemented to improve overall survival rates [2]. The advent of new chemotherapeutic agents offers hope for significant advances in the treatment of lung cancer.

The sensitivity of some neoplasms to hormonal control provides a rational basis for utilizing steroidal hormones as a biological platform for cytotoxic agents in cancer therapy, in order to diminish toxicity and en-

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hance specificity. Hybrid compounds, agents that combine two active molecules in one, such as steroid alkylators, have some advantages. Hybrid steroid alkylators may produce reduced toxicity, significantly lower than their cytotoxic components do alone, and increased anticancer activity. The designing of lactam steroids that contain –NH-CO- group inside the A or D steroid nucleus as biological platforms for carboxylic derivatives of N,N-bis(2-chloroethyl)aniline (nitrogen mustards) is based on the evidence that these esters are highly active against murine leukaemia [3-5] and rodent solid tumor systems including human xenografts [5-7]. Most of the unmodified steroid alkylators have been inactive in murine L1210 lymphoid and in P388 lymphocytic leukaemia, while the respective lactam steroid esters produced excellent results in these leukaemia systems [8,9].

Based on the above data and observations, in the present work we evaluate the activity of 2 homo-aza-steroid (lactam) vectors with the lactam group in A- or D- steroid ring derivatives of testosterone and androsterone respectively, esterified with 3 different derivatives of N,N-bis(2-chloroethyl)aniline (Figure 1) against NSCLC *in vitro* and *in vivo*. The alkylating moieties were also tested comparatively to the corresponding esters.

Materials and methods

Drug preparations

All of the compounds tested (Figure 1) were synthesized by previously described methods [10]. Stock

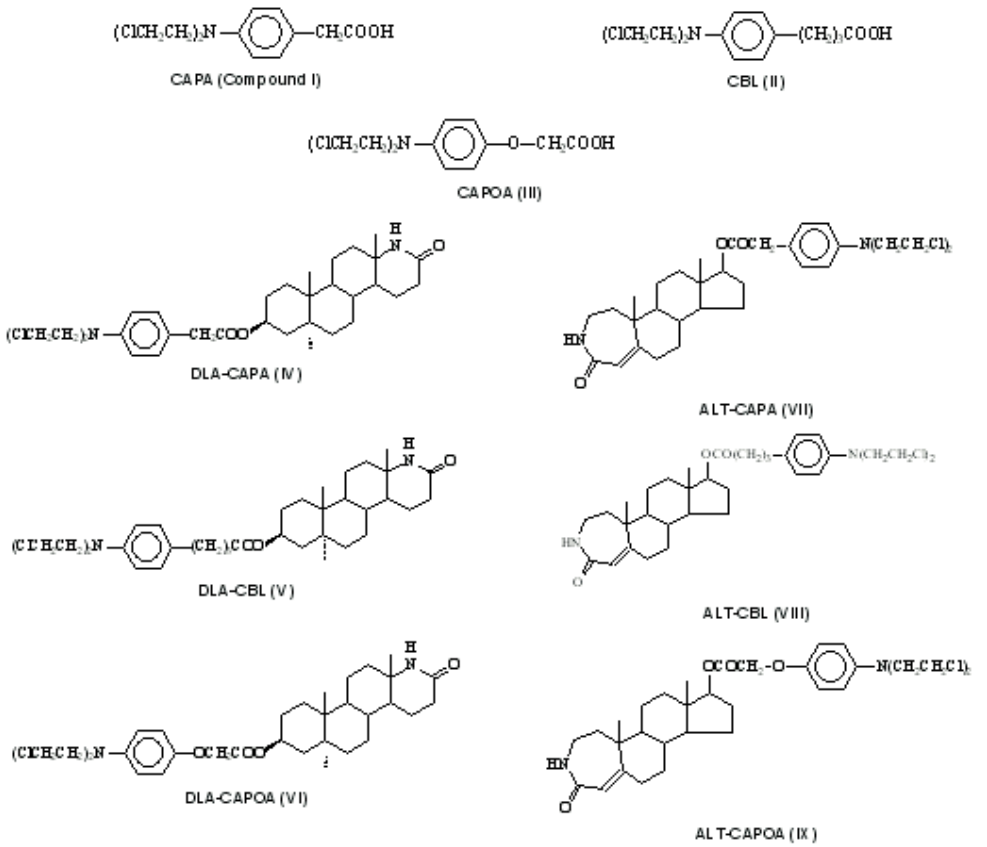

Figure 1. The chemical structures of the tested compounds are presented. CAPA (Compound I): p-N,N-bis (2chloroethyl) amino phenyl acetic acid, CBL (Chlorambucil, II): p-N,N-bis (2chloroethyl) amino butyric acid, CAPOA (III): p-N,N-bis (2chloroethyl) amino phenoxy acetic acid, DLA-CAPA (IV): 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-p-N,N-bis (2chloroethyl) amino phenyl acetate, DLA-CBL (V): 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-p-N,N-bis (2chloroethyl) amino phenyl butyrate, DLA-CAPOA (VI): 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-p-N,N-bis (2chloroethyl) amino phenoxy acetate, ALT-CAPA (VII): 17β-hydroxy-3-aza-A-homo-4α-androsten-4-one-p-N,N-bis (2chloroethyl) amino phenyl acetate, ALT-CBL (VIII): 17β-hydroxy-3-aza-A-homo-4α-androsten-4-one-p-N,N-bis (2chloroethyl) amino phenyl butyrate, ALT-CAPOA (IX): 17β-hydroxy-3-aza-A-homo-4α-androsten-4-one-p-N,N-bis (2chloroethyl) amino phenoxy acetate.

solutions of the tested compounds, were made immediately before use. The compounds were initially dissolved in a small amount of 10% dimethyl sulfoxide (DMSO). Prior to intraperitoneal (i.p.) administration the tested compounds were suspended in corn oil at the desired concentrations.

In vitro testing

The cytostatic and cytotoxic effects of the compounds under investigation were estimated on 10 human NSCLC cell lines: A549 lung carcinoma (ECCAC), NCI-H322 bronchioalveolar adenocarcinoma (ECCAC), NCI-H522 adenocarcinoma (ATCC), NCI-H23 adenocarcinoma (ATCC), EKVX adenocarcinoma (NCI), NCI-H226 squamous cell carcinoma (ATCC), NCI-H460 large cell carcinoma (ATCC), HOP-18 large cell carcinoma (NCI), HOP-92 large cell carcinoma (NCI), LXFL-529 large cell carcinoma (NCI). The cells were cultured in a concentration of 2-3 $\times 10^4$ cells/ml in RPMI 1640 medium, supplemented with 10% FCS, 2 mM L-glutamine, 1% antibiotics (gentamycin plus penicillin), and the cultures were maintained for 72 h in a 5% CO_2 incubator at 37° C. Growth medium in NCI-H522, NCI-H23 and NCI-H460 cell cultures were adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, 90%.

After 24 h, cells were treated with 0.1-100 ¼M of the tested compounds for 48 h. The viability of the cultured cells was estimated by the MTT assay [10]. MTT (Sigma, USA) was dissolved in PBS in a concentration of 5 mg/ml, filter-sterilized and stored at 4° C. MTT (0.2 ml of stock solution) was added to each culture (per ml) and incubated for 3 h at 37° C to allow metabolization. Formazan crystals 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 ELISA reader. The mean concentrations of each drug that generated GI50 and TGI as well as the drug concentrations that produced IC50 were calculated by the linear regression method [12,13]. Using 7 absorbance measurements [time 24 h (Ct24), control growth 72 h (Ct72), and test growth in the presence of drug at 5 concentration levels (Tt72x)], the percentage of growth was calculated at each level of the drug concentrations. The percent growth inhibition was calculated as: [(Tt72x-Ct24)/(Ct72-Ct24)]x100 for concentrations for which Tt72x≥Ct24, and [(Tt72x-Ct24)/Ct24]x100 for concentrations for which Tt72x <Ct24.GI50 was calculated from [(Tt72x-Ct24)/(Ct72-Ct24)]x100=50, TGI from [(Tt72x-Ct24)/(Ct72-Ct24)]x100=0 and IC50 from [(Tt72x-Ct24)/Ct24]x100=50. All the experiments were carried out in triplicate.

In vivo testing

Male and female DBA/2 and BDF1 (C57BL× DBA/2) mice, 6-9 weeks old, weighting 20-23 g, were used for toxicity studies. A nitrosoureas-resistant tumor, LLC, was utilized for antitumor testing. LLC cells were cultured in Dulbecco's modified Eagle's growth medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, and 10% fetal bovine serum, and the cultures were maintained for 72 h in a 5% CO_2 incubator at 37° C. Subcultures were prepared by diluting the suspension 1: 4 to 1: 6. C57BL mice were intramuscularly implanted with 2×10^6 cells/mouse.

The toxicity of the 6 homo-aza-steroid esters and of the 3 nitrogen mustards was figured out on BDF1 mice (groups of 10 animals per dose). For each compound 5 different doses were chosen. The number of surviving animals was determined after 30 days (Table 2). For chemotherapy testing the highest dose used was LD10/2 (LD10: lethal dose for 10% of the treated animals within 30 days). Drug treatment consisted of i.p. injections for all of the compounds tested. Control and each drug treated group consisted of 8 mice. Two different treatment schedules were applied. In the first treatment schedule, drugs were administered on days 1, 5, 9, after tumour inoculation at dose of LD10/2 x3, and in the second treatment schedule drugs were administered on days 1-7 after tumour inoculation at dose of LD10/4 x7. The antitumor activity was assessed from the percent increase in the median lifespan of the treated animals over the control [(T/C)%]. The minimum criterion for activity was considered a value of T/C >125%, according to the NCI requirements [14].

The present study adhered to the "Principles of Laboratory Animal Care" and to the United Kingdom Coordinating Committee on Cancer Research guidelines [15,16].

Results

In vitro activity

The results of the *in vitro* screening are presented analytically in Tables 1, 2 and 3. Both A- and D-lactam steroid alkylators displayed a satisfactory activity against the 10 human NSCLC cell lines, significantly higher than their respective alkylating components ($p < 0.001$). The 9 tested compounds demonstrat-

Table 1. *In vitro* cytostatic and cytotoxic effects induced by the tested compounds on 3 human non-small cell lung cancer cell lines expressed as: GI50 (drug mean concentration generating 50% growth inhibition), TGI (drug mean concentration producing total growth inhibition), IC50 (drug mean concentration inducing 50% cytotoxicity). Statistical significance levels were determined by Student's t-test (two-tailed; two sample with unequal variance). In all cases differences were significant for $p < 0.001$ or $p < 0.01$

Compounds	*Cell lines* *A549*			*NCI-H322*			*NCI-H522*		
	GI50	*TGI*	*IC50*	*GI50*	*TGI*	*IC50*	*GI50*	*TGI*	*IC50*
CAPA	32	65	>100	88	>100	>100	11	79	>100
CBL	63	>100	>100	>100	>100	>100	25	95	>100
CAPOA	36	69	>100	95	>100	>100	10	36	71
DLA-CAPA	15	31	50	20	30	64	14	28	46
DLA-CBL	31	64	97	25	52	78	23	51	97
DLA-CAPOA	7.1	19	43	17	36	70	5.5	14	27
ALT-CAPA	7.9	22	46	21	39	73	8.5	15	33
ALT-CBL	25	55	98	18	31	65	18	32	50
ALT-CAPOA	5.1	25	63	16	31	79	1.6	3.8	6.3

Table 2. The *in vitro* cytostatic and cytotoxic effects induced by the tested compounds on 3 human non-small cell lung cancer cell lines were determined by GI50, TGI, IC50. Statistical significance levels were determined by Student's t-test (two-tailed; two sample with unequal variance). In all cases differences were significant for $p < 0.001$ or $p < 0.01$

Compounds	*Cell lines* *NCI-H23*			*EKVX*			*NCI-H226*		
	GI50	*TGI*	*IC50*	*GI50*	*TGI*	*IC50*	*GI50*	*TGI*	*IC50*
CAPA	15	32	64	ND	ND	ND	58	>100	>100
CBL	27	96	>100	>100	>100	>100	90	>100	>100
CAPOA	11.5	22	55	72	>100	>100	43	98	>100
DLA-CAPA	12.5	25	50	16	31	63	19	32	66
DLA-CBL	20.5	50.5	99	19	42	85.5	27.5	58	>100
DLA-CAPOA	5.8	20.5	43.5	14.5	33.5	70.5	15.5	33.5	65
ALT-CAPA	8.5	20	42	12	29	53	18	30	69
ALT-CBL	15.5	36	77.5	17	37	73.5	24	41.5	90.5
ALT-CAPOA	3.1	20	45	15	39.5	79.5	12.5	31	63

Table 3. The *in vitro* cytostatic and cytotoxic effects induced by the tested compounds on 4 human non-small cell lung cancer cell lines were determined by GI50, TGI, IC50. Statistical significance levels were determined by Student's t-test (two-tailed; two sample with unequal variance). In all cases differences were significant for $p < 0.001$ or $p < 0.01$

Compounds	*Cell lines* *NCI-H460*			*HOP-18*			*HOP-92*			*LXFL 529*		
	GI50	*TGI*	*IC50*	*GI50*	*TGI*	*IC50*	*GI50*	*TGI*	*IC50*	*GI50*	*TGI*	*IC50*
CAPA	41.5	77.5	>100	ND	ND	ND	34.5	97	>100	ND	ND	ND
CBL	58.5	>100	>100	78	>100	>100	61.5	>100	>100	95	>100	>100
CAPOA	30.5	72.5	>100	ND	ND	ND	ND	ND	ND	45.5	98	>100
DLA-CAPA	5	31.5	98	15	33	63	17.5	39	75	7.5	24	61
DLA-CBL	37.5	85.4	>100	49	94	>100	42.5	92	>100	72.5	>100	>100
DLA-CAPOA	2.1	12.5	38.5	14	33.5	72.5	10	27	79	9.5	28	58
ALT-CAPA	3.2	21	85.5	11	28.5	55	12	27	59	8	20	53.5
ALT-CBL	29.5	60.4	>100	32.5	74	>100	30	72	>100	61	>100	>100
ALT-CAPOA	0.3	7.1	31.5	13	32.5	71	7.5	25	63	7.2	19.5	48.5

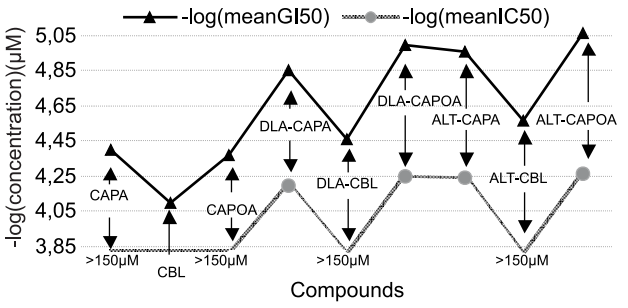

Figure 2. Mean cytostatic and cytotoxic activity of the tested compounds defined by log (mean GI50) and log (mean IC50) respectively. The compounds: DLA-CAPA, DLA-CAPOA, ALT-CAPA and ALT-CAPOA, presented significantly higher activity ($p < 0.01$, t-test).

ed a similar activity profile. ALT-CAPOA presented significant higher growth inhibitory (mean GI 50: 8.7 μM) and cytotoxic (mean IC 50: 54.9 μM) effect. Furthermore DLA-CAPA, DLA-CAPOA and ALT-CAPA also presented high activity (Figure 2). Based on the mean antiproliferative and cytotoxic effect of the tested compounds, these were ranked as follows: ALT-CAPOA > ALT-CAPA ≥DLA-CAPOA > DLA-CAPA > ALT-CBL > DLA-CBL > CAPA ≥CAPOA > CBL ($p<0.01$) (Figure 2).

In vivo activity

Among the tested compounds ALT-CAPOA exhibited higher antitumor efficacy producing a significant prolongation in the mean survival time (MST) (percent T/C=130%, $p < 0.01$) and generating 3: 8 (37.5%) 90-day disease-free survivors (cures) (Table 4). Based on the mean *in vivo* antineoplastic activity against LLC, expressed by %T/C, the tested compounds were ranked as follows: ALT-CAPOA > ALT-CAPA > ALT-CBL > DLA-CAPOA > DLA-CAPA > DLA-CBL > CAPOA > CBL ($p < 0.05$) (Table 4). A-lactam steroid esters of testosterone (ALT-) showed significantly ($p < 0.01$) higher antitumor activity than the respective A-lactam esters of androsterone (DLA-). CAPA due to its significant high toxicity was not further investigated *in vivo.* The tested nitrogen mustards were notably more toxic than the corresponding steroid esters (Table 4). Moreover, the tested compounds presented a moderate ($R^2=0.58$), but significant ($p < 0.05$) correlation between *in vivo* and *in vitro* activity pattern (Figure 3).

Table 4. *In vivo* antitumor activity of the 9 tested compounds against murine Lewis lung carcinoma (LLC). The percent lifespan increases of treated (T) to untreated (C) mice (T/C %) are demonstrated. Statistical significance levels were determined by Wilcoxon signed-rank test

Compound	*LD10 (mg/kg)*	*Treatment schedule*	*Dose (mg/kg/day)*	*MST (days)*	*T/C (%)*	*90-day DFS**
Control	–	–	*Corn oil*	*23.5*		*0/8*
CAPA	<1	Days 1,5,9	ND	–	–	–
		Days 1-7	ND	–	–	–
CBL	14	Days 1,5,9	7	25.6	109	0/8
		Days 1-7	3.5	24.9	106	0/8
CAPOA	30	Days 1,5,9	15	26.8	114	0/8
		Days 1-7	7.5	27.9	119	0/8
DLA-CAPA	28	Days 1,5,9	14	29.4**	125	0/8
		Days 1-7	7	30.8**	131	0/8
DLA-CBL	140	Days 1,5,9	70	25.4	108	0/8
		Days 1-7	35	26.3	112	0/8
DLA-CAPOA	80	Days 1,5,9	40	28.7	122	0/8
		Days 1-7	20	31.5**	134	0/8
ALT-CAPA	36	Days 1,5,9	18	33.8**	144	0/8
		Days 1-7	9	37.4**	159	0/8
ALT-CBL	120	Days 1,5,9	60	30.6**	130	0/8
		Days 1-7	30	33.1**	141	0/8
ALT-CAPOA	90	Days 1,5,9	45	26.8	114†	2/8
		Days 1-7	22.5	30.6**	130†	3/8

*90-day DFS: disease free survivors (cured); **The mean survival time (MST) values between treated and control mice were significantly different ($p < 0.01$); †MST of non-cured mice

Discussion

Epidemiological data suggest a clinical role for steroid hormones in lung cancer. Women with lung cancer have a significant longer survival than men; on the other hand, use of exogenous estrogen seems to increase the risk of lung cancer [17]. Lung cancer cells express steroid receptors in high rate. The glucocorticoid receptor and the vitamin D receptor are ubiquitously expressed. Among the sex steroid receptors estrogen, progesterone and androgen receptors are present in high percentage of NSCLC but they are absent or rarely found in small cell lung carcinoma [18,19].

These data suggest that NSLC is subject to hormonal control and thus it may provide an important biological target for hybrid steroid anticancer compounds, such as lactam-steroid alkylators. However, the homo-aza-steroid derivatives of androgens or estrogens do not clearly bind to respective receptors and their activity seems not to be strongly dependent on the existence of intracellular steroid receptors of target tissues. For example, the D-lactam derivative of estrone acts neither synergistically nor antagonistically to tamoxifen [6,20]. Nevertheless, Wall et al. [8] as well as Wampler and Catsoulacos [9] showed that unmodified (non-lactamic) steroid alkylating esters were inactive in the treatment of L1210 and P388 murine leukemias, while the respective lactam-steroid esters showed excellent antileukemic activity. Furthermore, comparative studies on the antineoplastic activity of homo-aza-steroid esters against experimental systems with alkylating agents used in current chemotherapy, such as mephalan, chlorambucil, cyclophosphamide, mechlorethamine, ThioTEPA and mitomycin C, showed that the tested homo-aza-steroid esters hold a superior or leastwise an equal anticancer activity [3,21].

The biological mechanism of action of lactam-steroid alkylators is not clear. The presence of the characteristic group –NHCO- of the homo-aza-steroid molecule was proven important, in order to lower acute toxicity and improve antitumor activity in cancer research [22,23]. Possibly the antineoplastic effects of these steroidal esters may be due to the multiple interactions of the –NHCO- group with similar groups or with structural specific domains which exist in the DNA and proteins. Catsoulacos et al. [24] suggested that the –NHCO- lactam group is 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 (-NHCO- → $-NH^{\ominus} + -C{=}O^{\oplus}$) (Figure 4). Furthermore, modifications of the –NHCO- lactam group by NH methylation ($-NCH_3CO-$) or by –CO- reduction led to derivatives with lower anticancer activity compared with the parent compounds [24-26].

In early studies on the action mechanisms of amino and azasteroids it was presumed that these compounds act due to a more fundamental mode than on sterol metabolism alone, interfering with mitochondrial respiration and/or oxidative phosphorylation [27]. In later studies it was indicated that the lactam ring of the aza-steroids can react as antagonist or agonist by its binding to certain cellular enzymes in a way similar with the indo- benzo- or other steroid lactams which effect on protein kinase C (PKC) enzymes with a relative specificity [28-30].

The alkylating component of these esters acts via the same biochemical pathway of other bifunctional alkylating nitrogen mustards [31]. Steroid esters can penetrate cellular membrane and produce high intracellular concentrations due to the lipophilic nature of the steroid carrier. It has been reported that a rate-limiting hydrolysis of the ester bond liberates the two active moieties (one steroid and one alkylat-

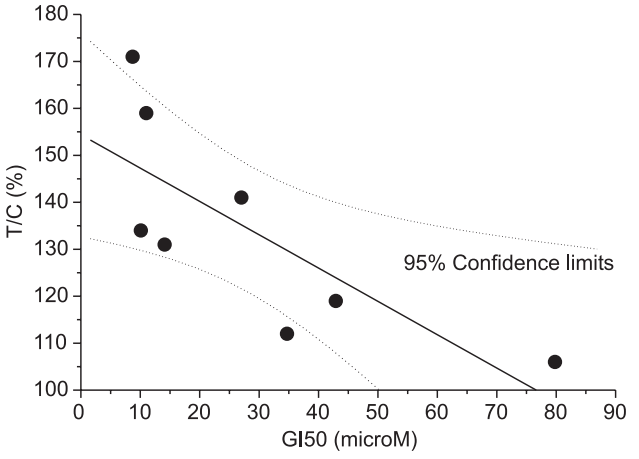

Figure 3. Correlation fit between *in vivo* antitumour activity (T/C%) and *in vitro* cytostatic activity (GI50) of the tested compounds (correlation index R^2=0.58, p=0.026).

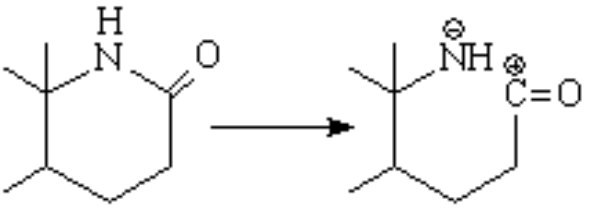

Figure 4. Hypothetical representation of intracellular biotransformation of lactamic nucleus to active groups capable to interact with cellular component.

ing) into the cellular microenvironment [32]. The stereoisomeric form and chemical structure of the steroid lactams and of alkylating components seem to determine the antileukemic effect of these compounds [33-35]. Our results indicate that the alkylator p-bis(2-chloroethyl)aminophenoxy acetic acid (CAPOA) is one of the most potent cytotoxic components and the esters of CAPOA were proven significantly effective. Moreover, the esters of A-lactam testosterone produced significantly higher antineoplastic activity against NSCC *in vitro* and *in vivo*, designating the importance of the steroid carrier structure in the treatment of specific neoplasms. On the other hand, the similar sensitivity patterns of the treated cell lines with the tested drugs, as well as the significant correlation between *in vivo* and *in vitro* activity patterns, indicated a common molecular basis in the activity profiles of the tested compounds.

We comparatively tested for antitumor activity 3 alkylating esters of D-lactam androsterone, 3 alkylating esters of A-lactam testosterone and the respective three alkylating nitrogen mustard components of these esters against NSCLC *in vitro* and *in vivo*. Our results indicate a high antitumor potential of lactam steroid alkylating esters depended on the chemical structure of the alkylating moiety as well as on the lactam steroidal carrier. Preclinical testing supports that the A-lactam testosterone ester of p-bis(2-chloroethyl) aminophenoxy acetic acid (ALT-CAPOA) generates well-tolerated toxicity as well as excellent antitumor activity against NSCLC. These significant results add antecedence for further investigation in order to introduce an important agent as ALT-CAPOA in clinical development for the treatment of NSCLC.

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