Effects of retinoic acid steroidal analogs on human leukemic HL60 cell proliferation *in vitro* and on angiogenesis *in vivo*

Evaggelia S. Arsenou[a], Evangelia P. Papadimitriou[b], Eleni Kliafa[b], Maria Hountala[b] and Sotiris S. Nikolaropoulos[a]

Retinoic acid (RA) can be regarded as a pharmacological agent commonly used for its ability to affect growth and differentiation of a variety of cell types, such as acute promyelocytic leukemic and endothelial cells. In the present work we studied the effect of all-*trans*-RA (ATRA) and its steroidal analogs EA-4, EA-136 and EA-137 on the growth of human promyelocytic HL-60 cells *in vitro*. The specific steroidal substrates were chosen in order to further investigate their ability to improve the pharmacological properties of conjugated antileukemic agents. ATRA decreased the number of HL60 cells from the first 24 h after its addition to the cell culture medium. The decrease was significant at concentrations higher than 10^{-5} M. All the analogs tested also decreased the number of HL60 cells with an IC_{50} similar to that of ATRA, except for EA-4 whose IC_{50} was almost two orders of magnitude lower than that of ATRA, 72 h after its addition to the cell culture medium. Since angiogenesis is important for the growth of hematological malignancies, we furthermore studied the effect of ATRA and its analogs on the formation of new capillaries in the *in vivo* chicken embryo chorioallantoic membrane (CAM). ATRA, EA-136 and EA-137 induced angiogenesis in the CAM, increased the layer of CAM keratinocytes, and resulted in a significant degree of extravasation. EA-4 had no effect on either angiogenesis or tissue structure in general. It seems that the retinoid EA-4 is a promising agent for the inhibition of human leukemia cell growth. *Anti-Cancer Drugs* 16:151–158 © 2005 Lippincott Williams & Wilkins.

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Laboratories of [a]Pharmaceutical Chemistry and [b]Molecular Pharmacology, Department of Pharmacy, School of Health Sciences, University of Patras, Greece.

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Correspondence to S. S. Nikolaropoulos, Laboratory of Pharmaceutical Chemistry, School of Health Sciences, Department of Pharmacy, University of Patras, 26500 Rion-Patras, Greece.
Tel: +30 2610 997 723; fax: +30 2610 992 776;
e-mail: nikolaropoulos_psf@mailbox.gr

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Introduction

All-*trans*-retinoic acid (ATRA), an active metabolite of vitamin A, is a differentiating agent increasingly utilized for the treatment of acne, psoriasis, skin photodamage and various malignancies [1–4], including acute promyelocytic leukemia (APL) [5]. ATRA treatment of patients with APL leads to 90% complete remission rate and differentiation of APL myeloblasts to neutrophils, but this differentiation therapy is transient and is commonly followed by relapse within 3–15 months, probably due to the development of resistance to ATRA [6]. Efforts to prevent relapses and to reduce toxicity have included co-treatment with α-tocopherol and the development of synthetic retinoids [7,8]. There is a large number of synthetic retinoids [7,9–12] and conjugates of ATRA with various chemical moieties, such as fenretinide [13,14], some retinamides [15], and conjugates with 3(2 H)-furanone and aniline mustard moieties [16]. Most of these compounds are less toxic than ATRA, but there is no significant enhancement of the anti-tumor activity.

Recent findings imply that the progression of hematological malignancies, like that of solid tumors, is dependent on angiogenesis [17,18]. The blockage of tumor angiogenesis has emerged as an attractive approach for the treatment of cancer [17–19]. Several reports have pointed to an effect of retinoids on angiogenesis [20]. Most studies suggest that ATRA and its derivatives have antiangiogenic effects [21–23]. ATRA interferes with angiogenesis regulatory pathways, suppressing vascular endothelial growth factor, stromelysin, collagenases and transforming growth factor-β [24]. It also positively modulates endothelial cell production of angiogenesis inhibitors TIMP-1 and TIMP-2 [25], and suppresses the basal expression as well as fibroblast growth factor (FGF)- or phorbol ester-mediated induction of cyclooxygenase-2 [26]. There is also evidence that ATRA inhibits the activation of blood coagulation in cancer patients [27]. However, pro-angiogenic effects of ATRA have also been reported both *in vitro* [20,28,29] and *in vivo* [28].

In the present work, we studied the effect of ATRA and three new steroidal ATRA analogs on the proliferation of HL60 leukemic tumor cells *in vitro* and on angiogenesis *in vivo*, using the chicken embryo chorioallantoic membrane (CAM) model. The ATRA analogs were prepared by the

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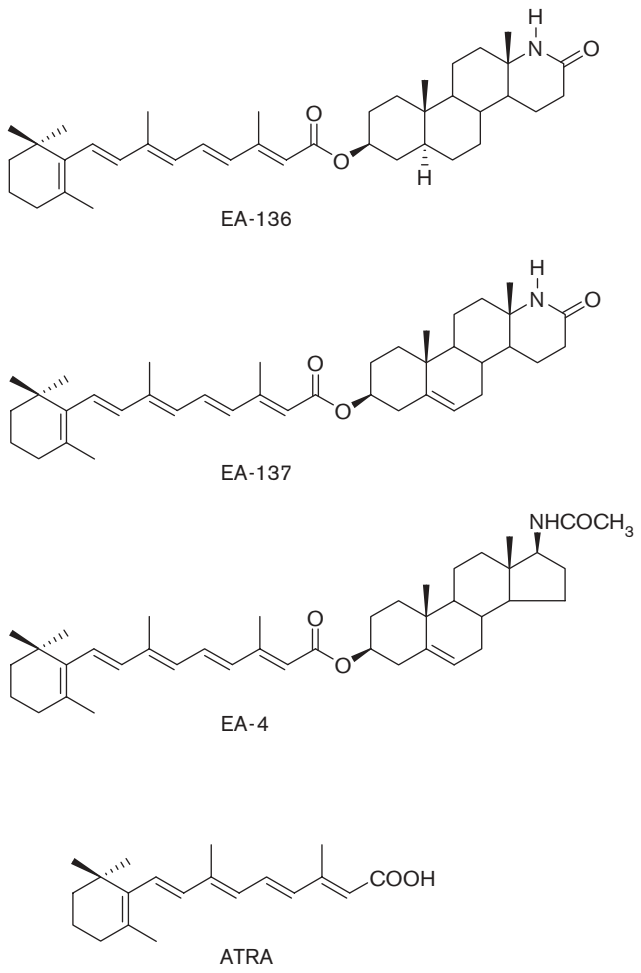
chemical combination of steroidal alcohols with ATRA via an esteric bond. The specific steroidal skeletons were chosen on the basis that these molecules had already been successfully used as carriers for cytotoxic entities, exhibiting reduced toxicity and significantly improved antineoplastic activity [30].

Methods

Synthesis of RA analogs

ATRA was purchased from Sigma (St Louis, MO). For the synthesis of EA-4, EA-136 and EA-137 (Fig. 1), the general method of esterification by using the asymmetric anhydride of ATRA with 2,4,6-trichlorobenzoyl chloride was followed (Fig. 2). According to this method the desired amount of the ATRA (1 mmol) was diluted in dry benzene in a round-bottom flask. 2,4,6-Trichlorobenzoyl chloride (1 mmol) and triethylamine (1 mmol) were added and the mixture was refluxed under argon for 1 h. A solution of the steroidal alcohol (1 mmol) in dry benzene and a catalytic amount of 4-dimethylaminopyridine (4-DMAP) were added to this mixture, and reflux was continued for 3–4 h. The benzene was totally removed by evaporation in vacuum and the remaining oily residue was diluted with DCM. The resulting mixture was extracted with a 5% HCl aqueous solution, the organic layer was washed with a 7% $NaHCO_3$ aqueous solution and finally with water, dried over Na_2SO_4, and the solvent was removed under reduced pressure. The residue was chromatographed on a silica gel column and the ester was isolated after elution with DCM:MeOH (98:2 v/v). The yield of the synthetic procedure for the esteric products varied between 75 and 96%. All the three compounds were re-crystallized from ethyl acetate and their structures were identified using IR, ^{1}H- and ^{13}C-NMR spectroscopy, and elemental analysis of C, H and N. The samples for the biological experiments were purified using HPLC instrumentation and methodology.

Fig. 1



Chemical structure of ATRA and its steroidal analogs.

Cell culture

HL60 cells were from the ATCC (Rockville, MD), and they were routinely cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cultures were maintained at 37°C under 5% CO_2 and 100% humidity.

Cell growth studies

The effect of ATRA and its structural analogs EA-136, EA-137 and EA-4 on HL60 cell growth was determined by directly measuring the number of cells, using a Neubauer hemocytometer and the Trypan blue exclusion assay. HL-60 cells were seeded at 10^6 cells/well in six-well tissue culture plates in complete culture medium. ATRA and its analogs were added to the medium of the cells at the indicated concentrations and the number of cells was determined 24, 48 and 72 h later. Growth inhibitory concentrations of 50% (IC_{50}) were calculated from interpolation of the graphical data.

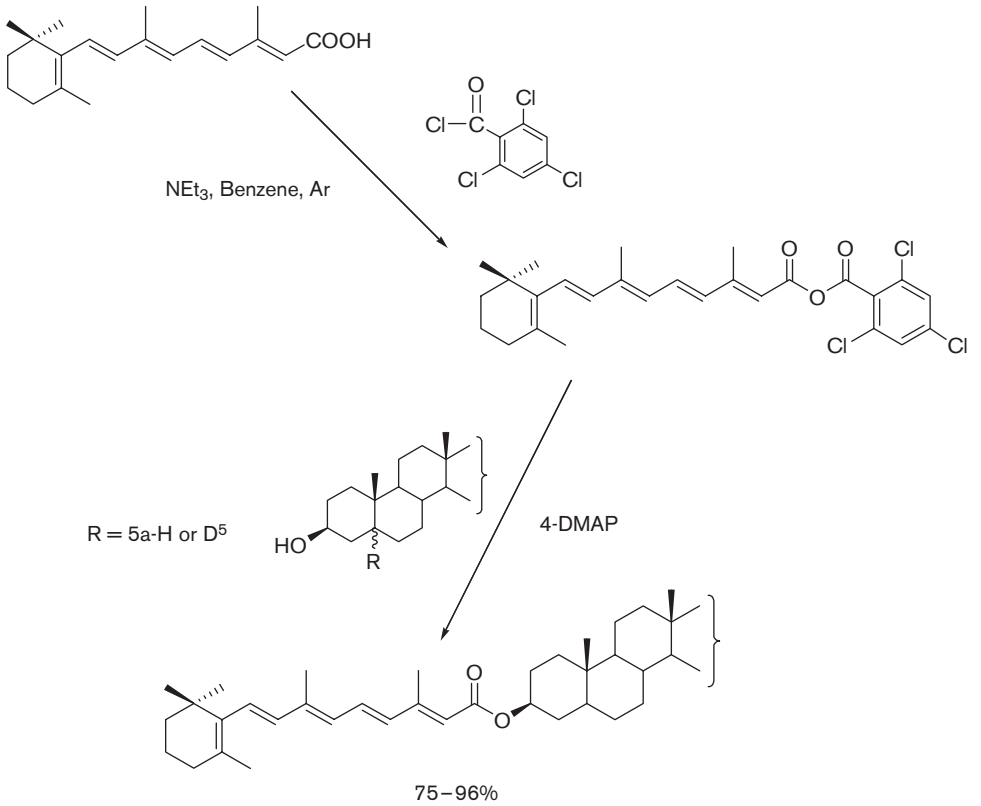
CAM assay

The CAM angiogenesis model was used as previously described [31]. Leghorn fertilized eggs (Pindos, Greece) were incubated for 4 days at 37°C, when a window was opened on the eggshell, exposing the CAM. The window was covered with tape and the eggs were returned to the incubator until day 9, when they were irradiated. The tested agents were diluted in 20 µl H_2O and applied on day 9 of chicken embryo development to an area of 1 cm^2 restricted by a plastic ring. After 48 h of incubation at 37°C, the CAMs were fixed *in situ* with saline-buffered formalin, excised from the eggs, dehydrated, embedded in paraffin, cut, placed on positively charged glass slides and after rehydration stained with standard hematoxylin & eosin staining.

Statistical analysis

The significance of variability between the results of each group and their corresponding controls was determined

Fig. 2



General procedure for the synthesis of ATRA's steroidal analogs.

by unpaired *t*-tests or ANOVA. Each experiment included triplicate measurements for each condition tested, unless otherwise indicated. All results are expressed as the mean ± SEM of at least three independent experiments.

Results

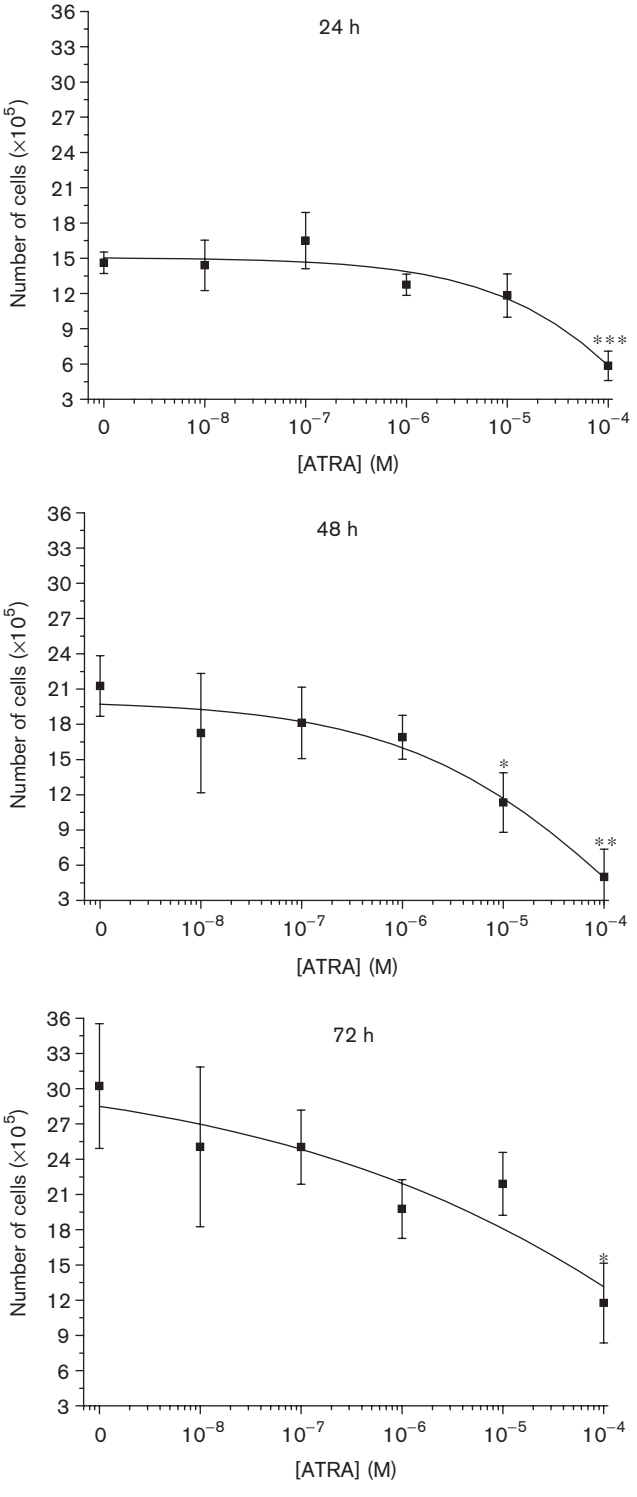
ATRA and its steroidal analogs decreased the number of HL60 cells

We first investigated the effect of ATRA on HL60 cell proliferation, 24, 48 and 72 h after its addition to the cell culture medium. HL60 cells were treated with several concentrations of ATRA (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M) and the number of viable cells was directly measured by Trypan blue exclusion using a standard Neubauer hemocytometer. As shown in Figure 3, ATRA decreased the number of cells in a concentration-dependent manner. The decrease was evident from the first 24 h after addition of ATRA into the culture medium of cells and was statistically significant mainly at a concentration of 10^{-4} M. Higher concentrations could not be used because of solubility problems. The IC_{50} values extrapolated from graphical data in all cases were $2-6 \times 10^{-5}$ M (Table 1).

The retinoid analog EA-137, tested at the same concentrations, had similar effects as ATRA. As shown in Figure 4, EA-137 decreased the number of cells in a concentration-dependent manner. The decrease was evident from the first 24 h after addition of the analog into the culture medium of cells and was statistically significant at concentrations higher than 10^{-5} M. The IC_{50} values extrapolated from graphical data in all cases were $1-2 \times 10^{-5}$ M, a little lower than the corresponding IC_{50} values for ATRA (Table 1).

EA-136 also caused a dose-dependent decrease in the number of HL60 cells, which was also evident from the first 24 h after its addition into the cell culture medium and was statistically significant at concentrations higher than 10^{-5} M (Fig. 5). However, in this case, the extrapolated IC_{50} values were similar or a little higher than the corresponding values of ATRA (Table 1).

Fig. 3



Effect of ATRA on the proliferation of HL-60 cells. Different concentrations of ATRA were added to the culture medium of HL60 cells and after different times of incubation, the number of cells was measured using a standard Neubauer hemocytometer. Results are expressed as the mean ± SEM of at least three independent experiments. Asterisks denote a statistically significant difference (unpaired *t*-test) from control, untreated cells. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table 1 **IC_{50}s (M) of ATRA and its analogs at various time points studied**

Time point (h)	ATRA	EA-137	EA-136	EA-4
24	5.8×10^{-5}	1.8×10^{-5}	10.0×10^{-5}	1.6×10^{-5}
48	5.0×10^{-5}	1.5×10^{-5}	2.3×10^{-5}	2.0×10^{-5}
72	5.5×10^{-5}	1.3×10^{-5}	3.0×10^{-5}	9.0×10^{-7}

The values were calculated from interpolations of the graphical data presented in Figures 3–6 using the Microcal Origin Program. SD values for the IC_{50} values were not calculated because they were estimated from graphical interpolations.

Finally, the analog EA-4 decreased the number of cells in a concentration-dependent manner. The decrease was evident from the first 24 h after addition of the analog into the cell culture medium and was statistically significant at concentrations higher than 10^{-5} M at 24 and 48 h. At 72 h after its addition to the HL60 culture medium, the decrease was significant at concentrations higher than 10^{-7} M and was higher than those observed with ATRA or the other two analogs (Fig. 6). The extrapolated IC_{50} values for EA-4 were $1-2\times10^{-5}$ M at 24 and 48 h and 9×10^{-7} M at 72 h (Table 1).

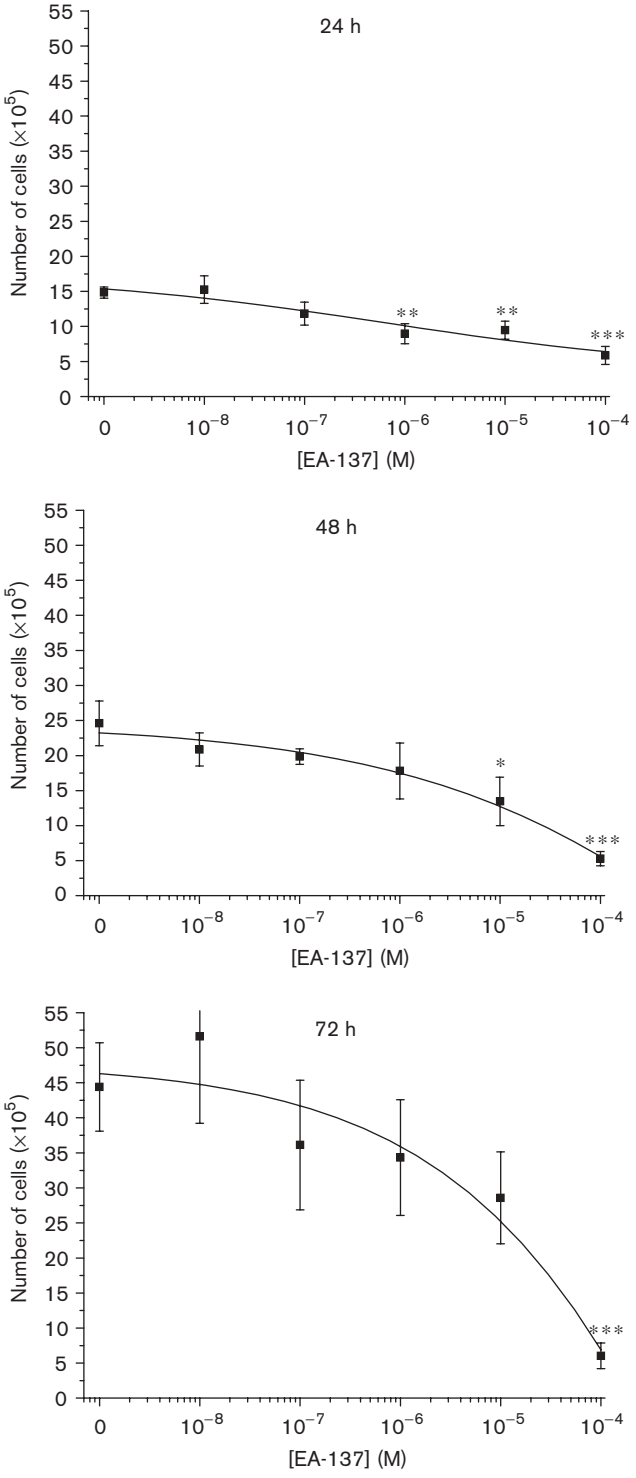
Effect of ATRA and its steroidal analogs on angiogenesis in the chicken embryo CAM *in vivo*

We also studied the effect of ATRA and its analogs on the formation of new blood vessels, using the *in vivo* chicken embryo CAM assay. ATRA or its analogs at a concentration of 10^{-4} M were applied on the CAM as described in Methods. The effect of the tested agents on angiogenesis and on tissue structure was estimated 48 h later in CAM paraffin sections. As shown in Figure 7, ATRA, EA-136 and EA-137 induced angiogenesis in the CAM (Fig. 7B, C and D, respectively) compared to the control, non-treated tissue (Fig. 7A). However, they also increased the number of connective tissue cells (especially ATRA and EA-137), as well as the layer of CAM keratinocytes at the border of the tissue and resulted in a significant degree of extravasation of blood cells. EA-4 had no effect on either angiogenesis or tissue structure in general (Fig. 7E).

Discussion

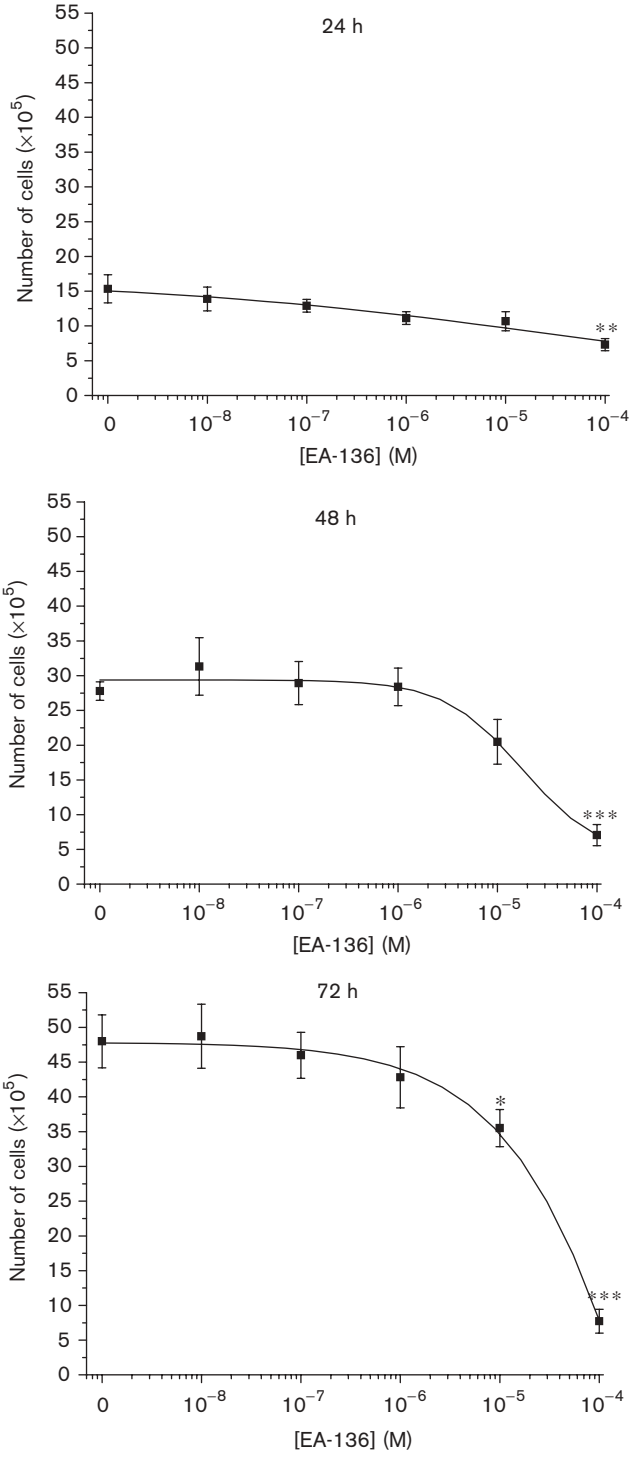
Retinoid and steroid receptors are both members of the same nuclear receptor superfamily [32]. Their activation initiates transcriptional pathways that mediate the activation or inhibition of several cellular events, such as differentiation, apoptosis, DNA or RNA synthesis, proto-oncogene synthesis and cell cycle control. An appropriate combination of ATRA with steroidal molecules could result in some synergy of action and/or decrease side-effects. In the present study, we synthesized three analogs EA-136, EA-137 and EA-4 by chemical conjugation of steroidal alcohols with ATRA via an esteric bond. This choice was based on the observation that steroids can successfully be used as carriers for cytotoxic agents [30], while chemical conjugation of such active molecules is one of the strategies researchers apply in

Fig. 4



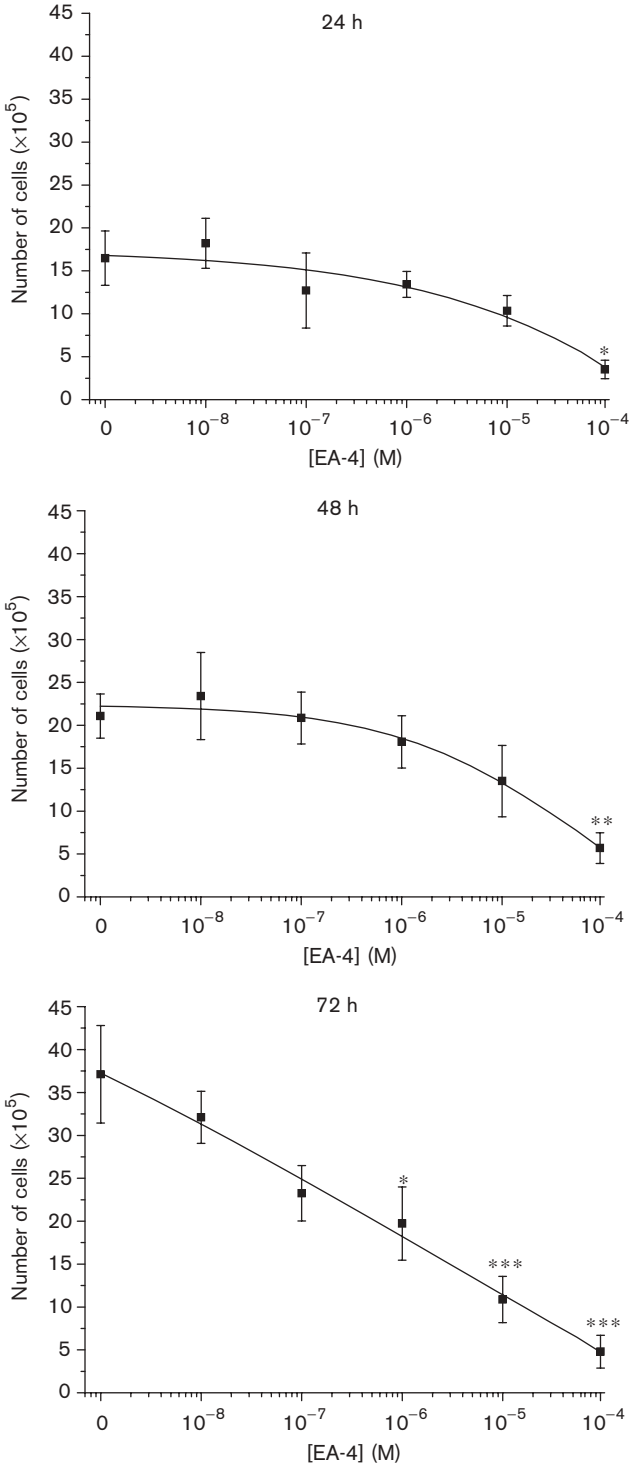
Effect of EA-137 on the proliferation of HL-60 cells. Different concentrations of the analog were added to the culture medium of HL60 cells and after different times of incubation, the number of cells was measured using a standard Neubauer hemocytometer. Results are expressed as the mean ± SEM of at least three independent experiments. Asterisks denote a statistically significant difference (unpaired *t*-test) from control, untreated cells. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Fig. 5



Effect of EA-136 on the proliferation of HL-60 cells. Different concentrations of the analog were added to the culture medium of HL60 cells and after different times of incubation, the number of cells was measured using a standard Neubauer hemocytometer. Results are expressed as the mean ± SEM of at least three independent experiments. Asterisks denote a statistically significant difference (unpaired *t*-test) from control, untreated cells. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Fig. 6



Effect of EA-4 on the proliferation of HL-60 cells. Different concentrations of the analog were added to the culture medium of HL60 cells and after different times of incubation, the number of cells was measured using a standard Neubauer hemocytometer. Results are expressed as the mean ± SEM of at least three independent experiments. Asterisks denote a statistically significant difference (unpaired *t*-test) from control, untreated cells. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

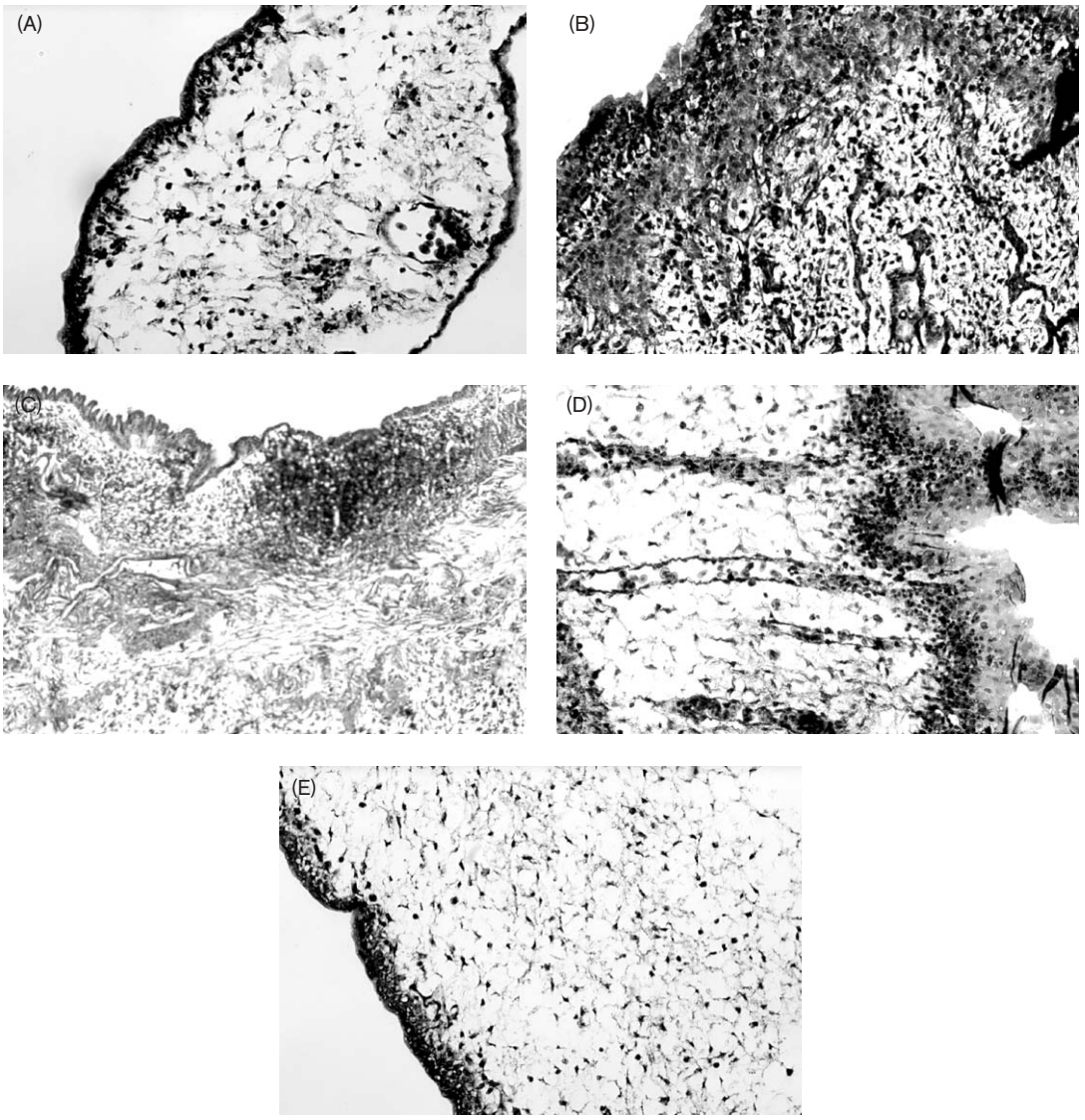
order to reduce toxicity, and increase selectivity and effectiveness towards cancer cells [16]. The specific moieties modified at the D-ring steroidal skeletons (D-lactams in EA-136 and EA-137, and D-amide in EA-4) were shown to be some of the most effective steroidal carriers of cytotoxic agents, e.g. nitrogen mustards. In several cases, they resulted not only in reduced toxicity, but also in significantly improved antineoplastic activity, leading to the suggestion that the existence of the lactamic or amidic NH-CO moiety enhances the activity of the final steroidal esteric derivatives [30,33,34]. In the present work, the amidic NH-CO moiety not only improved effectiveness, but also decreased unwanted effects in the chicken embryo CAM, although it is not yet clear through which mechanism the steroidal part of the molecule affects the effect of ATRA.

ATRA inhibited HL60 cell growth in agreement with several previous studies on the same or different leukemic cell lines [35,36]. Among the analogs tested, only the amidic analog EA-4 was more potent than ATRA for the inhibition of HL60 cell proliferation. In previous studies, it had been shown that the amidic steroid used in EA-4 also resulted in more potent and less toxic derivatives in relation to those derived from the lactamic steroids used in analogs EA-136 and EA-137 [30]. This is in line with the results of the present study.

Angiogenesis seems to be important for the progression of hematological malignancies [24]. ATRA stimulated angiogenesis in the chicken embryo CAM in agreement with previous results in the same *in vivo* system [28]. However, its effect was not specific on blood vessels, since it also increased proliferation of CAM fibroblasts and keratinocytes and induced extravasation and fibrin formation in line with the effect of ATRA-induced bFGF expression in the CAM [24]. The effect of bFGF on the CAM is not confined to the blood vessels, but also affects the mesenchyme, ectoderm and endoderm, leading to a dense fibroblast-rich connective tissue [37]. Moreover, the effects of ATRA on several different cell types have been well documented [38–40] and seem to be the main cause of its side-effects. The analogs EA-137 and EA-136 had similar effects as ATRA, while the analog EA-4 had no effect on angiogenesis, the proliferation of other types of CAM cells, or the structure of the tissue in general.

In conclusion, the retinoid EA-4 seems to be a promising agent for the inhibition of human leukemia cell growth, possibly with fewer effects on other cell types. Combination of ATRA with steroids may be of value for more effective management of cancer. Its amidic steroidal derivative seems to be more effective than the lactamic ones. Further studies are in progress in order to establish the increased effectiveness of the combination of ATRA

Fig. 7



Hematoxylin & eosin staining of CAM paraffin sections 48 h after treatment with ATRA or its analogs EA-137, EA-136 and EA-4. (A) Control, non-treated CAM, (B) CAM treated with ATRA 10^{-4} M, (C) CAM treated with EA-137 10^{-4} M, (D) CAM treated with EA-136 10^{-4} M and (E) CAM treated with EA-4 10^{-4} M. An increase in the number of connective tissue cells and the layer of CAM keratinocytes, as well as a significant degree of extravasation of blood cells can be observed in (B), (C) and (D).

with steroids, the structure–activity relationship of such molecules and their mechanism of action.

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