Liarozole, an Antitumor Drug, Modulates
Cytokeratin Expression in the Dunning AT-6sq
Prostatic Carcinoma Through In Situ
Accumulation of All-Trans-Retinoic Acid

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ABSTRACT: Liarozole showed antitumoral activity in the Dunning AT-6sq, an andro-
gen-independent rat prostate carcinoma. To investigate its potential mechanism of action,
the effects of the drug doses (ranging from 3.75 to 80 mg/kg b.i.d.) on endogenous plasma
and tissue all-trans-retinoic acid levels and on the differentiation status of the tumor cells
were evaluated. To follow modulation of differentiation, cytokeratins were localized in the
(un)treated tumors by immunocytochemistry and quantitatively determined by immuno-
blotting. Results showed that liarozole statistically significantly reduced tumor weight from
30 mg/kg upwards and induced accumulation of all-trans-retinoic acid both in plasma and
tumors. In the tumors, a statistically significant accumulation was already noted from 7.5 mg
liarozole/kg upwards. Concomitantly, the differentiation status shifted from a keratinizing
towards a non-keratinizing squamous carcinoma, which was further confirmed by the cy-
tokeratin profile of the carcinoma (presence of CK 8, 10, 13, 14, 18, 19). Immunoblotting
revealed an overall decrease in cytokeratin content, except for CK 8. These findings suggest
that the antitumoral properties of liarozole might be related to an increase in the degree of
tumor differentiation through accumulation of all-trans-retinoic acid. © 1995 Wiley-Liss, Inc.

KEY WORDS: liarozole, cytokeratin, all-trans-retinoic acid, prostate, carcinoma

INTRODUCTION

Liarozole is an imidazole derivative with antitumoral properties. It reduces tumor growth in Dun-
nig R3327 rat prostate adenocarcinoma models, including both the androgen-dependent (G and H) and
androgen-independent (PIF-1 and AT-6) tumors [1, 2]. It also inhibits subcutaneous and bone metastatic
umor growth of the androgen-independent PC-3ML-B^2 human prostate carcinoma in SCID mice [3].
ese experimental results have prompted clinical studies in advanced prostatic cancer. An open pilot
study in 42 patients with stage D metastatic prostate carcinoma in clinical relapse after orchiectomy
showed a reduction in PSA levels by at least 50% in half of the patients and a marked improvement in
pain in the majority of evaluated patients. Some remissions were also observed and the PSA responding

Received for publication May 10, 1994; accepted October 31, 1994.
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Abbreviations used: CK, cytokeratins; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography;
Pb, 0.1 M phosphate buffer, pH = 7.4; PSA, prostate-specific antigen; RA, all-trans-retinoic acid; SCID, severe combined immune
deficient mice; SDS, sodium dodecyl sulfate.
patients also tended to have a better survival [4]. Main side effects (dry skin and mucosa) resembled symptoms of hypervitaminosis A. This clinical trial as well as studies in healthy volunteers [5], demonstrated that interference with adrenal steroidogenesis was limited and that no change in adrenal androgen levels could be recorded. The antitumoral effects of liarozone in the androgen-independent rat prostate tumors, as well as in patients relapsing after androgen ablation, suggested the involvement of a non-androgen-related mechanism of action.

Recent data demonstrate that liarozone inhibits the cytochrome P-450-dependent breakdown of all-trans-retinoic acid (RA) [6], and exerts retinoid-mimetic effects in vivo [7]. In rats, liarozone increased the plasma half-life of exogenously administered RA and enhanced the endogenous plasma levels of the retinoid [6]. Also, in vitro analysis demonstrated the synergistic effect of liarozone on the anti-proliferative action of RA in MCF-7 cell culture, most likely through inhibition of RA metabolism [8].

RA is a nuclear transcription factor [9]. The six known subtypes of RA nuclear receptors seem to be differentially expressed in normal and tumor cells, thus explaining specific local effects [10]. In this way, RA triggers differentiation in vertebrate embryonal development [11,12] through multiple control of gene expression [9]. A morphological criterion for differentiation in epithelia is their cytokeratin (CK) expression profile. CKs form the intermediate filament cytoskeleton of epithelial cells [13]. Individual CK expression is cell-type specific, differentiation-dependent [14,15] and regulated by various hormones, including RA [16,17]. Besides their anti-proliferative and differentiation-inducing potencies, retinoids have antitumoral activities [18]. Recently, antitumoral effects of RA were demonstrated in acute promyelocytic leukemia [18], as well as in solid tumors, such as lung cancer, head and neck squamous cell carcinoma, and squamous cell carcinoma of the skin [19].

Consequently, the potential mechanism of the antitumoral activity of liarozone might be linked to an increase of endogenous plasma and/or tissue RA, modulating the proliferation and differentiation status of the tumor. In further exploring this hypothesis, we evaluated the dose-dependent antitumoral activity of liarozone in an androgen-independent prostate Dunning subline, R3327 AT-6sq, focusing on endogenous plasma and tissue RA levels and on the differentiation status of the cells. To follow differentiation modulation, cytokeratins in the (un)treated tumors were localized by immunocytochemistry and quantitatively determined by immunoblotting.

**MATERIALS AND METHODS**

**Animals and Test Compounds**

Seventy-two adult male F1 hybrid rats (Fisher female × Copenhagen male) were obtained from the breeding colony maintained at the Janssen Research Foundation, Beerse, Belgium. The rats were kept in a humidity- and temperature-controlled room with a 12 hr-light, 12 hr-dark schedule, and received a powdered diet, and tap water ad libitum. Liarozone (5-[3-chlorophenyl](1H-imidazol-1-yl)methyl]-1H-benzoimiazole) monohydrochloride was dissolved in 20% polyethylene glycol and administered by gavage, twice daily.

**Subcutaneously Implanted R3327 AT-6sq Tumor**

The R3327 AT-6sq tumor arose in a castrated male rat grafted with the parental R3327 H tumor. Tumor fragments of approximately 1 mm³ obtained under aseptic conditions from a subcutaneously grown R3327 AT-6sq tumor, were implanted subcutaneously in the dorsal flank of the rats under light ether anesthesia. The animals were allowed to recover, replaced in their cages, and then remained undisturbed until the tumor reached approximately 0.4 cm³. The animals were then randomly assigned to one of the experimental groups each comprising 8 to 10 rats. At day 0, treatment was initiated: castration, vehicle or decreasing dose of liarozone (80, 60, 30, 15, 7.5, and 3.75 mg/kg b.i.d.). Experimental details were reported in a previous article [2]. On day 35 of treatment, all animals from each treatment group were sacrificed by decapitation, exactly 3 hr after morning treatment. Tumor weights were recorded and the tumors partly frozen in liquid nitrogen for the measurement of RA concentrations or partly fixed for histological analysis (see further). Blood samples were collected in dark tubes containing 50 IU heparin. Blood was immediately centrifuged at 4°C, plasma was divided into two tubes and stored at −20°C until use in the RA assay.

**Quantification of RA in Blood Plasma**

RA was extracted from blood plasma and quantified by UV absorbance after HPLC separation as described previously [6]. Briefly, plasma samples (1 ml) were diluted with 1 ml water and mixed with 18 ml of acetonitrile to precipitate proteins. After centrifugation (1,000g, 5 min), supernatants were recovered, diluted with 18 ml of 40 mM acetic acid and passed through 100 mg Bond Elut C18 minicolumns (Analytchem International, Harbor City, CA). After washing the minicolumns with 4 ml of 40% acetonitrile in water, the absorbed material was eluted with 3 ml of
methanol. The eluates were then evaporated in vacuo and the residues dissolved in 200 μl of mobile phase for reverse-phase HPLC analysis. Samples (150 μl) were analyzed on a 10 μm C18 Bondapak column (3.9 × 300 mm, Millipore, Bedford, MA). The mobile phase consisted of methanol-water (85:15) containing 30 mM ammonium acetate and flowed at a rate of 1 ml/min. The effluent was monitored by UV absorbance detection at 350 nm and RA (retention time 5–6 min) was quantified by peak-height measurement. A standard curve was prepared by extracting rat plasma to which known amounts of RA had been added. The detection limit was 0.5 ng/ml with an absolute recovery of 80 to 85% over a concentration range of 0.5 to 200 ng/ml.

**Quantification of RA in Tumoral Tissue**

Tumoral tissue (500 mg) was cut into small pieces with scissors and homogenized with an Ultraturrax (Janke und Kunkel, GMBH & Co., Staufen, Germany) homogenizer in 2 ml of ice-cold 0.9% saline containing 0.05% EDTA and 0.05% ascorbic acid. The homogenates were then diluted with 1 ml of water and mixed with 27 ml of acetonitrile to precipitate proteins. After centrifugation (1,000g, 5 min), supernatants were recovered, acidified below pH 3 with 2 ml of 18 N acetic acid and diluted with 35.5 ml of water to reduce the acetonitrile concentration to 40%. The mixtures were passed through 500 mg Bond Elut C18 minicolumns and processed as described for plasma samples [6], except that RA was quantified by peak height measurement. A standard curve was prepared by extracting control tumor tissue to which known amounts of RA had been added. The detection limit was 0.5 ng/500 mg. Recovery for the extraction procedure following tissue homogenization was ± 60%.

**Histological and Immunocytochemical Examination of AT-6sq Tumors**

**Fixation and staining protocol.** Tumor fragments were either frozen in isopentane cooled on dry ice or fixed in 4% paraformaldehyde in PB. Fixed tissue was included in JB4 (Polysciences, Warrington, PA); 2 μm sections were stained with haematoxylin and erythrosin or with the Gomori one-step trichrome method. Automatic determination of areal densities of epithelial tissue. The areal densities of epithelial tissue and total surface of the tissue sections were determined by means of automated image analysis [20]. Briefly, the system consisted of a light microscope (objective × 10), a Plumbicon black-and-white camera and a Quantimet 900 image analysis computer (Cambridge Instruments, Leica, Paris, France). The object stage of the microscope was automatically steered in three dimensions. In this way, complete tissue sections (n = 3 per tumor; 8–10 tumors examined per treatment group), circumscribed by coordinates which were introduced in the computer, could be automatically scanned. The mean densitometrical values, corresponding to the total number of epithelial cells, could then be calculated for each tumor and was expressed as densitometrical value per μm². To relate these data with the data of densitometry of immunoblotting experiments, the data of the treated samples were pooled as follows: tumor samples of 80 and 60 mg/kg treatment, tumor samples of 30 and 15 mg/kg treatment, 7.5 and 3.75 mg/kg treatment.

**Immunocytochemical localization of intermediate filament proteins.** Frozen sections of tumor tissue (5 μm thick) were cut on a cryostat and air-dried before fixation in methanol (−20°C, 5 min) and aceton (three times, 5 sec) according to Verhagen et al. [21] and Ramaekers et al. [22]. After being washed in PB for 10 min, the tissue sections were incubated with 0.1% lysozyme (Sigma, St. Louis, MO) in PB at room temperature (30 min), and subsequently overnight with the primary monoclonal antibody. Antibodies and their respective dilutions are listed in Table I. Their corresponding epitopes are reviewed by Ramaekers et al. [14]. All antibodies were obtained from Ramaekers et al., except for RPN166 (Amersham, Buckinghamshire, UK). After washing in PB (three times, 10 min), the slides were incubated with the peroxidase-conjugated goat anti-mouse IgG (1:20, 30 min) (Janssen Biochemica, Beerse, Belgium). The antigens were visualized with 3,3'-diminobenzidine (1.5 mg/ml) and H2O2 (1%) as the chromogen and substrate, respectively. Replacing the first antibody by PB served as the control and did not reveal any signal.

**Extraction of Intermediate Filament Proteins, Gel Electrophoresis, and Immunoblotting**

Instead of scoring the immunocytochemical reaction, a more accurate quantification of the intermediate filament proteins, CKs and vimentin, was performed by means of densitometrical analysis of immunoblots. Because the remainder of the tumor samples from each group of the liarozole treated animals was not sufficient for the intermediate filament extraction, tumor pieces were pooled to obtain 250 mg tumor material. Tumor specimens from 80 mg/kg treatment were pooled with those from the 60 mg/kg treated rats, those from 30 mg/kg with those from the 15 mg/kg treated rats and those from 7.5 mg/kg with those from the 3.75 mg/kg treated rats. Tumor material of the orchietomized group and of the control groups were obtained in sufficient quantities to be analyzed separately. The extraction of 250 mg tumor
TABLE 1. Monoclonal Antibodies Used for Immunochemical Localization of Cytoke-ratin and Vimentin

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution for immunocytochemistry</th>
<th>Dilution for immunoblot</th>
<th>Ig subtype</th>
<th>Intermediate filament protein recognized</th>
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<td>1/25</td>
<td>IgG1</td>
<td>CK 4</td>
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<td></td>
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<tr>
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<td>1/10</td>
<td>IgG1</td>
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<td>1/4</td>
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<td>CK13</td>
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Fig. 1. Effect of liarozole treatment b.i.d. on growth of R3327 AT-6sq rat prostate carcinoma as determined by tumor weight. Results are expressed as mean ± SEM. Asterisks indicate statistically significant difference (P < 0.05) versus control.

from each of the control, orchiectomized, and liarozole treatment groups (80 and 60 mg/kg, 30 and 15 mg/kg, 7.5 and 3.25 mg/kg) was done according to Achtsaetter et al. [23]. All extraction buffers contained as protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml pepstatin, 2 µg/ml aprotinin, 2 µg/ml leupeptin. The samples were diluted 1/4 with SDS-electrophoresis sample buffer, boiled for 5 min and analyzed on a 7.5% SDS-polyacrylamide gels [24]. Molecular weight markers were: rabbit skeletal muscle myosin MW 200,000; E. coli beta-galactosidase MW 116,250; rabbit muscle phosphorylase B MW 97,400; bovine serum albumin MW 66,200; hen egg white ovalbumin MW 45,000 (Bio-Rad, Richmond, California). After the run, the proteins were transferred to polyvinylidene difluoride membranes using a semi-dry blotting apparatus. Total protein staining

Fig. 2. Effect of liarozole treatment on the retinoic acid concentration in blood plasma (a) and in R3327 AT-6sq rat prostate carcinoma (b). Results are expressed as mean ± SEM. Asterisks indicate statistically significant difference (P < 0.05) versus control.

on a duplicate blot was done with AuroDye™ (Amersham, Buckinghamshire, UK). Immunodetection with the different antibodies was done with the primary antibodies and their respective dilutions (Table 1) and using the Protoblot™ Western Blot AP Detection System (Promega, Madison, WI).

The immunodetected bands were quantitated by digitizing the blots with a RX5 video camera attached to a Macintosh (Apple, Brussels, Belgium) IIFX computer. Integration of the density was done with the Image 1.28c software (NIH, Bethesda, MD). All sig-
Fig. 3. Effects of liarozole treatment on the histology of R3327 AT-6sq rat prostate carcinoma. In control tumors (a) and in tumors from castrated animals (b) a high degree of keratinization was noted in the centre of the epithelial cell clusters; at the highest doses of liarozole, 80 mg/kg (c), almost no squames were detectable. The presence of squames increased gradually with lower doses of liarozole going from 60 (d), 30 (e), 15 (f), 7.5 (g), to 3.75 (h) mg liarozole/kg. In all tumors a squamous growth pattern was observed.
nals were corrected for background. The densitometrical values were expressed as the percentage of control tumors relative to the amount of epithelial cells.

**Statistical Analysis**

Data from tumor weight, plasma and tumor RA concentrations, and number of epithelial cells, were analyzed for significance by the Mann-Whitney U-test (two-tailed) using the Stat View II software (Abacus Concepts, Inc., Berkeley, CA) with all references to statistical significance being at least at the $P \leq 0.05$ level.

**RESULTS**

**Effects of Liarozole on Tumor Weight (Fig. 1), Plasma, and Tumor RA Concentrations (Fig. 2)**

Liarozole reduced mean tumor weight at all doses, with a minimal inhibition of 77% or more at 30, 60, and 80 mg/kg b.i.d. (Fig. 1). The level of significance was reached after administration of 30 ($P = 0.020$), 60 ($P = 0.010$), and 80 mg/kg b.i.d. ($P = 0.018$). Castration resulted in a non-significant increase of tumor weight. Mean increase of size was 52%. Plasma RA concentrations significantly increased up to three-fold, above the detection limit (0.5 ng/ml) 3 hr after the last administration of 30, 60, and 80 mg liarozole/kg (Fig. 2a). In the tumors, a dose-related accumulation of RA was observed, from 7.5 mg/kg b.i.d. upwards, and up to 6-fold above the detection limit (1 ng/g) after 80 mg/kg (Fig. 2b). In castrated and control animals, plasma and tumor, RA concentrations were not above detection level.

**Effect of Liarozole on the Morphology of the Dunning AT-6sq Tumor (Fig. 3)**

In vehicle-treated and castrated rats, the grayish-white to pale yellow AT-6sq tumors were indurated by the presence of keratinized pearls. Microscopic examination revealed a typical keratinizing squamous cell carcinoma type of differentiation (SQC) (Fig. 3a,b). Tumor cells were arranged in epithelial cell clusters, with intermittent fibrous tissue and irregular tissue architecture. In the center of all epithelial cell clusters, squamous pearls were abundantly present. As in a typical squamous epithelium, several cell layers were distinguished: a basal cell compartment in contact with the underlying tumor stroma and several above lying cell layers resembling a stratum spinosum (Fig. 3a,b). The central layers of the epithelial cell clusters were comparable to cells of a stratum granulosum with varying degrees of squame muration (Fig. 3a,b). In the centre of the epithelial cell clusters, pure squames are visible which correspond with the macroscopically apparent keratinized pearls. Although the tumors from castrated animals were larger (+52%), the relative amount of epithelial cells was smaller (77% of the epithelial cell number in control tumors) though not statistically different when compared to control tumors (Table II).

In both treated and untreated tumors, necrotic cells were only occasionally observed. Treatment with liarozole did not induce apoptosis based on the absence of typical cellular or nuclear fragmentation. Liarozole dose-dependently reduced desquamation (Fig. 3c–h). At the higher doses of liarozole (60–80 mg/kg b.i.d.) almost no squames were present. The tissue architecture did not change upon treatment; 80–60 mg/kg treated tumors still had a SQC nature (Fig. 3c,d). As in control tumors, a cell layer, with the typical characteristics of a stratum granulosum, lined the inner core of the epithelial cell clusters. In treated tumors, the relative amount of epithelial cells was slightly lower though not statistically different from that in control tumors (Table II).

**Effect of Liarozole on CK Expression in the Dunning AT-6sq Tumor as Detected by Immunocytochemistry (Fig. 4)**

In control tumors, the basal cell layers were positive for CK 8 (Fig. 4a). The squames, abundantly present in centres of the tumor epithelial cell clusters, were strongly positive for CK 10 (Fig. 4c). CK 13 was present in the suprabasal layers (Fig. 4e) while the suprabasal layer close to the inner core was immunopositive for CK 14 (Fig. 4g). No clear immunopositive staining for CK 18 (data not shown) and CK 19 (Fig. 4i) was observed. Expression of CKs in tumors from castrated animals was comparable with the tu-
Fig. 4. Effects of liarozole treatment on CK immunopositivity. No difference was noted for CK 8 between the control tumors (a) and tumors treated with 80 mg liarozole/kg (b). However, CK 10 was heavily expressed in control tumors (e) in contrast to tumors treated with 80 mg liarozole/kg (d). CK 13 and 14 were present in few cell layers of the different epithelial cell clusters of the control tumors (CK 13, e and CK 14, g), while more cell layers were stained in the 80 mg/kg treated tumors (CK 13, f and CK 14, h). At the lowest doses of liarozole, 2.75 mg/kg (i), many faint immunopositive cells for CK 19 were noted in contrast to control tumors (i) which did not show a clear-cut positive signal.
mors from control rats, but the tumor cells from castrated rats expressed vimentin in addition.

In tumors treated with 80 and 60 mg liarozone/kg, CK 8 expression (Fig. 4b) was not different from control tumors. In the tumors from liarozone treated rats, few CK 10 positive cells (Fig. 4d) were found and, when present, staining intensity was weak, in contrast to control tumors. The CK most typical for a keratinizing squamous cell carcinoma, namely CK 10 was suppressed with doses of liarozone between 15 and 80 mg/kg. At the higher doses (80 and 60 mg/kg), more cell layers in the individual epithelial cell clusters were positive for CK13 and CK 14 (Fig. 4f, h) as compared to tumors from control animals (Fig. 4e, g). The lower doses (7.5 and 3.75 mg/kg) had clearly less anti-keratinizing effects. At these lowest doses of liarozone, CK 18 (data not shown) and CK 19 were expressed in many epithelial cell clusters but with low immunostaining intensity (Fig. 4j).

CK 4 was only occasionally expressed in both untreated and treated tumors. Positivity for CK 4 could not be related to that of CK 13. Briefly, treatment with liarozone resulted in a seemingly higher number of cells immunostained for CK 13, 14, 18 (data not shown), and 19, while the number of cells immunopositive for CK 10 decreased.

**Effect of Liarozone on CK Levels in the Dunning AT-6sq Tumor as Detected by Densitometry of Immunoblots (Fig. 5–7)**

Total protein staining revealed changes in both low and high molecular weight intermediate filament proteins (Fig. 5). Densitometrical values of the respective bands in immunoblots (Fig. 6) were expressed as percentage of the same protein band in the control tumors and relative to the respective number of epithelial cells (Fig. 7). Liarozone treatment induced an inhibition of CK 10, 13, 14, and 19, while expression of CK 8 did not show many differences. Vimentin in tumors from orchietomized animals was found in both epithelial and stroma cells, and thus could not be related to the amount found in control tumors.

**DISCUSSION**

The present study confirms the antitumoral activity of liarozone in a Dunning AT-6sq prostate adenocarcinoma model, when dosed by oral gavage twice daily. This is in keeping with results calculated from tumor volumes and growth curves [2]. Furthermore, the present article demonstrates an increase in plasma and tumor RA levels after chronic liarozone treatment. At the same time, a down-regulation in CK expression was noted in tumor tissue with a shift in differentiation status from a keratinizing towards a non-keratinizing squamous epithelium.

The liarozone-induced increase of RA has been described before in a rat model of vaginal keratinization [7]. At 5–20 mg liarozone/kg, once daily for 3 days to ovariectomized rats with estrogen-induced vaginal keratinization, liarozone administration resulted in increased RA plasma levels with concomitant desquamation. This dekeratinization has been described to be a typical RA effect [25]. Also, in the present study, the most obvious histological difference after liarozone treatment was a dose-dependent decrease in desquamation as reflected by the absence of keratinized squames and a strong decrease in CK 10 expression.

Liarozone treatment did not induce apoptosis as defined by morphological shrinkage of the cells and nuclear fragmentation. Thus, the presently observed decrease in tumor volume and weight does not seem to be induced by programmed cell death but rather by slowing down of the cell cycle.

The keratinized squamous cell carcinoma character
of the untreated AT-6sq tumors was proven by the expression of, in descending order of prevalence, CK 10, 14, 13, 8, 19. Our results confirm previous studies that monoclonal antibodies against human cytokeratins do cross-react with rat cytokeratins [26]. The AT-6sq CK profile is typical for a squamous carcinoma [27]. This CK profile reflects that CK profiles are more related to the type of tumor tissue differentiation rather than to the original normal tissue architecture [28]. As such, it is not remarkable that the AT-6sq squamous tumors do not express the CKs of simple or complex epithelia in the same proportions as their cells of origin, in this case rat prostatic epithelium. In human prostate, the epithelium is characterized by CK 7, 8, 18, 19 in the luminal cells and by CK 5 and 15 in the basal cells [21, 29, 30]. The CK pattern in rat epithelium is comparable to that of the human system, but less well specified [21, 22].
Although liarozole treatment induced a shift in CK expression as revealed by immunocytochemistry, the CK pattern remained typical for a squamous cell carcinoma. However, the presence of many CK19 positive cells in the different cell layers, though weakly stained, could be indicative for differentiation towards adenocarcinoma [31]. CK 14 was however present in any tumor irrespective of treatment and is generally considered an indicator of keratinocyte-like cells or stem cells, being absent from simple luminal epithelial cells [32, 33]. Moreover, the stratum granulosum-like cells remained clearly visible after liarozole treatment.

As noted in other types of squamous cell tumors, expression of CK 4 was not related to the presence of CK 13, as was also the case for CK 8-immunopositivity in relation to the presence of CK18 and CK19. It seems that although these CKs, normally, form heteropolymers by the association of one acidic CK and one basic type CK, in certain cancers these typical combinations might not exist [34].

Coexpression of CKs and vimentin as noted in carcinoma cells from AT-6sq tumors in castrated animals, may be indicative for a relatively high malignant nature of the tumor cells, as also suggested before in other tumor systems [35–37]. Although principally androgen-independent, the AT-6sq tumors from castrated animals are always slightly (but not significantly) larger than those growing in non-castrated rats, reflecting, in addition, the more aggressive character of these tumors.

In the tumor cells, liarozole induced a shift in CK profile and inhibited CK expression. The induced shift in differentiation by liarozole as determined by differences in amount and type of CKs, densitometric quantification of immunoblots was preferred, rather than scoring the immunocytochemical reaction. This approach was chosen in particular since liarozole treatment induced a decrease in the number of epithelial cells relative to the stroma. Although this decrease was not significant, it might explain some discrepancies between the amount of CK as determined by densitometry on the one hand, and scoring the number of immunopositive cells on the other hand. Other differences might be related to epitope exposure, being more masked in the tissue sections than in the immunoblots. For example, in control tumors no CK 18 and CK 19 immunopositive cells were seen, while most cell layers in tumors treated with the lowest doses of liarozole (7.5 and 3.75 mg/kg) were weakly positive. However, immunoblotting revealed a decrease in CK 19 content in liarozole treated tumors as compared to control tumors.

The modulation of CK expression by retinoids in human epidermal squamous carcinoma cells and in normal epithelial cells seems to be differentially regulated [38, 39]. It has been shown that in normal epidermal cells, retinoids do increase CK19 levels while it down regulated this CK type in squamous carcinoma cells. This opposite action might be explained by differentially expressed retinoid receptors [39]. A down-regulation of CK in tumor cells, more specifically in squamous cell carcinoma, is considered as an induction of differentiation by some authors [17, 40]. It has been shown in neuroblast cell division systems that following withdrawal of growth factors, neuronal cells either can be directed towards apoptosis or, alternatively, can differentiate into mature neuronal cells [41]. Thus, it is likely to assume that liarozole arrested AT-6sq growth by inducing a differentiation shift towards a non-keratinizing squamous carcinoma with a concomitant down-regulation of CK expression.

In conclusion, this study demonstrates that the anti-tumor activity of liarozole in the Dunning AT-6sq prostate model was accompanied by tumor RA accumulation and by changing the tumor differentiation status from a keratinizing towards a non-keratinizing squamous cell carcinoma with a concomitant decrease of CK content.

ACKNOWLEDGMENTS

The authors thank Dr. W. Wouters for his critical review of the manuscript. We also thank I. Gevers for secretarial help, B. Xhonneux, M. Adriaensen, B. Janssens, M. Callens, J. Goosens, and W. Cools for their
technical assistance and L. Leijssen and his colleagues for the photographic layout.

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