Urol Res (1999) 27:17–22

ORIGINAL PAPER

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The cytostatic effect of 9-*cis*-retinoic acid, tretinoin, and isotretinoin on three different human bladder cancer cell lines in vitro

Received: 17 July 1997 / Accepted: 26 June 1998

Abstract Retinoids have been shown to have activity in both preclinical and clinical bladder cancer studies but their exact role in its treatment and prevention remains obscure. In this study cytostatic activity of a novel 9-*cis*-retinoic acid (9-*cis*-RA) was compared with two other retinoids: tretinoin and isotretinoin, in three different bladder cancer cell lines: RT4 (well differentiated), 5637 (moderately differentiated) and T24 (poorly differentiated). The three retinoids were incubated at concentrations of 0.3, 3 and 30 μg/ml with bladder cancer cells in microtitre plates for 3 and 6 days. The cytostatic effect was estimated by using luminometric measuring of ATP activity of viable cells in suspension. Compared with the older retinoids, tretinoin and isotretinoin, the highest concentration of 9-*cis*-RA had a cytostatic efficacy in all three bladder cancer cell lines tested. A clear dose–response relationship was observed in isotretinoin-treated cultures after 6 days and in all 9-*cis*-RA-treated cultures. Tretinoin was either ineffective or had a stimulating effect on poorly differentiated tumour cells. To conclude, isotretinoin and 9-*cis*-RA had a cytostatic effect on human bladder cancer cells in vitro. However, the possibility of stimulating cancer growth at small doses, at least with tretinoin, and toxicity at high doses must be considered when planning clinical trials.

Key words Superficial bladder cancer · Bioluminescence · Retinoids · Cancer therapy

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Introduction

Retinoids, a class of compounds structurally related to vitamin A, act by binding to specific receptors and modulating definite gene pathways that ultimately control cell differentiation and development [2]. Some epidemiological studies have suggested that low vitamin A intake or low serum retinoid levels are associated with increased risk of bladder cancer, but other reports have not been confirmatory [14]. Studies using experimental urinary bladder cancer in rats and/or mice have shown that various retinoids have either chemopreventive activity or are inactive against bladder cancer [7]. Retinoids have been shown to have activity in both preclinical and clinical bladder cancer studies [1, 6, 10, 13] but their exact role in treatment and prevention remains to be defined. The most effective retinoid for use in bladder cancer is unknown and toxicity has been a problem and limiting factor for clinical use. Recently more potent and less toxic retinoids have been developed, 9-*cis*-retinoic acid (9-*cis*-RA) being one of the most promising ones due to its high affinity to the retinoid X receptors [6].

In the present study, the direct in vitro cytostatic effects of 9-*cis*-RA were compared with older retinoids, tretinoin (all-*trans*-retinoic acid) and isotretinoin (13-*cis*-retinoic acid), using well-, moderately and poorly differentiated bladder cancer cell strains.

Materials and methods

Cell lines

Following cell lines (from ATCC) were used:

RT4 (HTB-2):	well-differentiated transitional cell papilloma of bladder.
5637 (HTB 9):	moderately well differentiated transitional cell carcinoma of bladder.

T24 (HTB 4): poorly differentiated transitional cell carcinoma of bladder.

The cells were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal calf serum.

Culture conditions

Tretinoin (all-*trans*-retinoic acid), Isotretinoin (13-*cis*-retinoic acid) and 9-*cis*-RA (all three a kind gift from Roche) were used as tumour growth inhibitors at concentrations of 0.3, 3 and 30 μg/ml. All these retinoids were freshly dissolved in absolute ethanol. A volume of 10 μl of each concentration was pipetted on the bottom of each well of the microtitre 96-well plates. On each concentration of the three tested retinoids six parallel cultures were made. The plates were incubated at room temperature until dry. After that cell suspension with desired cell count (15 000 cells/plate) was pipetted into the wells. The cells were incubated in microtitre plates in the incubator for 3 and 6 days. The control plates were treated correspondingly but without retinoids.

The anti-tumour activity was estimated using luminometric measuring of the adenosine triphosphate (ATP) activity of viable cells in a suspension described by Kangas et al. in 1994 [5]. In brief, separate plates for all incubation times are prepared. Drugs and cells (100 μl) are added and incubated under due conditions. At each desired time the plate to be measured is removed from the incubator, 100 μl 1% cold TCA (trichloric acetic acid) is added to the plates and mixed about 15 times with Finnpipette R. Aliquots of 100 μl are transferred to tubes containing buffered ATP monitoring reagent R. After a short vortexing the ATP level is read in a luminometer. ATP monitoring agent R containing purified firefly luciferase and TRIS-HCl buffer (pH 7.75) were needed for the light reaction. TCA was used to destroy the cell membranes and to release the intracellular ATP. Results are expressed as means ± 95% confidence levels. The significance of differences in means between the control and the experimental groups was tested by a two-way analysis of variance.

Results

Microscopically the various cell strains became attached on the culture plates during the experiments.

Tretinoin had not any cytostatic effect on human transitional cell carcinoma line T24, the most malignant cell line (Fig. 1A, B). In the cell line 5637 the smallest concentration of 0.3 μg/ml was ineffective, but concentrations of 3 and 30 μg/ml were cytostatic in both 3- and 6-day cultures ($P < 0.001$). In the cell line RT4, tretinoin was dose-dependently cytostatic in both 3- and 6-day cultures in all concentrations ($P < 0.01$ and $P < 0.001$, respectively).

Isotretinoin was clearly cytostatic in cell line T24 at concentration of 30 μg/ml ($P < 0.001$) and in cell line 5637 at concentrations of 3 and 30 μg/ml ($P < 0.001$) (Fig. 2A, B). In the cell line RT4 isotretinoin was dose-dependently effective at all concentrations ($P < 0.001$).

9-*cis*-RA had a cytostatic effect on cell line T24 at concentration of 30 μg/ml in both 3- and 6-day day cultures (Fig. 3A, B). In the cell line 5637 with concentrations of 3 and 30 μg/ml and in the cell line RT4 with all concentrations, 9-*cis*-RA was dose-dependently cytostatic ($P < 0.001$).

Discussion

Compared with the older retinoids tretinoin and isotretinoin, 9-*cis*-RA had at least an analogous or more potent cytostatic efficacy in all three bladder cancer cell lines tested in our study. This is in accordance with previous reports which have shown 9-*cis*-RA to be also highly effective in other cell cultures [4, 6]. In addition, to binding to RA receptors (RAR), 9-*cis*-RA, which is a stereoisomer of retinoic acid, is a high-activity ligand for retinoid X-receptors (RXR). In contrast, tretinoin interacts 40-fold less effectively with RXR compared with RAR [6]. This might partly explain the lesser effect of tretinoin on the T24 cell line compared with 9-*cis*-RA.

There are only a few reports about the influence of 9-*cis*-RA on cell proliferation and differentiation [2, 4, 6]. To our knowledge, the present study is the first to compare its cytostatic efficacy with that of older retinoids in bladder cancer cell lines. The method of estimating anti-tumour activity we used is based on luminometric measuring of ATP activity of viable cells in the suspension described by Kangas et al. [5]. This method has been proven to be accurate when correlated with changes in cell count and tritiated thymidine incorporation [9]. Furthermore, a similar method has been used to compare the activities of different cytostatics on the Walker 256 carcinosarcoma cell line [9] and the cytostatic effect of different strains of Bacillus Calmette-Guerin alone and in combination with mitomycin C and interferon-α [9].

In the present study, a clear dose–response relationship was observed in all tested retinoids in two of three bladder cancer cell lines. However, in the cell line T24 only the highest concentration of 30 μg/ml of isotretinoin or 9-*cis*-RA was effective while the lower concentrations of tretinoin, isotretinoin or 9-*cis*-RA were either ineffective or even had a stimulating effect on tumour cells. Furthermore, the cytostatic effect of 9-*cis*-RA for 3 days incubation on RT4 was somewhat less than that for 6 days. The reason for this phenomenon needs further experiments but it can be speculated that 9-*cis*-RA might have no time-dependent cytostatic action on the most benign cell line studied. Previously very low concentrations of retinoids have been shown to stimulate proliferation of malignant (leucaemic) cells rather than induce their differentiation [6]. This provokes a question in clinical use whether retinoids in continuous therapy may be metabolized more rapidly with increased urinary excretion, lowering their plasma levels to a range that may stimulate proliferation of tumour cells without inducing their differentiation. Our experimental setup might partly explain these results in that during the initiation of the experiments the cells were not exponentially growing, and despite the fact that the cells attached well as observed microscopically, the retinoids might have an effect on the degree of cell attachment. Further studies are needed to clarify these aspects.

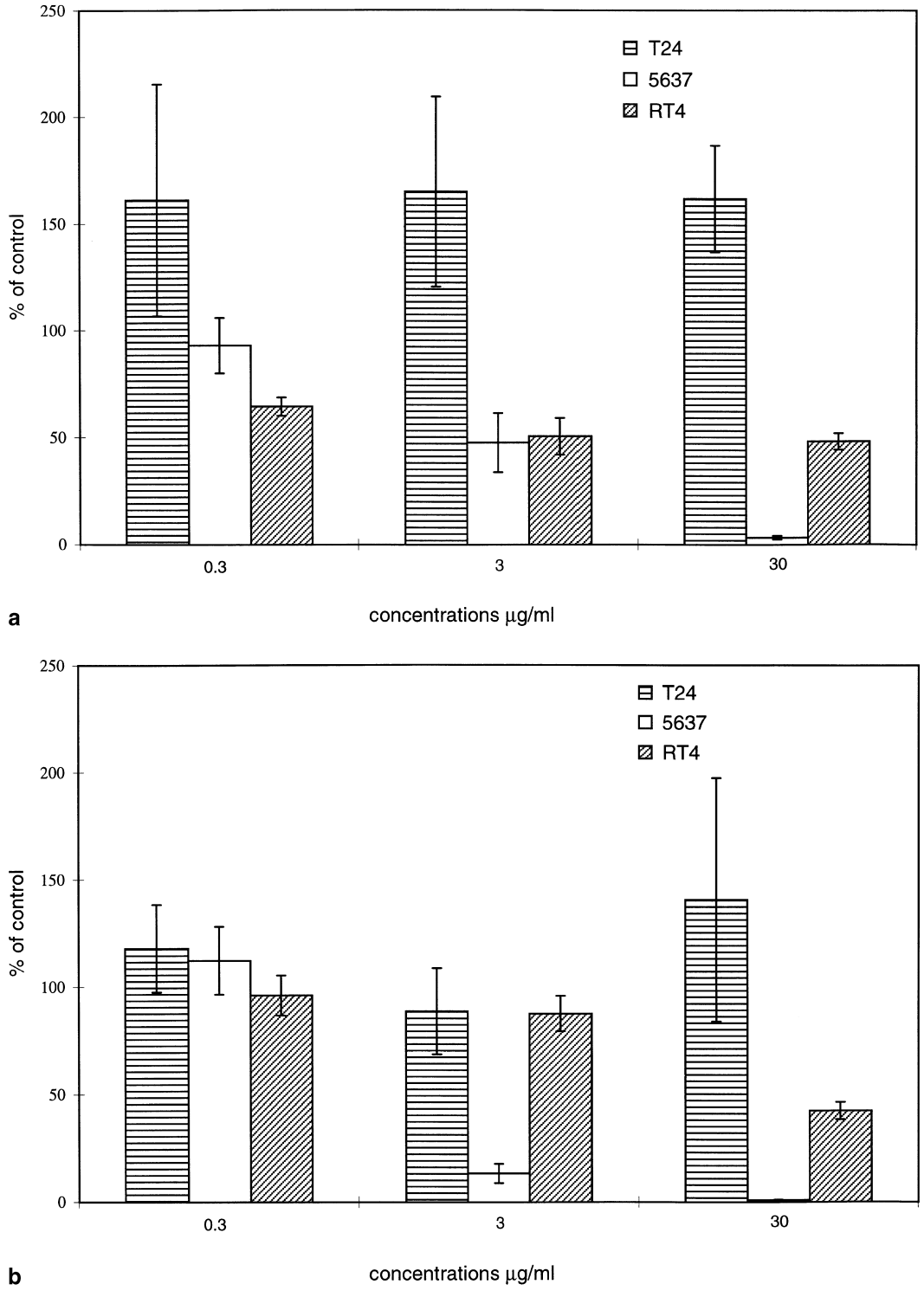

Fig. 1 Cytostatic effect of tretinoin on luminometric measurements of adenosine triphosphate (ATP) activity of viable T24, 5637 and RT4 cells compared with controls in 3-day (**a**) and 6-day (**b**) cultures

Previous clinical studies have given conflicting results of the efficacy and toxicity of retinoids in the treatment of patients with recurrent superficial bladder cancer [1, 3, 8, 11, 15]. The study testing isotretionin was terminated early because of drug toxicity and lack of efficacy [3]. On the other hand, etretinate, which was found to prevent recurrence of superficial bladder cancer, was well tolerated at the final maintenance dose of 25 mg/day [1]. Similar results with etretinate in randomized patients with superficial bladder cancer were observed in a multicenter Swiss study [11, 12], but not in a Danish study including a high-risk population of patients who had experienced at least two recurrences of

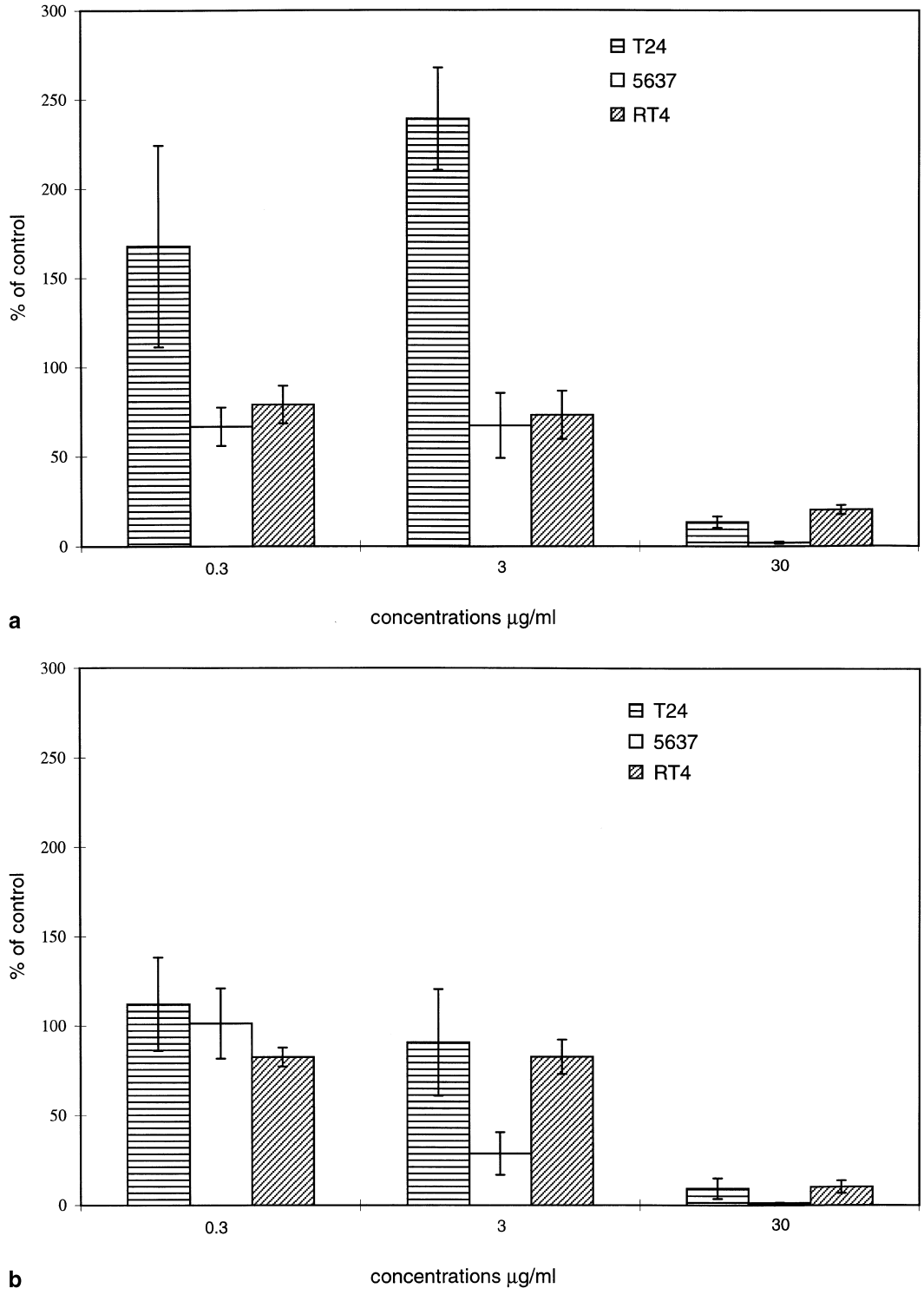

Fig. 2 Cytostatic effect of isotretinoin on luminometric measurements of ATP activity of viable T24, 5637 and RT4 cells compared with controls in 3-day (**a**) and 6-day (**b**) cultures

non-invasive bladder cancer during the preceeding 18 months [8]. Further development of new less toxic retinoids and more studies to test them both in vitro and in vivo are needed.

It is concluded that in bladder cancer cell cultures some retinoids have a clear cytostatic efficacy. The new 9-*cis*-RA, with a wider mechanism of action, looks a promising alternative offering chances of chemoprevention of recurrence of superficial bladder cancer. Further studies are, however, needed to test its efficacy and safety in vivo.

Acknowledgements This study was supported by grants from the Medical Research Council of the Academy of Finland and Turku University Hospital.

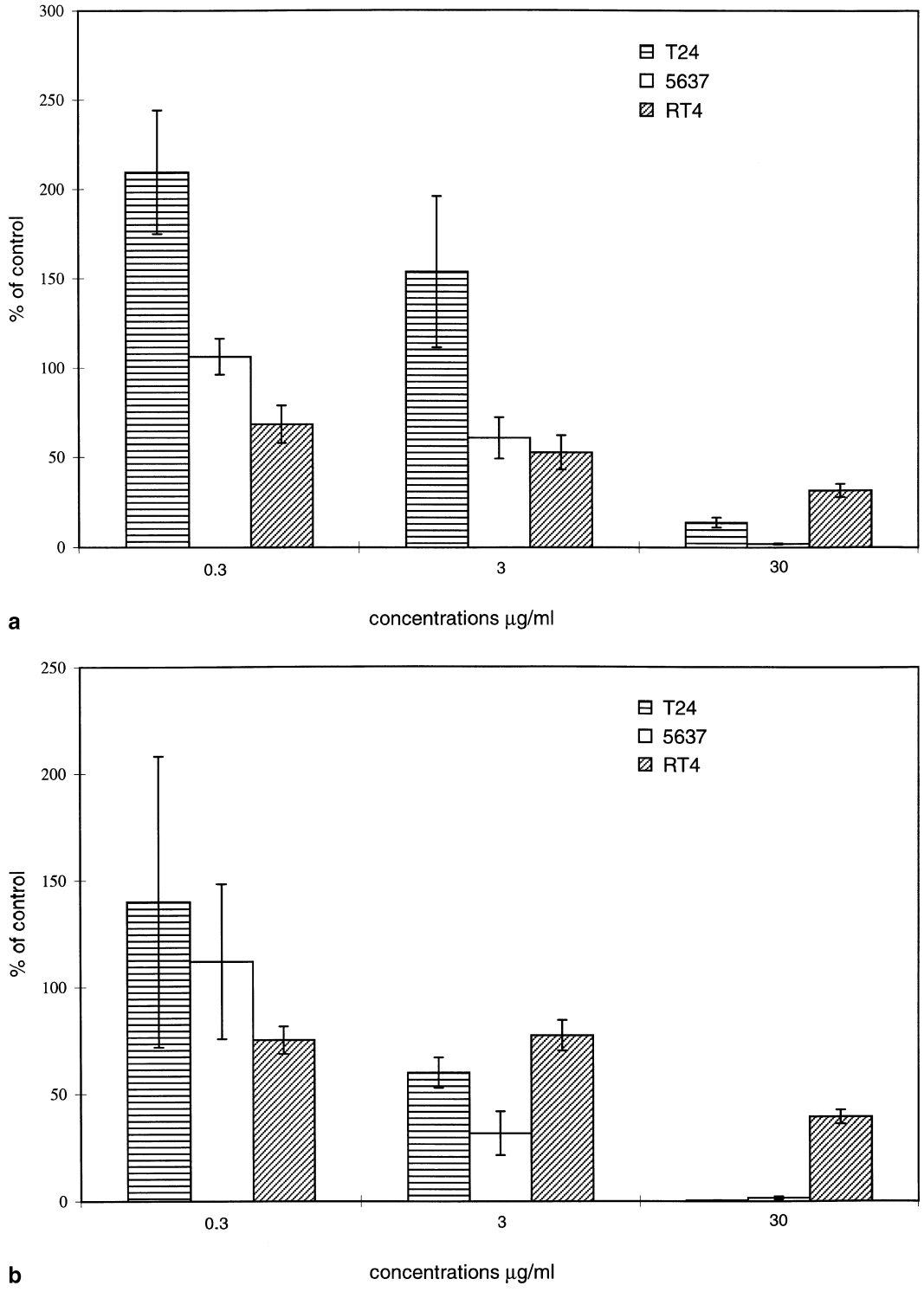

Fig. 3 Cytostatic effect of 9-*cis*-retinoic acid on luminometric measurements of ATP activity of viable T24, 5637 and RT4 cells compared with controls in 3-day (**a**) and 6-day (**b**) cultures

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