ORIGINAL ARTICLE

Preclinical evaluation of the homo-aza-steroid ester 13β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-p-bis(2-chloroethyl)aminophenoxy acetate for the treatment of malignant melanoma

D.T.P. Trafalis[1,4], C. Camoutsis[1], P. Karamanakos[2], A. Arvanitis[3], E. Tegou[1], N. Ziras[4], A.E. Athanassiou[4]

[1]*Laboratory of Pharmaceutical Chemistry, School of Pharmacy, University of Patras, Patras;* [2]*1st Department of Surgery, "Laikon" District General Hospital, School of Medicine, University of Athens, Athens;* [3]*Department of Dermatology, Veterans Hospital "NIMTS", Athens;* [4]*1st Department of Medical Oncology, "Metaxa" Cancer Hospital, Piraeus, Greece*

Summary

Purpose: To investigate the in vitro and in vivo activity of an homo-aza-steroid alkylating ester, namely 13β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-p-bis (2-chloro ethyl) aminophenoxy acetate (HASE), in comparison with dacarbazine (DTIC) in the treatment of malignant melanoma.

Materials and methods: Cytotoxicity was assessed in vitro by the MTT assay using a panel of 6 malignant melanoma human cell lines, with or without the presence of rat liver microsome assay. B16 melanoma-bearing mice were used to evaluate in vivo the antitumor activity of the tested compounds.

Results: In all cases of in vitro screening, HASE displayed significantly higher (p <0.0001) cytostatic and cytotoxic activity than DTIC. Moreover, the antitumor activity of HASE in B16 melanoma-bearing mice was satisfactory, prolonging the mice lifespan at 67%, compared to 43% achieved by DTIC. Furthermore, HASE significantly inhibited the tumor growth (tumor growth rate: <42%) as this was defined by tumor volume and weight differences, presenting higher antitumor effect than DTIC.

Conclusion: HASE displayed superior in vitro and in vivo activity than DTIC in the treatment of melanoma. Thus, HASE may be considered as a significant candidate anticancer agent for further development.

Key words: B16 melanoma, dacarbazine, homo-aza-steroid ester, human cell lines, melanoma

Introduction

The incidence of cutaneous melanoma is increasing faster than in any other tumour and the death rate from melanoma has doubled over the past 3 decades [1,2]. Advanced melanoma is associated with an extremely poor median survival of the order of 6-12 months [1,2]. Despite the availability of numerous chemotherapeutic and biological agents, stage IV melanoma remains a rapidly fatal disease and chemotherapy is employed mostly with palliative intent. A few drugs have shown significant activity against melanoma. DTIC (Figure 1) is considered as the most active agent achieving, alone or in combination, complete or partial responses in approximately 20% of the patients [2]. Moreover, this is the only FDA-approved chemotherapeutic agent for the treatment of metastatic melanoma [2].

The evidence that the hybrid steroid compounds of anticancer agents produce reduced toxicity, significantly lower than their cytotoxic components do alone, and increased anticancer activity, prompted the designing and developing of such steroids, mostly alkylating esters. In this combination, steroid hormones function as carriers for transporting the alkylating agents to specific targeted tissue.

The utilization of modified steroids, namely homo-aza-steroids, that contain –NH-CO- group inside the A or D steroid nucleus, as biological carriers for carboxylic derivatives of N,N-bis(2-chloroethyl) aniline led to the synthesis of highly active compounds against murine leukaemia [3-5] and rodent solid tumor systems including human xenografts [5-7]. Most of the unmodified steroid alkylating esters have been inactive in murine L1210 lymphoid and in P388 lymphocytic leukaemia, while the respective homo-aza-steroid esters gave excellent results in these leukaemia systems [8,9].

Among the tested steroid esters, HASE (Figure 1) produced excellent antitumour activity and very promising results [4]. In the present work we evaluated the effect of the homo-aza-steroid alkylator in question against malignant melanoma in vitro and

I in vivo, and in comparison with the currently used anticancer agent DTIC.

Materials and methods

Drug preparations

HASE was synthesized by previously described methods [10]. Stock solutions of the tested compounds were made immediately before use. HASE was initially dissolved in a small amount of 10% dimethyl sulfoxide (DMSO). Suspension in corn oil at the de-

Homo-aza-steroidal alkylating ester (HASE)

Dacarbazine (DTIC)

Figure 1. Chemical structures of the homo-aza-steroidal ester: 13β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-p-bis(2-chloroethyl)aminophenoxy acetate (HASE) and of dacarbazine: 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (DTIC).

sired concentration followed prior to subcutaneous (s.c.) administration to animals. DTIC (Aventis Pharma) was diluted in its appropriate solvent for intravenous (i.v.) use.

In vitro testing

The cytostatic and cytotoxic effects of the compounds under investigation were estimated on 6 human malignant melanoma cell lines: GR-M (ECACC), A375 (ECACC), RPMI-7951 (ATCC), MALME-3M (ATCC), HMM-02 and HMM-06. The HMM-02 and HMM-06 are primary cell lines isolated by Arvanitis et al. [11] from pleural fluid of two Caucasian females, 25 and 36 year-old, respectively, with metastatic melanoma. These 2 cell lines were characterized immunophenotypically and karyotypically, and presented an epithelial morphology. The cells were cultured in a concentration of 3×10^4 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. After 24 h, the cells were treated with 1-100 μM of the tested compounds (HASE and DTIC) for 48 h. In addition, a set of cell cultures were treated with the tested drugs in the presence of 10% (v/v) of rat liver microsome assay (S9 fraction), in order to produce metabolic activation of the drugs to active species. The liver homogenate fraction S9 was obtained from male Wistar rats (200-250 g) that were injected with 500 mg/kg of Aroclor 1254, and prepared according to previously described methods [12,13]. The viability of the cultured cells was estimated by the MTT assay [14]. 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 [12]. 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 by the linear regression method [13,15,16]. Using 7 absorbance measurements [time 24h (Ct24), control growth 72h (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)]
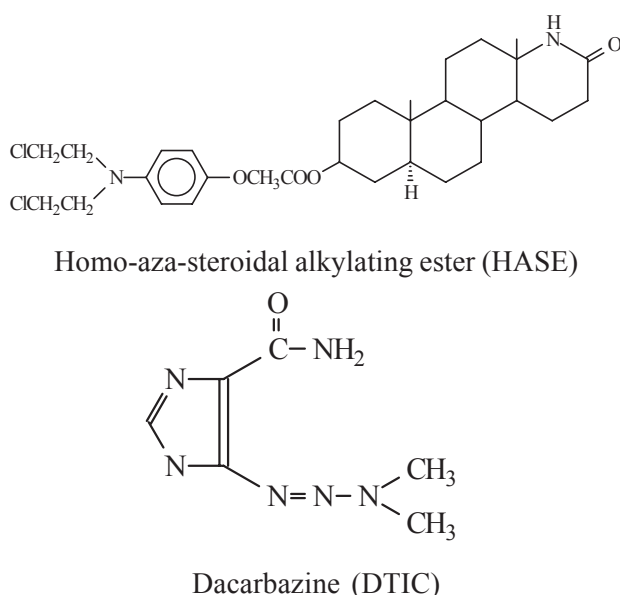
×100 for concentrations for which Tt72x≥Ct24, and [(Tt72x-Ct24)/Ct24]×100 for concentrations for which Tt72x<Ct24. GI50 was calculated from [(Tt72x-Ct24)/(Ct72-Ct24)]×100=50, TGI from [(Tt72x-Ct24)/(Ct72-Ct24)]×100=0 and IC50 from [(Tt72x-Ct24)/Ct24]×100=50. All the experiments were carried out in triplicate.

In vivo testing

Male and female DBA/2 and BDF1 (C57BLx DBA/2) mice, 6-9 weeks old, weighting 20-23g, were used for toxicity studies. A drug-insensitive tumor (melanoma B16) was utilized for antitumor testing. 0.2 ×10^6 B16 melanoma cells were injected s.c. in C57 black mice (C57BL/6) as previously described [17]. Moreover, the maximum tolerated total dose (MTTD) of the evaluated drugs was determined on animals bearing B16 melanoma and on tumor-free animals.

The toxicity of the tested homo-aza-steroid ester and of DTIC 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. 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 s.c. injections, and a number of different treatment schedules and dose intensities for DTIC and HASE, during days 6-12 after tumor inoculation, were tested. Control and each drug-treated group consisted of 8 mice. The treatment schedules 30 mg/kg DTIC on days 6,9 and 12 and 20 mg/kg HASE on days 6,9 and 12 after tumor inoculation, presented as most effective. The treatment started when the estimated tumor volume reached about 40 mm^3 after tumor inoculation (day 6). The antitumor activity was assessed from the percent increase in 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 [18].

Furthermore, the antitumor activity was evaluated by comparing the tumor volume of the treated group with that of the control group. The tumor diameter was serially measured with vernier calipers and the tumor volume (V) was calculated as: V=1/2XaXb^2 where a is the long axis and b the short axis (mm). The tumor wet weights of the treated (Tw) and control (Cw) groups were measured on the last day of each experiment and the percentage of tumor growth inhibition was calculated as follows: tumor growth rate (TGR)(%)= (Tw/Cw)×100, and tumor growth inhibition rate (TGIR)(%)= [1-(Tw/Cw)]×100.

TGR ≤42% is considered as an indication of significant antitumor activity and is defined as sufficient activity for further development of a candidate drug by the Division of Cancer Treatment (NCI) [17].

Statistical significance levels on differences between drug-treated groups and control were determined by Student's t-test (two-tailed; two-sample with unequal variance).

The present study adhered to the "Principles of Laboratory Animal Care" according to the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines [19,20].

Results

In vitro activity

The results of the in vitro screening are presented in Table 1. HASE displayed a satisfactory activity against the 6 human melanoma cell lines with mean GI50: 6.8 μM, TGI: 23 μM and IC50: 50 μM. The antiproliferative and cytotoxic effect of HASE was significantly (p <0.0001) superior than that of DTIC (mean GI50: 30 μM, TGI: 97 μM and IC50: >100 μM). Simultaneous treatment of cell lines with DTIC or HASE and S9 fraction induced significantly higher (p <0.0001) antiproliferative and cytotoxic effect, due to metabolic microsome activation of the tested drugs into active species. In this latter case, HASE was also more effective than DTIC (p <0.0001) (HASE mean GI: 5.1μM, TGI: 15.8 μM, IC50: 32.6 μM; and DTIC mean IG50: 13.4 μM, TGI: 36.3 μM, IC50: 83.8 μM). The sensitivity patterns of the cell lines treated with the tested drugs were somewhat similar, indicating a common molecular basis in the activity pattern of both compounds tested. RPMI-7951 cell line appeared as the most sensitive whereas MALME-3M as the most resistant one.

In vivo activity

In mice treated with DTIC and with HASE, the TGR and TIGR, when were calculated by tumor wet weights on day 20 after tumor transplantation were: TGR= 58.1 (p <0.05) and 41.9% (p <0.01), compared to controls, respectively; TGIR: 41.9 (p <0.05) and 58.9% (p <0.01), respectively (Table 2). The TGR and TGIR were also determined (including zeros) by tumor volumes on day 20 after tumor transplantation, and were TGR= 55 (p <0.05) and 41.1% (p <0.01); TGIR: 45 (p <0.05) and 58.9% (p <0.01) (Table 2, Figure 2).

Table 1. In vitro cytostatic and cytotoxic effects induced by HASE and DTIC, with or without the presence of rat liver microsomal assay (S9 fraction), expressed as: GI50 (drug mean concentration that generates 50% growth inhibition), TGI (drug mean concentration that produces total growth inhibition), IC50 (drug mean concentration that induces 50% cytotoxicity). Statistical significance levels on differences between drug-treated and untreated tumour cells (control) were determined by Student's t-test (two-tailed; two- sample with unequal variance). In all cases differences were significant for $p < 0.001$

			Cell lines			
w/o S9 fraction	GR-M	A375	RPMI-7951	MALME-3M	HMM-02	HMM-06
HASE						
GI50 (µM)	4.7	8.1	3.2	14.3	6.6	3.9
TGI (µM)	17.1	27.3	13.4	42.3	23.5	14.8
IC50 (µM)	43.4	62.1	28.2	78.6	54.2	33.5
DTIC						
GI50 (µM)	27.3	36.9	14.6	45.6	33.6	22.1
TGI (µM)	98.6	>100	63.6	>100	>100	85.9
IC50 (µM)	>100	>100	>100	>100	>100	>100
plus S9 fraction	GR-M	A375	RPMI-7951	MALME-3M	HMM-02	HMM-06
HASE						
GI50 (µM)	3.1	6.8	2.5	10.7	4.8	2.7
TGI (µM)	8.5	17.9	9.9	30.8	17.9	9.6
IC50 (µM)	26.9	39.6	19.8	51.3	36.5	21.7
DTIC						
GI50 (µM)	11.6	14.6	7.4	26.9	10.6	9.1
TGI (µM)	31.2	38.4	22.1	62.4	36.8	26.9
IC50 (µM)	74.5	95.2	48.9	>100	85.6	61.3

After treatment with HASE, a significant increase in the lifespan of mice inoculated with B16 melanoma was registered compared to untreated mice (HASE T/C: 167%, $p < 0.001$) (Figure 3). The mice treated with DTIC also demonstrated a significantly prolonged lifespan compared to control mice (T/C: 143%, $p < 0.001$) (Figure 3). However, treatment with DTIC was less potent than treatment with HASE ($p < 0.001$).

The mean survival of the control group was 28 days, of the group treated with DTIC (30 mg/kg on days 6,9,12) 39.97 days, and of the group treated with HASE (20 mg/kg on days 4,7,10) it was 46.8 days.

MTTD of DTIC and HASE 5-day consecutive treatment in C57BL/6 mice bearing melanoma B16 250 mg/kg and 200 mg/kg, respectively. Moreover, acute toxicities as expressed by LD10 were 80 mg/

Table 2. In vivo antitumor activity against murine malignant melanoma B16 and toxic effects induced by HASE and DTIC in C57BL/6 mice

	Tumour wet weight (g)[a]	Tumour volume (mm³)[b]	Animal weight difference (g)[c]	MTD (mg/kg)[d]	LD10 (mg/kg)[e]
Control	2.15	2350	0	–	–
DTIC	1.25*	1310*	−1.2	35	95
HASE	0.90**	990**	−1.5	25 / 30[f]	80

a. Tumour wet weight was measured on day 20 after tumor inoculation. Statistical significance levels were determined by Student's t-test (two-tailed; two-sample with unequal variance; *p <0.05, **p <0.01).
b. Tumour volume was measured on day 20 after tumour inoculation. Statistical significance levels were determined as in (a).
c. Average animal weight difference: weight on toxicity evaluation day (day 20) minus weight on day starting treatment (day 6) of test groups minus average weight change of control group. Statistical significance levels on differences between drug-treated groups and control were determined by Wilcoxon signed-rank test.
d. Maximum tolerated doses of DTIC and HASE in C57BL/6 mice bearing melanoma B16 or of HASE in normal mice (f) expressed as mg/kg for 5 days of consecutive treatment.
e. Lethal dose for 10% of treated animals within 30 days.
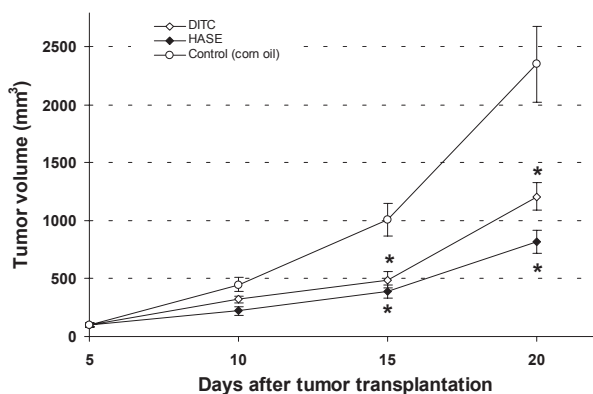
Figure 2. Tumour volume changes of mice bearing B16 melanoma. Statistical significance levels on differences were determined by Student's t-test (two-tailed; two sample with unequal variance; *p <0.01).

kg for HASE and 95 mg/kg for DTIC. These data suggest that DTIC and HASE produce similar toxicity levels on mice (Table 2).

Discussion

Research on, and anticancer evaluation of hybrid compounds (agents which combine two molecules in one), such as modified (homo-aza-) steroids and carboxylic derivatives of N,N-bis(2-chloroethyl) aniline, engaged this laboratory in earlier and later studies. Wall et al. [8] as well as Wampler and Catsoulacos [9] demonstrated that unmodified steroid alkylating esters were inactive in the treatment of L1210 and P388 murine leukemias, while the respective homo-aza-steroid esters showed excellent antileukemic activity. In the present study HASE produced relatively equal toxicity compared with DTIC, where-
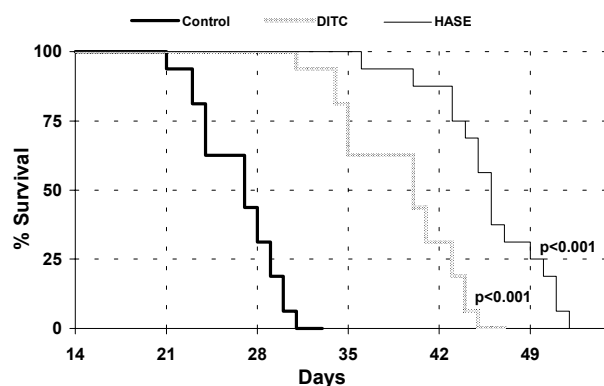
Figure 3. Survival curves of mice bearing B16 melanoma. The percent lifespan increases of treated (T) to untreated (C) mice were: T/C$_{DTIC}$: 143 and T/C$_{HASE}$: 167 (p <0.001). Statistical significance levels were determined by Wilcoxon signed-rank test.

as displayed significantly higher antitumor activity than DTIC in the treatment of malignant melanoma in vitro and in vivo.

Based on the aforementioned primary data, several homo-aza-steroids were prepared and used as biological carriers for alkylating agents. 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 [21,22]. Possibly, the antineoplastic effects of these steroid esters may be due to the multiple interactions of the –NHCO- group with similar groups or with structural specific domains which exist in DNA and proteins. Catsoulacos et al. [23] 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 (-NH-CO- → -NH$^{\ominus}$+ -C=O$^{\oplus}$). Furthermore, modifications of the –NHCO- lactam group by NH methylation (-NCH$_3$CO-) or by –CO- reduction led to derivatives with lower anticancer activity than that of the parent compounds [23-25].

In early studies on the action mechanisms of amino and aza-steroids 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 [26]. As it was indicated by later studies, 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 [27-29].

Although epidemiological data clearly show a survival benefit for female patients with metastatic melanoma, the mechanism of influence of sex steroid hormones on the biological behavior of human melanoma still remains a controversial issue. In general, independently of the percentage of tumor positivity to steroid receptors, malignant melanoma is considered as a hormone-independent tumor [30]. Phase II trials showed that the clinical course of advanced malignant melanoma was indifferent to competitive and additive endocrine treatment [31]. It is suggested that the apparent binding capacity of sex steroids in human melanoma tissues is the result of interactions other than with the respective steroid receptors [32,33]. Moreover, it was demonstrated that dehydroepiandrosterone (an important androgen metabolite) upregulates the activity of PKC in B16 melanoma [34]. On the other hand, the homo-aza-steroid derivatives of androgens or estrogens do not bind to

respective receptors and their activity is independent of the existence of intracellular steroid receptors on target tissues, while the D-lactam derivative of estrone acts neither synergistically nor antagonistically to tamoxifen [6,35].

The exact molecular mechanism of action of homo aza-steroid alkylators is still unknown. The alkylating component of these esters effects via the same biochemical pathway of other bifunctional alkylating mustards [36]. It is believed that these compounds can generate high intracellular concentrations due to the lipophilic nature of the steroid carrier. It has been reported for other steroid alkylators that a rate-limiting hydrolysis of the ester bond liberates the two active moieties (one steroid and one alkylating) into the cellular microenvironment [37]. As it has been reported, the stereoisomeric form and chemical structure of the steroid lactams and of alkylating components determine the antileukemic effect of these compounds [22,38-40]. Our antitumor agent screening studies showed that the alkylator p-bis(2-chloroethyl) aminophenoxy acetic acid (CAPOA), is one of the most potent cytotoxic components. In this study, the homo-aza-steroid ester of CAPOA was proven significantly effective against malignant melanoma and clearly superior to DTIC, both in vitro and in vivo.

DTIC actually functions as an alkylating agent. It is active against a broad spectrum of murine solid and ascitic tumors, but its clinical effectiveness is limited to Hodgkin's disease, malignant melanoma, and soft tissue sarcomas. DTIC exerts its alkylating properties by means of hepatic microsomal mediated activation [41]. Indeed, as it was shown in our results, the cytostatic and cytotoxic activity of DTIC was overdublicated with the microsomal assay. Intriguing was the significant increment of cytostatic and cytotoxic effect of HASE in the presence of microsomal S9 fraction. This is consistent with the findings for other substituted aniline mustards and could be related to a double mechanism of drug action: (I) a direct nucleophilic reaction with DNA via aziridinium intermediate product of the mustard, and (II) epoxidation of the benzene ring by the microsomes with a subsequent attack on the DNA [13,42]. 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 [3,43].

Malignant metastatic melanoma is a tumor with very poor prognosis. Though biologic and immune therapies have shown promising results, effective cytotoxic therapies are further searched for. Thus, we believe that HASE might be a considerable candidate for the treatment of human malignant melanoma.

References

1. Beddingfield FC. The melanoma epidemic: Res Ipsa Loquitur. Oncologist 2003; 8:459-465.
2. Hunckharek M, Caubet JF, McGarry R. Single-agent DTIC versus combination chemotherapy with or without immunotherapy in metastatic melanoma: a meta-analysis of 3273 patients from 20 randomized trials. Melanoma Res 2001; 11:75-81.
3. Catsoulacos P, Politis D, Wampler GL. A new steroidal alkylating agent with improved activity in advanced murine leukemias. Cancer Chemother Pharmacol 1979; 3:67-70.
4. Catsoulacos P, Camoutsis C, Wampler GL. Effect of a Δ5-homo–aza-steroidal ester in P388 and L1210 murine leukemias. Oncology 1982; 39:59-60.
5. Catsoulacos P. Activity of 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13, 17 - lactam p–bis (2-chloroethyl)aminophenoxy acetate (NSC-294859) on experimental tumor and leukemia systems. Oncology 1983; 40:290-292.
6. Catsoulacos P, Wampler GL. Activity of 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam(p-bis(2-chloroethyl)amino)-phenyl)acetate (NSC-290205) in murine solid tumors. Oncology 1982; 39:109-112.
7. Catsoulacos P. Further studies on the anti-neoplastic activity of 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam(p–bis(2-chloroethyl)amino)-phenyl)acetate(NSC-290205). Cancer Lett 1984; 22:199-202.
8. Wall ME, Abernethy SG, Carroll JFI, Taylor DJ. The effects of some steroidal alkylating agents on experimental animal mammary tumor and leukemia systems. J Med Chem 1969; 12:810-818.
9. Wampler GL, Catsoulacos P. Antileukemic effect of homo-aza-steroidal-ester of [p-[bis(2-chloroethyl)amino]acetic acid. Cancer Treat Rep 1977; 61:37-41.
10. Catsoulacos P, Boutis L. Aza-steroids. Beckmann rearrangement of 3β-acetoxy-5α-androstan-17-one oxime acetate with boron fluoride. Alkylting agents. Chim Ther 1973; 8:215-217.
11. Arvanitis A, Trafalis D, Camoutsis C, Tegou E, Stavrianeas N. Effect of the steroidal lactam alkylating ester NSC-294859 on primary human melanoma cell lines. 20th World Congr Dermatol (Paris). Ann Dermatol Venereol 2002; 1(Suppl): 634.
12. Maron D, Ames BN. Revised methods for the salmonella mutagenicity test. Mutation Res 1983; 113:173-215.
13. Voutsinas G, Kappas A, Demopoulos NA, Catsoulacos P. Mutagenic activity of the antitumor agent homo-aza-steroidal ester of p–N,N-bis(2-chloroethyl)aminophenoxy acetic acid (NSC-294859) in the salmonella microsome assay. Mutagenesis 1993; 8:431-435.
14. Finlay GJ, Wilson WR, Baguley BC. Comparison of in vitro activity of cytotoxic drugs towards human carcinoma and leukaemia cell lines. Eur J Cancer Clin Oncol 1986; 22:655-662.
15. Alley MC, Scudiero DA, Monks PA et al. Feasibility of

drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res 1988; 48:589-601.

16. Boyd MR, Paull KD. Some practical considerations and applications of the National Cancer Institute in vitro anticancer drug discovery screen. Drug Devel Res 1995; 34:91-109.

17. Corbett TH, Valeriote FA. Rodent models in experimental chemotherapy. In: Kallman RF (ed). The use of rodent tumors in experimental cancer therapy: Conclusions and recommendations. Pergamon, 1987, pp 233-247.

18. NCI Monograph 55. NIH Publication 1986, No 80, p 193.

19. Principles of laboratory animal care. NIH Publication 1985, No 85, p 23.

20. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia. Br J Cancer 1998; 77:1-10.

21. Catsoulacos P, Politis D, Wampler GL. Antitumor activity of homo-aza-steroidal esters of [p-bis(2-chloroethyl) amino]phenyl]acetic acid [p-bis(2-chloroethyl)amino] phenyl] butyric acid. Cancer Chemother Pharmacol 1983; 10:129-132.

22. Catsoulacos P, Catsoulacos D. Antitumor activity of homo-aza-steroidal esters of p-N, N-bis(2-chloroethyl) amino phenoxy acetic acid. Anticancer Res 1993; 13:1203-1208.

23. Catsoulacos P, Camoutsis C, Papageorgiou A, Adamiak-Margariti E. Cytostatic effect of homo-aza-steroidal esters in vivo and in vitro structure-activity relationships. Anticancer Res 1992; 12:1617-1620.

24. Dalmases P, Gomez-Belinchon JI, Bonet J-J, Giner-Sorolla A, Schmid FA. Antineoplastic agents. II. A nitrogen mustard derivative of N-methylated steroidal lactam. Eur J Med Chem 1983; 18: 541-543.

25. Dalmases P, Cervantes G, Quintana J, Bonet J-J. Antineoplastic agents. V. Nitrogen mustards of systematically modified steroidal ring A lactams. Eur J Med Chem 1984; 19:465-467.

26. Kabara JJ, Holzschu DL, Catsoulacos P. Structure function activity of azasterols and nitrogen containing steroids. Lipids 1976; 11:7555-7562.

27. Ma D, Wamg G, Wang S, Kozikowski AP, Lewin NE, Blumberg PM. Synthesis and protein kinase C binding activity of benzolactam-V-7. Bioorg Med Chem Let 1999; 9:1371-1374.

28. Endo Y, Yokohama A. Role of the hydrophobic moiety of tumor promoters. Synthesis and activity of 2-alkylated benzolactams. Bioorg Med Chem Let 2000; 10:63-66.

29. Endo Y, Shimazu M, Fukasawa H et al. Synthesis computer modeling and biological evaluation of novel protein kinase C agonists based on a 7-membered lactam moiety. Bioorg Med Chem Let 1999; 9:173-178.

30. Kokoschka EM, Spona J, Knobler R. Sex steroid hormone receptor analysis in malignant melanoma. Brit J Dermatol 1982; 107:54-59.

31. Rose C, Pedersen L, Mouridsen HT. Endocrine treatment with anti-estrogen, anti-androgen or progestagen of advanced malignant melanoma: three consecutive phase II trials. Eur J Cancer Clin Oncol 1985; 21:1171-1174.

32. Flowers JL, Seigler HF, McCarty KSSr, Konrath J, McCarty KSJr. Absence of estrogen receptor in human melanoma as evaluated by a monoclonal antiestrogen receptor antibody. Arch Dermatol 1987; 123:764-765.

33. Lorea P, Decaestecker C, Goldschmidt D et al. Correlation between gender and cytomorphonuclear characteristics in human melanoma and in vitro evidence of sex steroid-induced modifications in the morphonuclear characteristics of three human melanoma cell lines. Melanoma Res 1997; 7:382-392.

34. Kawai S, Yahata N, Nishida S, Nagai K, Mizushima Y. Dehydroepiandrosterone inhibits B16 mouse melanoma cell growth by induction of differentiation. Anticancer Res 1995; 15:427-431.

35. Leclercq G, Devleeschouwer N, Pairas G, Camoutsis Ch, Catsoulacos P. Effect of an homo-aza-steroid on estrogen receptor. Meth Find Exptl Clin Pharmacol 1983; 5:365-367.

36. Papageorgiou A, Ivanov IG, Markov GG, Koliais SI, Boutis L, Catsoulacos P. Interaction of homo-aza-steroidal ester of [p-[bis-(2-chloroethyl)amino] acetic acid (ASE) with DNA of Ehrlich ascites tumor cells. FEBS Lett 1983; 153:194-198.

37. Shepherd RE, Huff K, McGuire WL. Brief communication: Estrogen receptor interaction with antitumor agent estradiol mustard. J Nat Cancer Inst 1974; 53:895-897.

38. Camoutsis C, Trafalis DTP. An overview on the antileukemic potential of D-homo-aza- and respective 17β-acetamido- steroidal alkylating esters. Invest New Drugs 2003; 21:47-54.

39. Catsoulacos P, Catsoulacos D. Conjugated systems of homo-aza-steroidal esters in cancer chemotherapy. Anticancer Res 1994; 14:2525-2528.

40. Catsoulakos P, Catsoulakos D. Hybrid anticancer compounds. Steroidal lactam esters of carboxylic derivative of N, N-bis (2-chloroethyl) aniline. Anticancer Res 1991; 11: 1773-1778.

41. Hayward IP, Parson PG. Epigenetic effects of the methylating agent 5-(3-methyl-1-triazeno)-imidazole-4-carboxamide in human melanoma cells. Aust J Exp Biol Med Sci 1984; 62:597-606.

42. Leo A, Panthananickal A, Hansch C, Theiss J, Shimkin M, Andrews AW. A comparison of mutagenic and carcinogenic activities of aniline mustards. J Med Chem 1981; 24:859-864.

43. Catsoulacos P, Papageorgiou A, Margariti E, Mourelatos D, Mioglou E. Comparison of current alkylating agents with a homo-aza-steroidal ester for antineoplastic activity. Oncology 1994; 51: 74-78.