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ORIGINAL ARTICLE

A preclinical survey on the efficacy of lactandrate in the treatment of colon carcinoma

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Summary

Purpose: *There has been a recent and dramatic increase in the pace of drug development for colorectal cancer which holds promise to further improve curative therapy. We tested lactandrate, an alkylating ester of D-lactam androsterone, for antineoplastic activity against colon adenocarcinoma in vitro and in vivo.*

Materials and methods: *The cytostatic and cytotoxic activity of lactandrate were evaluated in vitro against 9 human colon adenocarcinoma cell lines. The in vitro testing was performed with the sulforhodamine B (SRB) colorimetric assay and the mean concentrations of each drug that generated 50% (GI50) or total (100%) growth inhibition (TGI), as well as the drug concentrations that produced cytotoxicity against 50% of the cultured cells (IC50) were calculated. The in vivo antitumour effect was determined against two rodent colon carcinomas, the Colon 26 and the relatively chemoresistant Colon 38 carcinoma, as well as against the human xenograft CX-1 colon carcinoma.*

Results: *Lactandrate displayed a satisfactory activity against the 9 human colon cancer cell lines, inducing significant growth inhibition and cytotoxicity. Lactandrate induced antiproliferative activity against colon cancer cell lines linearly correlated with the carcinoembryonic antigen (CEA) production. There was a non-linear polynomial correlation between CEA production and the cytotoxic effect of lactandrate. The more differentiated cell lines DLD-1 and HCC2998 appeared more resistant to the cytostatic effect of lactandrate. In vivo, the compound produced a significant antitumour activity against Colon 26 and Colon 38, as well as a moderate antitumour effect against CX-1 colon carcinoma.*

Conclusion: *Preclinical research supports the high in vitro and in vivo antitumour potential of lactandrate against colon carcinoma. Therefore, lactandrate represents an important candidate drug for further clinical development.*

Key words: colon carcinoma, homo-aza-steroids, human cell lines, in vitro, in vivo, lactam steroid alkylator

Introduction

Colorectal cancer is the third most frequent cause of death from malignancies among men and women in the Western world. Although colorectal cancer could be characterized as a preventable disease, it is estimated that 148,300 persons in the USA were diagnosed with colorectal cancer and 56,600 died from the disease in 2002. The crude incidence of colorectal cancer in the European Union is 58/100 000 per year, and the mortality is 30/100 000 per year. In the USA the 5-year relative survival for patients diagnosed with cancers of the colon and rectum during 1985–1989 were 60 and 57%, respectively, while in Europe the corresponding numbers were 48 and 44% [1-3].

Although surgical resection is still the only curative maneuver in the treatment of colon cancer,

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efforts of the past decades have proved that systemic chemotherapy in the adjuvant setting definitely improves the curative rate for patients with localized disease. Traditional alkylating and non-classical agents are relatively inactive and 5-fluorouracil has been the mainstay of treatment for metastatic colorectal cancer over the past 40 years. Moreover, platinum compounds have shown limited single-agent activity. Recently, major developments in the treatment of colon cancer have emerged and oxaliplatin has become an important option in the treatment of colorectal cancer. However, oxaliplatin has modest activity as a single agent in advanced colorectal cancer. The development of new drugs remains a focus of intense investigation [4-6].

Under the rationale that steroid receptors may serve to localize and concentrate appended drug species, mainly in hormone-responsive cancers, a number of conjugates of mustards and steroids have been developed. The designing and use of lactam 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 (nitrogen mustards), generated a pioneer group of steroidal alkylators with high anticancer effect and low toxicity, introducing novel patterns of mechanisms of action [7-9].

Hereby we present the activity of a very active D-homo-aza-steroid alkylating ester, namely 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam,p–bis(2-chloro ethyl)aminophenylacetate (Lactandrate, Figure 1), against 9 human colon adenocarcinoma cell lines *in vitro* and against 2 murine and one human xenograft colon tumour models *in vivo*.

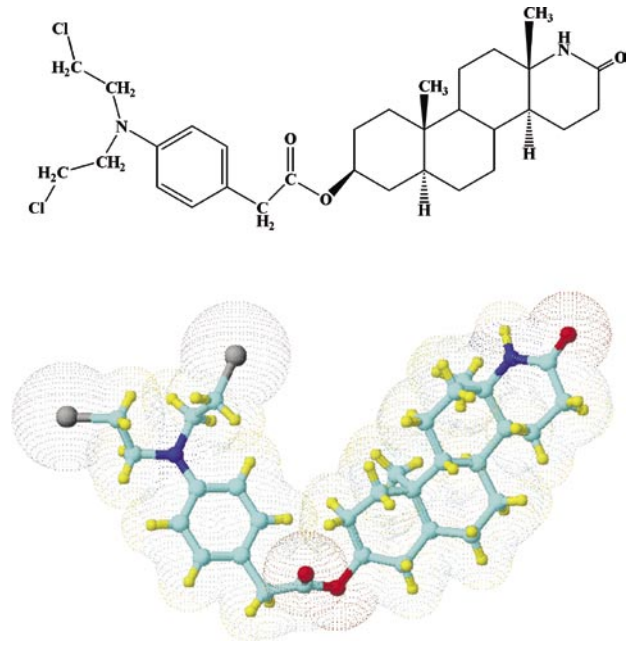

Figure 1. Chemical structure of 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam,p–N,N-bis(2-chloroethy l)aminophenylacetate (Lactandrate).

Materials and methods

Drug preparations

Lactandrate (Figure 1) was synthesized by previously described methods [10]. Stock solutions of the tested compound were made immediately before use. The compound was initially dissolved in a small amount of 10% dimethyl sulfoxide (DMSO). Prior to intraperitoneal (i.p.) administration it was suspended in corn oil at the desired concentrations.

Cell lines and NCI in vitro testing

The cytostatic and cytotoxic effects of lactandrate were estimated on the following 9 human colon carcinoma cell lines: COLO 205 with 1.5 to 4.1 ng/10^6 cells/10 days carcinoembryonic antigen (CEA) secretion; DLD-1 with 0.5 ng/10^6 cells/10 days CEA secretion and good cell differentiation; the well cell differentiated HCC-2998 with 0.1-0.5 ng/10^6 cells/10 days CEA secretion; HCT-116 with 1.0 ng/10^6 cells/10 days CEA secretion; HCT-15 with 5.4 ng/10^6 cells/10 days CEA secretion; HT29 with 5.0-9.0 ng/10^6 cells/10 days CEA secretion; KM12 with 1.0-2.0 ng/10^6 cells/10 days CEA secretion; KM20L2 with 4.0-6.0 ng/10^6 cells/10 days CEA secretion; and SW-620 with 0.15 ng/10^6 cells/10 days CEA secretion.

The cells were cultured in a density of 3-4 $\times 10^4$ cells/ml in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate and 1% antibiotics (gentamycin plus penicillin) except SW-620 cell line that was cultured in Leibovitz's L-15 medium with 2 mM L-glutamine, 10% fetal bovine serum and 1% antibiotics (gentamycin plus penicillin), as well as HCT and HT29 cell lines that were cultured in McCoy's 5a medium with 1.5 mM L-glutamine, 10% fetal bovine serum and 1% antibiotics (gentamycin plus penicillin). The cultures were maintained for 72 h in a 5% CO_2 incubator at 37° C.

After 24 h, cells were treated with 0.1-100 μM of the tested compound for 48 h. The viability of cultured cells was estimated by the SRB assay which was per-

formed as described previously [11]. In brief, cells were split into 96-well plates. After incubation, anchorage-dependent cells were directly fixed by the slow addition of 50 μL of 50% trichloroacetic acid (TCA) solution per well. Anchorage-independent cells were fixed by pre-centrifugation (150 *g*, 1 min at 20° C) and the drop-wise addition of 50% TCA. Fixation proceeded for 1 h at 4° C. After fixation, plates were washed 5 times with tap water, and were air-dried. One hundred microliters of SRB solution (0.4% in 1% acetic acid) were added to each well of the 96-well microplates. Staining was done at room temperature for 30 min. Residual dye was washed out with 1% acetic acid and was air-dried. To each well, 100 μL of Tris solution (10 mM, pH 10.5) was added. Optical density (O.D.) was measured in a microtiter plate reader at 540 nm. Each drug concentration was tested in triplicate at least 3 times.

The *in vitro* cytostatic effects of lactandrate were determined with the calculation of GI50 and TGI, while cytotoxicity was assessed by IC50. The mean concentrations of the drug that generated GI50 and/or TGI, as well as the drug concentrations that produced IC50 were calculated by the linear regression method. 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)]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.

NCI in vivo testing

Lactandrate was tested for *in vivo* antitumour activity against the two rodent colon tumours Colon 26 and Colon 38 and the human xenograft CX-1. Testing was carried out according to NCI established protocols [12].

BALB/c×DBA/2F$_1$ (CD2F$_1$) mice were inoculated i.p. with Colon 26 adenocarcinoma cells (5×10^5/mouse). Mice weighed 20-22 g and were divided into control and drug treatment groups (10 mice/group). Colon 26 adenocarcinoma cells were inoculated i.p. on day 0, and lactandrate was administered i.p. on days 1, 5 and 9 at dosages of 1.5-48 mg/kg. The experiment was conducted on day 70. Median survival time (days) of control and each test group was estimated.

Colon 38 tumors were grown subcutaneously (s.c.) from 1 mm^3 fragments implanted in one flank of B6D2F1 (BDF1) mice weighing 20–22 g (6–8 weeks of age). When tumors reached a diameter of approximately 4–6 mm (7–8 days), mice were divided into control and drug treatment groups (10 mice/group), with similar average tumor volumes in each group. Lactandrate was administered i.p. using intermittent (q7d×2) schedules (days 7 and 14) at dosages of 1.5-48 mg/kg. The mice were monitored closely and tumor diameters were measured with callipers 3 times a week. The day 20 was considered as the endpoint for evaluation of antitumour activity. Tumor volumes or weights were calculated as $0.52\times a^2\times\beta$, where a and β are the minor and major tumor axes, and data were plotted on a semi-logarithmic graph as mean tumor volumes (±SEM) *versus* time after treatment.

CX-1 is a human colorectal adenocarcinoma cell line that was originally isolated from HT-29 in a nude mouse metastasis model. Athymic NCr nu/nu male mice, 6-8 weeks old and weighing 20-25 g kept in pathogen-free conditions, were used. $2x10^6$ viable CX-1 tumor cells from *in vitro* culture suspended in 0.2 ml saline were grafted s.c. into the right flank region of mice or transplanted intra-renally. Mice were divided into groups of 6 prior to treatment, when tumors reached a volume of 50-60 mm^3 (9 days after implantation). Lactandrate was administered s.c. using intermittent (q4d×4) schedules (days 1, 5, 9 and 13) at dosages of 12.5-100 mg/kg. The tumor volumes and the mean tumor weight change were calculated. The experiments were typically terminated on day 15.

For evaluation of the antitumor effect, the percent increase in median survival time (MST) in Colon carcinoma 26, or the percent decrease of median tumor weight (volume) in Colon carcinoma 38, or the percent of mean tumor weight change in CX-1 carcinoma of the treated animals (T) over the controls (C) were estimated (T/C%).

Activity thresholds were designated according to NCI Screener Instruction 14. Decision Network 2 (DN2) activity criteria require a T/C% ≤10 against Colon 38 and CX-1 carcinomas and Material Classification 1 (MC1) requirement for minimum reproducible activity is ≤ 42 and ≤ 20, respectively.

The animal experiments were performed according to the "Principles of Laboratory Animal Care" [12].

Statistical analysis

For the *in vitro* experiments Student's t-test was used and for the *in vivo* experiments significance

levels were determined by chi-square and Wilcoxon signed-rank tests.

Results

Figures 2a-c analytically describe the *in vitro* testing results. Lactandrate displayed a satisfactory activity against the 9 human colon cancer cell lines, inducing significant growth inhibition (Student's t-test, $p < 0.0001$, mean GI50±SD: 9.5 ±5.8 μM, range: 2-20 μM). TGI induced by lactandrate was also significant (Student's t-test, $p < 0.0001$, mean TGI±SD: 23.7 ±8.7 μM, range: 4-31.6 μM). The cytotoxic effect of lactandrate, as estimated by IC50 values, was effective on all tested cell lines (Student's t-test, $p < 0.0001$, mean IC50±SD: 58.1±23.7 μM, range: 7.9-100 μM). Based on the mean antiproliferative and cytotoxic effect of lactandrate on the tested cell lines, as shown in Figures 2a-c, DLD-1 and HCC2998 cells appeared relatively resistant to the cytostatic effect and KM20L2 cells presented relative resistance to the cytotoxic effect (IC50) of lactandrate (Student's t-test, $p < 0.01$). On the other hand, in all cases, HT29 cells appeared to be the most sensitive.

Furthermore, as far as its cytostatic effect was concerned, the *in vitro* chemosensitivity to lactandrate was moderately significantly correlated by linear regression with the increase of CEA secretion (correlation index $R = -0.71$, $p < 0.03$) (Figure 3). However, the TGI and the cytotoxic effects (IC50) of lactandrate on colon cancer cells did not present linear correlation with the CEA secretion, but a polynomial of third class (correlation index $R^2 = 0.92$, $p < 0.002$) (Figure 3).

In vivo, lactandrate generated excellent antitumor results against the Colon 26 carcinoma, prolonging significantly the survival of female and male mice that were treated with 24 mg/kg of lactandrate (female T/C%: 213, male T/C: 193+, $p < 0.001$) and producing 3/10 and 8/10 tumor-free 70 days survivorship, respectively (Table 1). Lactandrate produced a significant tumour volume reduction of Colon 38 carcinoma *in vivo* (T/C%: 27, $p < 0.01$) at the dose of 24 mg/kg (Table 2, Figure 4), corresponding to the NCI requirement for minimum reproducible activity (MC1, T/C% ≤ 42) for further development. Moreover, lactandrate induced significant attenuation of CX-1 tumour weight and volume at the dose of 100 mg/kg (T/C%: 38, $p < 0.01$) (Table 3, Figure 5), but this effect did not fall into NCI activity requirements for further development. However, it has to be noted that lactandrate was not tested sufficiently against CX-1 carcinoma.

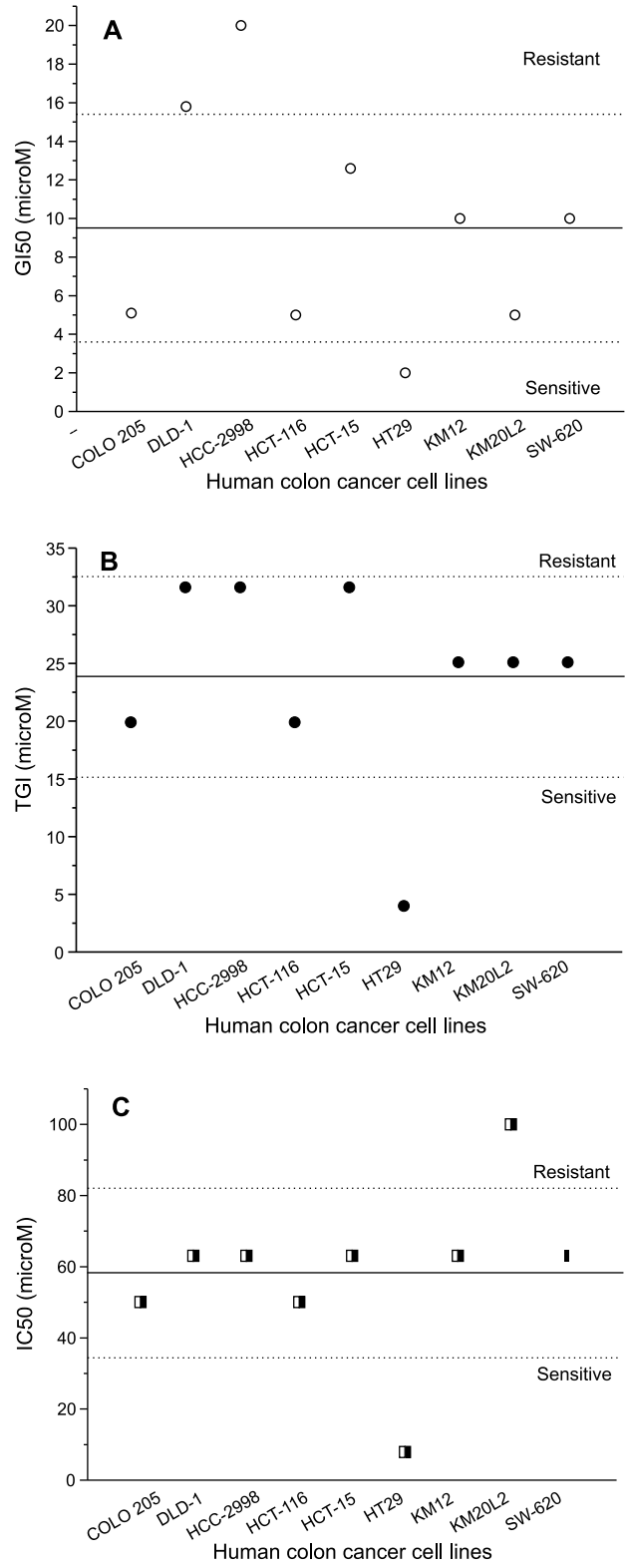

Figure 2. *In vitro* cytostatic and cytotoxic effects induced by lactandrate on 9 human colon carcinoma cell lines expressed as: **a.** GI50 (drug mean concentration that generates 50% growth inhibition); **b.** TGI (drug mean concentration that produces total growth inhibition); **c.** IC50 (drug mean concentration that induces 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 $p < 0.001$.

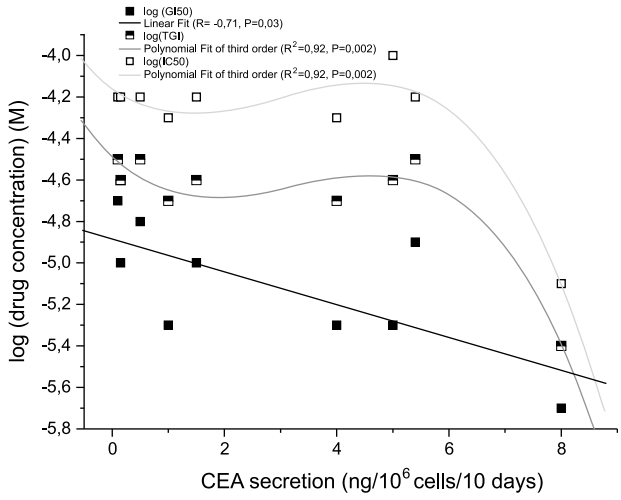

Figure 3. The *in vitro* cytostatic effect of lactandrate is linearly correlated with the increase of CEA secretion (correlation index R= – 0.71, $p < 0.03$). The TGIs and the IC50s induced by lactandrate on colon cancer cells present polynomial of third class correlations with the CEA secretion (correlation index R^2=0.92, $p < 0.002$).

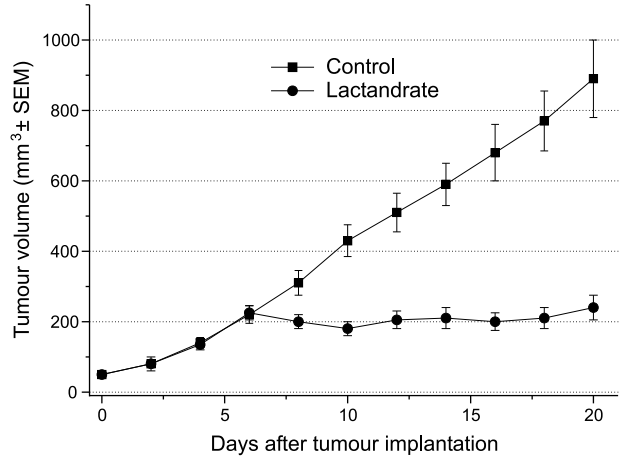

Figure 4. Inhibition effect of lactandrate at dose of 24 mg/kg on Colon 38 carcinoma growth as indicated by the mean tumour volume reduction ($p < 0.001$).

Table 2. *In vivo* antitumor effect of intraperitoneal (i.p.) lactandrate on Colon 38 tumour

Dose (mg/kg)	*Treatment schedule*	*T/C%**
control	–	100
1.5	q7dx2	121
3.0	q7dx2	77
6.0	q7dx2	48
12.0	q7dx2	39
24.0	q7dx2	27

*treated/control %

Table 3. Subcutaneous (s.c.) lactandrate antitumour effect on CX-1 human xenograft *in vivo*

Dose (mg/kg)	*Treatment schedule*	*T/C%**
control	–	100
12.5	q4dx4	99
25.0	q4dx4	109
50.0	q4dx4	77
100.0	q4dx4	38

*treated/control %

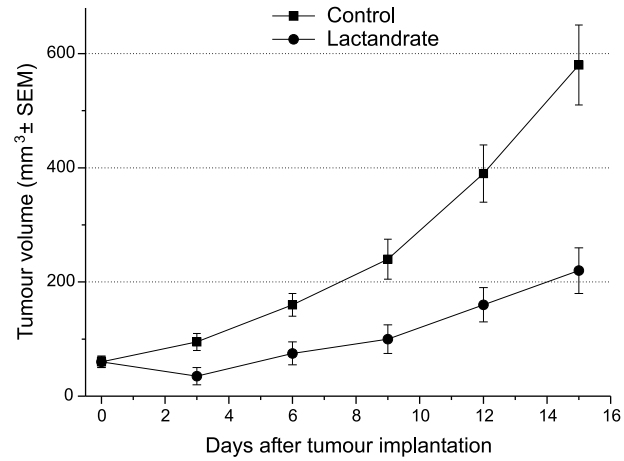

Figure 5. Inhibition of CX-1 human colon cancer xenograft subcutaneous growth was induced by the treatment with 100mg/kg of lactandrate ($p < 0.001$).

Table 1. Median survival of lactandrate-treated $CD2F_1$ mice bearing Colon 26 adenocarcinoma

Dose (mg/kg) (i.p.)	*Treatment schedule*	*MST§ (days)*	*T/C%†*	*70-day tumor-free survivors*	*Animal weight* difference (g)*
Females					
control	–	28.5	100	0/10	0.0
6	Days 1,5,9	56.0	196	3/10	– 0.9
12	Days 1,5,9	49.0	171	2/10	– 1.6
24	Days 1,5,9	60.8	213	3/10	– 2.2
Males					
control	–	36.1	100	0/10	0.0
6	Days 1,5,9	50.0	166	4/10	– 0.7
12	Days 1,5,9	70+	193+	4/10	– 1.2
24	Days 1,5,9	70+	193+	8/10	– 2.4

§median survival time, †treated control %, *average weight change (weight on toxicity evaluation day minus weight on initial day of treatment) of test group minus average weight change of control group

Discussion

Evidence indicating that hybrid steroid compounds of anticancer agents produce reduced toxicity, significantly lower than the cytotoxic components alone, and increased anticancer activity has prompted the design and development of such steroids, mostly alkylating esters. In this combination, steroid hormones function as carriers for the transport of alkylating agents to specific targeted tissue. The utilization of modified lactam steroids, namely homo-aza-steroids, as biological carriers for nitrogen mustards, led to the synthesis of highly active compounds against murine leukaemia [13-15] and rodent solid tumour systems, including human xenografts [15-17]. Most of the unmodified steroid alkylating esters are inactive in murine L1210 lymphoid and P388 lymphocytic leukaemia, and the respective homo-aza-steroidal esters gave superior results in these leukaemia systems [18,19]. These aza-steroid esters were synthesized with the rationale that they could produce high intracellular concentrations of alkylating moieties and could target specific components, biochemical systems or pathways of tumour cells.

In early studies on the mechanisms of action of amino- and aza-steroids, it was presumed that these compounds acted via a more fundamental mode than on sterol metabolism alone, interfering with mitochondrial respiration and/or oxidative phosphorylation [20]. As indicated by later studies, the lactam ring of the aza-steroids can react as an antagonist or agonist by binding to certain cellular enzymes in a manner similar to the indo-, benzo- or other steroid lactams which target protein kinase C (PKC) enzymes with a relative specificity [21-23].

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 (-NH-CO-) of the homo-aza-steroid molecule has been proven to be important in lowering the acute toxicity and improving antitumour activity in cancer research [24,25]. Possibly, the antineoplastic effects of these steroid esters may be due to the multiple interactions of the -NH-CO- group with similar groups or with structurally specific domains that exist in DNA and proteins. Catsoulacos et al. [26] have suggested that the -NH-CO- lactam group is transformed by a metabolic process or, at least, by an enzymatically catalysed reaction to active species which strongly interact with similar groups in DNA and proteins (-NH-CO- $\rightarrow$ $-NH^- + -C{=}O^+$). Furthermore, modifications of the -NH-CO- lactam group by NH methylation ($-NCH_3CO-$) or by -CO- reduction leads to derivatives with lower anticancer activity than that of the parent compound [26-28]. Moreover, the alkylating component of these esters acts via the same biochemical pathway as other bifunctional alkylating mustards [29]. 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 [30]. It has also been reported that the stereoisomeric form and chemical structure of the steroid lactams and alkylating components determine the antileukaemic effect of these compounds [25,31-33].

A relationship between the degree of differentiation, the degree of penetration, the metastatic capacity and stage of disease, and CEA expression in colon cancer has been demonstrated [34,35]. It is suggested that the serum level of CEA is mainly determined by tumor differentiation and stage of disease [36]. Our results show that lactandrate induces antiproliferative activity against colon cancer cell lines linearly correlated with the CEA production. On the other hand, there is a non-linear polynomial correlation between CEA production and cytotoxic effect of lactandrate, indicating that the chemosensitivity to lactandrate depends on the high CEA secretion. Additionally, the more differentiated cell lines DLD-1 and HCC2998 appear to be more resistant to the cytostatic effect of lactandrate.

The Colon 38 tumour model is fairly refractory to standard clinical topoisomerase II inhibitors, as well as to antimetabolites and alkylating agents. *In vivo* Colon 38 tumor models have been shown to be the best predictors for clinical utility [37]. It is notable that lactandrate produced a significant antitumour activity against this resistant tumour, presenting a rather different pattern of mechanisms of action than standard alkylating agents. Moreover, CX-1 is a highly metastatic, moderately differentiated, low CEA-producing cell line. Lactandrate induced a moderate antitumour effect against CX-1 colon carcinoma [38,39]. These results correspond to the *in vitro* activity of lactandrate.

Epidemiological studies show a strong link between postmenopausal hormone replacement therapy and decreased incidence of colorectal cancer. It is demonstrated that cytoplasmic estrogen and androgen receptors are present in colon carcinomas. Estrogen use reduces the risk of colon cancer and the related mortality. Selective regulation of genes in colon cancer cells by estradiol is involved in induced cancer cell apoptosis. Moreover, there is a possible role of

androgens and their receptors in colon carcinogenesis [40-42]. These data underscore a possible importance of the steroidal component of lactandrate and other steroidal alkylators in the treatment of colon cancer.

There has been a recent and dramatic increase in the pace of drug development for colorectal cancer which holds promise to further improve curative therapy as part of a multidisciplinary approach in the postsurgical adjuvant setting. Lactandrate exhibits very good activity against colon cancer and therefore it represents an important candidate drug for further development. It is hoped that, with the development of new drugs, the response rates and survival for patients with colorectal cancer will further improve, compared with present-day figures.

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