Cell biology of retinoids in cancer : the modulating role of liarozole-fumarate

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Cell biology of retinoids in cancer

Cell biology of retinoids in cancer **the modulating role of liarozole-fumarate**

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. Dr. A.C. Nieuwenhuijzen Kruseman,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 19 februari 1998 om 14.00 uur
door

Jimmy Van heusden

geboren op 27 april 1971 te Wilrijk

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Voor mijn ouders

Voor Danny

Voor Inge

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Abbreviations

APL	acute promyelocytic leukemia
ATRA	all- <i>trans</i> -retinoic acid
BrdU	5-bromo-2'-deoxyuridine
CARD	catalyzed reporter deposition
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
DAPI	4'-6-diamidine 2-o-phenylindole
DCC-FBS	dextran-coated charcoal-treated fetal bovine serum
DMEM	Dulbecco's modified Eagle's medium
DR	direct repetition
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
IEF	isoelectric focusing
LRAT	lecithin:retinol acyltransferase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2 <i>H</i> -tetrazolium bromide
N-CAM	neural-cellular adhesion molecule
NADPH	reduced form of β -nicotinamide adenine dinucleotide phosphate
PBS	phosphate buffered saline
PI	propidium iodide
PLL	poly-L-lysine
PPAR	peroxisome proliferator-activated receptor
PSA	prostate specific antigen
RA	retinoic acid
RalDH	retinal dehydrogenase
RAR	retinoic acid receptor
RARE	retinoic acid responsive element
RBP	retinol-binding protein
REH	retinyl ester hydrolase
RoDH	retinol dehydrogenase
RT	room temperature
RXR	retinoid X receptor
SEM	standard error of the mean
S.E.M.	scanning electron microscopy
SD	standard deviation
SDS	sodium dodecyl sulfate
TR	thyroid hormone receptor
Tris	Tris(hydroxymethyl)-aminomethane
TSA	tyramide signal amplification
TTR	transthyretin
VDR	vitamin D receptor

1

General introduction

Retinoids are a group of natural and synthetic compounds that share structural and/or functional similarity to vitamin A. Vitamin A (retinol) has long been recognized as an indispensable nutrient, playing a central role in many biological processes such as vision, reproduction, and immune function. Besides retinoids are well-known to modulate cellular growth and differentiation. Therefore it is not surprising that retinoids are being evaluated for use in the treatment of cancer, aiming at reversing the process of malignancy by inducing differentiation and inhibiting uncontrolled growth. All-*trans*-retinoic acid (ATRA), a metabolite of vitamin A, is probably the most potent naturally occurring retinoid. Clinical successes with ATRA in differentiation therapy are however limited. One explanation for this limited success is the rapid emergence of resistance to the drug, believed to result from increased ATRA catabolism. Inhibitors of ATRA catabolism might therefore prove valuable in the treatment of cancer.

cycle of hydrolysis and re-esterification before storage in the stellate cells. When needed, the stored retinyl esters are hydrolyzed to retinol and secreted from the liver in association with retinol-binding protein (RBP). In the blood plasma, RBP-retinol complexes to transthyretin (TTR) and serves as a source of retinoid for extrahepatic tissues. Also extrahepatic tissues have been shown to play a significant and important role in the storage and mobilization of retinol. As such they may contribute to vitamin A homeostasis (Soprano and Blaner, 1994).

Tissue uptake of retinol

The mechanism by which retinol is taken up by target cells remains to be elucidated (Soprano and Blaner, 1994). At least in some cells, a receptor for RBP has been described (Blomhoff *et al.*, 1991; Dew and Ong, 1994). Despite the attractiveness of such a model for receptor-mediated uptake of retinol, it has been reported that holo-RBP spontaneously and rapidly dissociates to RBP and retinol (Noy and Xu, 1990a, 1990b). The free retinol would then be available for cellular uptake by non-specific mechanisms. It has been postulated by Noy and Blaner (1991) that the quantity of apo-CRBP, present in the target cell, determines the amount of retinol that is taken up by the cell.

Intracellular metabolism of retinol to retinoic acid (RA)

Once inside the cell, retinol can be either stored as retinyl esters or converted to RA. RA is now recognized as a major, but not the exclusive, physiological active metabolite of retinol. The different steps involved in its formation have so far not been unequivocally established. Many tissues and cells possess the ability to convert retinol into RA (Napoli, 1994). Figure 2 illustrates a model, as proposed by Napoli (1996), for RA biosynthesis and catabolism and stresses the involvement of retinoid binding proteins. In this model, the ratio apo-CRBP/holo-CRBP determines the flux of retinol to RA or to retinyl esters (Napoli, 1996). CRBP-bound retinol serves as a substrate for a family of microsomal retinol dehydrogenases (RoDH) (Posch *et al.*, 1991) that catalyze the conversion to retinal. Retinal remains bound to CRBP and is oxidized irreversibly to RA. This conversion involves cytosolic retinal dehydrogenases (RalDH) (Posch *et al.*, 1992), although several cytochrome P450-dependent enzymes also have been shown to catalyze this reaction (Raner *et al.*, 1996). RA binds to cellular RA binding protein (CRABP) and is further metabolized (see below).

Figure 2: A model proposed for the biosynthesis of ATRA from retinol. ARAT, acyl CoA:retinol acyltransferase; CRABP, cellular RA binding protein; CRBP, cellular retinol binding protein; RA, retinoic acid; RBP, retinol-binding protein; REH, retinyl ester hydrolase; TTR, trans-thyretin; LRAT, lecithin:retinol acyltransferase; RalDH, retinal dehydrogenase; RoDH, retinol dehydrogenase (adapted from Napoli, 1996, see text for details).

Other sources of RA

RA can also originate from eccentric cleavage of β -carotene (Wang *et al.*, 1992; Parker, 1996). Another possibility is that RA is present in trace quantities in the diet or is formed in small quantities in the intestine. RA circulates in blood bound to serum albumin and as such can serve as a source of RA for several tissues (Smith *et al.*, 1973; Kurlandsky *et al.*, 1995). The molecular mechanisms for RA uptake into the cell are not clear.

ATRA: metabolism and mechanism of action

ATRA has been shown to control cell growth and differentiation in a number of cell types. The responses elicited by ATRA are however very diverse. It has only recently become clear how a relatively simple molecule like ATRA can elicit such a diversity of biological responses.

ATRA metabolism and isomerization

Diversity in the action of ATRA exists because of complexity at different levels of the ATRA signalling pathway. A first and important level in this complex ATRA signalling pathway is represented by the existence of natural isomers and metabolites of ATRA, whose synthesis may be modulated in a cell-specific fashion (De Luca, 1991; Leid *et al.*, 1992; Keaveny and Stunnenberg, 1995; Chambon, 1996; Napoli, 1996). Naturally occurring isomers and metabolites of ATRA (Fig. 3) include 13-*cis*-RA (Eckhoff *et al.*, 1991; Tang and Russell, 1990), 9-*cis*-RA (Heyman *et al.*, 1992; Levin *et al.*, 1992), 9,13-di-*cis*-RA (Tzimas *et al.*, 1994a; Sass *et al.*, 1995), 4-hydroxy-ATRA (Skare *et al.*, 1982), 4-oxo-ATRA (Eckhoff *et al.*, 1991; Tang and Russell, 1991), 18-hydroxy-ATRA (Hänni and Bigler, 1977; Fiorella and Napoli, 1994), 3,4-didehydro-ATRA (Thaller and Eichele, 1990), 5,6-epoxy-ATRA (McCormick *et al.*, 1978; Barua *et al.*, 1991), and retinoyl- β -glucuronide (Barua and Olson, 1986; Barua *et al.*, 1991; Eckhoff *et al.*, 1991).

Figure 3: Chemical structures of naturally occurring metabolites and isomers of ATRA.

Oxidation

The oxidative metabolism of ATRA to more polar metabolites has been known for some time. However, it was not until the advent of high-performance liquid chromatography (HPLC) that this topic could be investigated in detail. Hänni and Bigler (1977) identified two metabolites of ATRA in rat faeces as 4-oxo-ATRA and 18-hydroxy-ATRA. McCormick *et al.* (1978) identified 5,6-epoxy-ATRA as a metabolite of ATRA in the intestinal mucosa of vitamin A-deficient rats, and Frolik *et al.* (1978, 1979) identified both 4-hydroxy-ATRA and 4-oxo-ATRA as metabolites of ATRA in the hamster trachea in organ culture and in subcellular fractions of the liver. Roberts *et al.* (1980) studied the metabolism of ATRA in hamster liver microsomes. It was shown that ATRA is first converted to 4-hydroxy-ATRA, which is then converted to 4-oxo-ATRA and further catabolized to more polar metabolites. The formation of 4-hydroxy-ATRA and the further catabolism of 4-oxo-ATRA were found to require NADPH. In addition, the enzymatic activity was found to be mainly present in the microsomal fraction, shown to require oxygen, and was strongly inhibited by carbon monoxide. Together, these findings suggested the participation of cytochrome P450-dependent enzyme(s) in ATRA catabolism (Roberts *et al.*, 1979a, 1979b, 1980).

A number of cytochrome P450-dependent enzymes have been shown to be capable of 4-hydroxylating ATRA (Table I). Purified cytochromes P450 2C7 and P450 2B1 from rat liver microsomes were shown to catalyze the conversion of ATRA to polar metabolites, including 4-hydroxy-ATRA (Leo *et al.*, 1984). The P450 2C8 isoform from human liver microsomes can oxidize ATRA to 4-hydroxy-ATRA and 4-oxo-ATRA (Leo *et al.*, 1989) and, in addition, can also convert retinol into 4-hydroxy-retinol. Many cytochrome P450 isoforms, including 2B4, 1A2, 2E1, 2E2, 2C3, 2G1, and 3A6, from rabbit liver microsomes catalyze the 4-hydroxylation of ATRA, with the isoforms 1A2 and 2B4 being the most potent ones (Roberts *et al.*, 1992). A member of the cytochrome P450 3A subfamily has also been implied in the 4-hydroxylation of ATRA (Martini and Murray, 1993). Although these findings demonstrate that a number of purified cytochrome P450-dependent enzymes are able to catalyze the 4-hydroxylation of ATRA, the characterization of the ATRA 4-hydroxylase at the molecular level was achieved only very recently. White *et al.* (1996) cloned a cytochrome P450-dependent enzyme from zebrafish, namely P450RA1, that was strongly induced by ATRA. P450RA1 was shown to catalyze the conversion of ATRA to 4-hydroxy-ATRA and 4-oxo-ATRA. It seems that its human counterpart is highly homologous, indicating a conserved role in ATRA catabolism (White *et al.*, 1996).

Table 1: Cytochrome P450-dependent ATRA 4-hydroxylases

isozyme	species	tissue	reference
2B1, 2C7	rat	liver	Leo <i>et al.</i> (1984)
2C8	human	liver	Leo <i>et al.</i> (1989)
1A2, 2B4	rabbit	liver	Roberts <i>et al.</i> (1992)
3A	rat	liver	Martini and Murray (1993)
P450RAI	zebrafish	fins	White <i>et al.</i> (1996)

Other pathways by which ATRA may be oxidized is via epoxidation to 5,6-epoxy-ATRA (McCormick *et al.*, 1978; Barua *et al.*, 1991) and hydroxylation to 18-hydroxy-ATRA (Hänni and Bigler, 1977; Fiorella and Napoli, 1994; Han and Choi, 1996). The significance of these pathways in the catabolism of ATRA remains to be established.

Isomerization

ATRA readily undergoes isomerization. Pharmacological doses of ATRA isomerize to 13-*cis*-RA in rats (Cullum and Zile, 1985; Napoli *et al.*, 1985; Kojima *et al.*, 1994) and mice (Kalin *et al.*, 1981). Conversely, 13-*cis*-RA was found to isomerize to ATRA (Puglisi and DeSilva, 1978; Kalin *et al.*, 1982; Vane *et al.*, 1982; McCormick *et al.*, 1983). Also in human serum 13-*cis*-RA has been identified (Tang and Russell, 1990; Eckhoff *et al.*, 1991) and isomerization of ATRA to 13-*cis*-RA and vice versa has been reported (Goodman *et al.*, 1982; Muindi *et al.*, 1994; Takitani *et al.*, 1995). The existence of 9-*cis*-RA was reported by Levin *et al.* (1992) and Heyman *et al.* (1992). In rats, ATRA isomerizes to 9-*cis*-RA to a limited extent (Kojima *et al.*, 1994), while in mice and rats 9-*cis*-RA isomerizes to ATRA, 13-*cis*-RA and 9,13-dicis-RA (Tzimas *et al.*, 1994a). In man, isomerization of 9-*cis*-RA into ATRA was observed to only a limited extent (Sass *et al.*, 1995) and 9,13-dicis-RA has not been identified. Isomerization of ATRA into 9-*cis*-RA and 13-*cis*-RA, and vice versa, occurs non-enzymatically (Shih *et al.*, 1986; Urbach and Rando, 1994; Shih *et al.*, 1997).

Glucuronidation

The free carboxyl group of ATRA is important for its biological activity. The only known naturally occurring derivative where the carboxyl group is blocked is retinoyl- β -glucuronide (Barua and Olson, 1986). β -Glucuronides of retinol, 13-*cis*-RA, 9-*cis*-RA, 4-oxo-RA and 5,6-

epoxy-RA have also been identified (Napoli *et al.*, 1982; Eckhoff *et al.*, 1991; Creech Kraft *et al.*, 1991a, 1991b; Sass *et al.*, 1994). Unlike other ATRA derivatives, retinoyl- β -glucuronide is water soluble and is considered a product of detoxification. Retinoyl- β -glucuronide is biologically active (Zile *et al.*, 1987; Janick-Buckner *et al.*, 1991; Mehta *et al.*, 1991) but could not be demonstrated to bind to cellular retinoid binding proteins or nuclear receptors (Mehta *et al.*, 1992; Sani *et al.*, 1992). It seems that its activity is derived from the hydrolysis to ATRA (Tzimas *et al.*, 1994b; Foerster *et al.*, 1996; Nau *et al.*, 1996).

Cellular RA binding proteins (CRABPs)

Another level that adds to the complexity of RA signalling is the existence of cellular RA binding proteins, CRABPs (Ong *et al.*, 1994; Li and Norris, 1996). Both isoforms of CRABP, CRABPI and CRABPII, bind RA with high affinity. There is a strong preference for ATRA over the other RA isomers and CRABPI binds ATRA with higher affinity than does CRABPII, suggesting that they may have different functions. Natural metabolites of ATRA, including 4-hydroxy-ATRA, 4-oxo-ATRA, 18-hydroxy-ATRA and 3,4-didehydro-RA, can also bind to CRABPs (Fiorella *et al.*, 1993). Binding of 9-*cis*-RA occurs with much lower affinity. There is little, if any, detectable binding of 13-*cis*-RA and 9,13-dicis-RA to either isoform of CRABP (Ong *et al.*, 1994; Li and Norris, 1996). The function of CRABPs is not clear. It has been proposed that CRABPs sequester ATRA in the cytoplasm, functioning as a buffer system to limit the availability of ATRA to its nuclear receptors (Boylan and Gudas, 1991). Besides, CRABPs have been suggested to transport ATRA from the cytoplasm to the nucleus (Takase *et al.*, 1986; Gustafson *et al.*, 1996). Furthermore, CRABPs have also been shown to facilitate ATRA catabolism (Ong *et al.*, 1994; Li and Norris, 1996). However, the physiological importance of CRABP-facilitated catabolism was recently challenged by showing that CRABPI null mutant mice had a normal phenotype (de Bruijn *et al.*, 1994; Gorry *et al.*, 1994). Also CRABPII null mutant mice did not exhibit any overt phenotypic defects (Fawcett *et al.*, 1995; Lampron *et al.*, 1995). Double mutant mice for both CRABPI and CRABPII were also essentially normal (Lampron *et al.*, 1995). Moreover, these null mutant mice did not show increased sensitivity to RA. It has been suggested that CRABPs may function in maintaining physiological levels of intracellular ATRA under conditions of limited supply of vitamin A. The exact function of CRABPs thus remains as yet elusive.

Nuclear retinoid receptors

It is now well established that ATRA signals are mediated by nuclear retinoid receptors. Two families of retinoid receptors exist, i.e. the RA receptors (RARs) and the retinoid X receptors (RXRs), which are structurally and functionally related to the members of the nuclear receptor superfamily (Mangelsdorf *et al.*, 1995). This superfamily also includes receptors for steroids, thyroid hormone, and vitamin D (Mangelsdorf *et al.*, 1995; Chambon, 1996) as well as a number of "orphan receptors" for which specific ligands have not yet been identified. Each retinoid receptor family consists of three subtypes, i.e. α , β , and γ , encoded by separate genes located on different chromosomes (Chambon, 1996). Each subtype can occur as different isoforms as a result of differential promoter use and alternative splicing. The major difference between RARs and RXRs lies in their ligand binding potential. In addition to ATRA and 9-*cis*-RA, RARs also bind ATRA metabolites including 4-oxo-ATRA (Pijnappel *et al.*, 1993; Kim *et al.*, 1994; Chambon, 1996). The ability of the natural stereoisomer 13-*cis*-RA to bind to RARs is still controversial (Åström *et al.*, 1990; Crettaz *et al.*, 1990; Matsushima *et al.*, 1992; Repa *et al.*, 1993; Huggenvik and Sharma, 1995). On the contrary, RXRs only bind 9-*cis*-RA (Heyman *et al.*, 1992; Levin *et al.*, 1992). In contrast to the receptors for steroid hormones, which bind to DNA as homodimers, the retinoid receptors interact with DNA as heterodimers. RARs can only dimerize with RXR. RXR serves as a heterodimer partner for the thyroid hormone receptor (TR), the vitamin D receptor (VDR), the peroxisome proliferator-activated receptor (PPAR), and a number of orphan receptors, e.g. NURR1, NGFIB, and LXR (Pfahl and Chytil, 1996). In addition, RXR can also function as a homodimer in the presence of 9-*cis*-RA (Zhang *et al.*, 1992). The unique capability of RXRs to form homodimers in response to 9-*cis*-RA and the ability of RXR to form heterodimers with a number of other nuclear receptors, suggest that RXRs act as important regulators in the cross talk among retinoids and other hormones.

Upon ligand-binding, the RXR/RAR heterodimer functions as a transcription factor (Fig. 4) that transactivates gene expression by binding to short DNA sequences known as RA-responsive elements (RARE) (Leid *et al.*, 1992; Mangelsdorf *et al.*, 1994; Keaveny and Stunnenberg, 1995; Chambon, 1996). RAREs are located in the promoter-region of a large number of RA target genes, such as for example RARs and laminin B1. Most RARE consist of the direct repetition (DR) of two core consensus hexamer sequences (A/G)G(T/G)TCA separated by a number of nucleotides. RXR/RAR heterodimers bind DR spaced by 1 (DR1), 2 (DR2) or 5 (DR5) nucleotides with increasing efficiency (Chambon, 1996). It has been established that RXR/RAR heterodimers bind DR with a certain polarity (Fig. 4) (Glass, 1996).

Indeed, RXR binds the 5'-upstream hexameric sequence while RAR binds the 3'-downstream hexamer half site on DR2 and DR5. Furthermore, on DR1 the RXR/RAR heterodimer can also bind with the reverse polarity i.e. with RXR occupying the 3'-downstream hexamere (5'-RAR/RXR-3'). Unlike most other members of the nuclear receptor superfamily, RARs are able to suppress transcription of RA target genes in the absence of ligand binding. The polarity of binding (Fig. 4) to the DR determines the transactivation or transrepression potential of the RXR/RAR heterodimer. The transactivator/repressor function of RXR/RAR heterodimers is further fine tuned by a family of coactivators and corepressors (Horwitz *et al.*, 1996).

A large number of retinoid responsive genes have been identified, but the specific mechanisms by which retinoids affect cellular growth and differentiation have not been fully elucidated in any model system.

In conclusion, the complex RA signalling pathway is explained by the existence of various naturally occurring RA metabolites, which can undergo a number of conversions, the existence of several CRABPs, nuclear retinoid receptors, response elements, coactivators and corepressors.

Figure 4: Schematic representation of RAR/RXR transactivation and transrepression.

Retinoids, cancer and differentiation therapy

Cancer is characterized by abnormal growth and a derailment of differentiation. Besides, it has also been well established that retinoids are able to inhibit cell proliferation and induce differentiation in a large number of cell types. Therefore, it is not surprising that retinoids have been extensively explored for potential use in cancer chemoprevention and therapy.

The aim of therapy with retinoids, also known as differentiation therapy, is to arrest or reverse the process of malignancy to a more "normal" phenotype by the induction of differentiation (Scott, 1997).

Biological activity of ATRA

In vitro, retinoids and more particularly ATRA have been demonstrated to inhibit cell proliferation of both normal and malignant cells (Lotan, 1980; Amos and Lotan, 1990). In addition, in some cell types, ATRA was able to induce terminal differentiation that was accompanied by a loss of neoplastic character. The induction of terminal differentiation was most striking in human HL-60 promyelocytic leukemia cells (Breitman *et al.*, 1980; Strickland *et al.*, 1983), and in mouse F9 teratocarcinoma cells (Strickland and Mahdavi, 1978; Strickland and Sawey, 1980). In HL-60 cells, ATRA induced differentiation of promyelocytes to mature granulocytes, concomitant with growth inhibition. In F9 teratocarcinoma cells, ATRA induced differentiation to a variety of cell lineages depending on the culture conditions used (Strickland and Mahdavi, 1978; Strickland *et al.*, 1980; Hogan *et al.*, 1981; Rogers *et al.*, 1990). Recently, it has also been recognized that retinoids induce programmed cell death (apoptosis), but little is known about the mechanisms involved (Kalemkerian and Ramnath, 1996).

Also *in vivo* the usefulness of retinoids has been extensively demonstrated (Moon *et al.*, 1994). In animal models, retinoids were highly effective in inhibiting the process of carcinogenesis and in chemoprevention (Moon *et al.*, 1994). Furthermore, epidemiological studies have reported that a low intake of vitamin A is correlated with a higher tumor incidence (Hong and Itri, 1994; Lotan, 1996). Taken together, these data indicate a potential role of retinoids in the treatment and prevention of human cancer.

ATRA: clinical results

By far the most encouraging results have been obtained in patients with acute promyelocytic leukemia (APL) (Menger *et al.*, 1988; Castagne *et al.*, 1990; Chen *et al.*, 1991; Smith *et al.*, 1992; Warrell *et al.*, 1991). ATRA treatment resulted in complete remissions in a large number of patients. In addition, some of these studies showed that the remissions were due to induction of differentiation of promyelocytes to mature polymorphonuclear leukocytes. Besides the beneficial effect of ATRA in APL patients, only limited success has been obtained in epithelial cancers.

13-*cis*-RA showed efficacy in the treatment of premalignant oral leukoplakia (Hong *et al.*,

1986) and in the prevention of second primary tumors in patients with head and neck cancer (Hong *et al.*, 1990). On the contrary, RA was not effective in the treatment of most primary solid cancers, including squamous cell carcinoma of the head and neck, lung and malignant melanoma (Hong and Itri, 1994). To improve the clinical effectiveness of retinoids, combination therapy with interferons has been used (Lippman *et al.*, 1997). This kind of combination therapy has been shown valuable in the treatment of squamous cell carcinoma of the skin (Lippman *et al.*, 1992a) and cervix (Lippman *et al.*, 1992b). In conclusion, retinoids have so far shown only limited success and ongoing clinical trials will have to establish their potential use in cancer therapy. Furthermore, the clinical use of ATRA is significantly hampered by its toxicity and the prompt emergence of ATRA resistance.

Toxicity

Drug toxicity is a major problem in ATRA therapy. Low doses of ATRA are usually well tolerated by adults, but at higher doses general signs of toxicity, also known as hypervitaminosis A, become evident. Major side effects influencing the quality of life include cheilitis, mucocutaneous reactions, manifesting as erythema, rash, and desquamation, and headache (Lee *et al.*, 1993). The limited success of ATRA therapy may in part be explained by the fact that the toxicity of high doses of ATRA limits its therapeutic use.

Resistance

As already mentioned, ATRA therapy has been shown highly effective in the treatment of patients with APL. However, clinical remissions are usually short-lived because most patients relapse due to the prompt emergence of ATRA resistance (Castaigne *et al.*, 1990; Warrell *et al.*, 1993; Lazzarino *et al.*, 1996). Several pharmacokinetic studies have shown that the plasma concentration of ATRA, with continuous dosing, progressively declines, suggesting that ATRA induces its own catabolism (McPhillips *et al.*, 1987; Muindi *et al.*, 1992a, 1992b, 1994; Smith *et al.*, 1992; Rigas *et al.*, 1993). This phenomenon was also observed in patients with solid tumors (Lee *et al.*, 1993; Muindi *et al.*, 1994). Furthermore, the autoinduction of catabolism was not observed with chronic dosing of 13-*cis*-RA (Brazell *et al.*, 1983) or 9-*cis*-RA (Adamson *et al.*, 1995; Miller *et al.*, 1996). One approach to overcome ATRA resistance is the development of compounds that are able to inhibit the catabolism of ATRA. Such inhibitors would be able to maintain high ATRA levels despite the presence of increased catabolism.

Liarozole-fumarate, an inhibitor of ATRA catabolism

Liarozole-fumarate (Fig. 5), a new antitumor drug, has been identified as an inhibitor of the cytochrome P450-dependent catabolism of ATRA (Van Wauwe *et al.*, 1990, 1992; Wouters *et al.*, 1992; Krekels *et al.*, 1996, 1997).

In vitro, liarozole-fumarate by itself has no effect, but in combination with ATRA the drug enhances the biological activity of this retinoid. In MCF-7 human breast cancer cells, liarozole-fumarate enhanced the antiproliferative activity of ATRA by 10-fold (Wouters *et al.*, 1992). Under basal growth conditions, MCF-7 cells were unable to catabolize ATRA, but the ATRA catabolism could be induced rapidly by pretreatment with ATRA (Wouters *et al.*, 1992; Krekels *et al.*, 1997). This resulted in its conversion to more polar metabolites, as shown by HPLC (Wouters *et al.*, 1992). Liarozole-fumarate was able to inhibit this induced ATRA catabolism with an IC_{50} value of 440 nM, resulting in a decreased amount of polar metabolites (Wouters *et al.*, 1992; Krekels *et al.*, 1997). Thus, the enhancement by liarozole-fumarate of the antiproliferative activity of ATRA in MCF-7 cells was most probably due to inhibition of ATRA catabolism. In F9 mouse teratocarcinoma cells, which are known to have an active ATRA catabolism (Williams and Napoli, 1985, 1987), liarozole-fumarate was also able to enhance the activity of ATRA on the induced expression of plasminogen activator (De Coster *et al.*, 1992).

In addition, the activity of ATRA and β -carotene on cellular transformation and gap junctional communication was potentiated in 10T1/2 mouse embryonal fibroblasts (Acevedo and Bertram, 1995) and retinoid-induced apoptosis was enhanced in DU145 human prostate cancer cells (Hall, 1996).

Figure 5: Chemical structure of liarozole-fumarate [$\pm$ -5-[(3-chlorophenyl) (1H-imidazole-1-yl)methyl]-1H-benzimidazole (E)-2-butenedioate (2:3)].

In vivo, liarozole-fumarate increased the biological half-life of exogenously injected ATRA in rats (Van Wauwe *et al.*, 1990) and of orally administered ATRA and 13-*cis*-RA in cancer patients (Westarp *et al.*, 1993; Achkar *et al.*, 1994; Miller *et al.*, 1994). Moreover, liarozole-fumarate was able to enhance endogenous ATRA levels (Van Wauwe *et al.*, 1990; Smets *et al.*, 1995), thereby mimicking similar morphological and biochemical effects on rat vaginal epithelium as observed after RA treatment (Van Wauwe *et al.*, 1992). These retinoid-mimetic effects of liarozole-fumarate could be explained by its inhibition of ATRA catabolism (Van Wauwe *et al.*, 1992).

The antitumoral activity of liarozole-fumarate was investigated in several rat Dunning R3327 prostate adenocarcinoma sublines. The drug reduced tumor growth in both androgen-dependent, sublines G and H, and androgen-independent, sublines PIF-1 and AT-6, prostate tumors (Van Ginckel *et al.*, 1990; Dijkman *et al.*, 1994; Smets *et al.*, 1995). In the Dunning AT6sq subline, it was shown that liarozole-fumarate increased endogenous ATRA levels both in plasma and in the tumor (Smets *et al.*, 1995). Concomitantly, a shift in the differentiation status was noticed which was further confirmed by a strong decrease in cytokeratin 10, a typical ATRA-linked phenomenon (Smets *et al.*, 1995). Tumor homogenates of the Dunning G subline were shown to have an active ATRA catabolism, that could be inhibited by liarozole-fumarate (Krekels *et al.*, 1996). Also the subcutaneous and bone metastatic tumor growth of androgen-independent PC-3ML-B2 human prostate cancer cells was inhibited by liarozole-fumarate in severe combined immunodeficient mice (Stearns *et al.*, 1993). Taken together, these findings suggested that the antiproliferative activity of liarozole-fumarate was related to its potential to inhibit ATRA catabolism.

In clinical trials, liarozole-fumarate has been evaluated in advanced prostate cancer patients (Boccardo *et al.*, 1994; Mahler *et al.*, 1994; Wouters, 1994; Seidmon *et al.*, 1995; Bryson and Wagstaff, 1996). In some patients with measurable disease a partial response was observed, as determined by regression of soft-tissue metastasis. Because measurable disease is rare, prostate specific antigen (PSA) was regarded as the most suitable alternative in the follow-up of advanced prostate cancer patients (Mahler *et al.*, 1994). Liarozole-fumarate induced a $\geq 50\%$ decrease of PSA levels in about 30% of unselected patients (Mahler *et al.*, 1994; Bryson and Wagstaff, 1996).

A number of patients experienced less bone pain and most patients showed side effects similar to hypervitaminosis A, reinforcing the hypothesis that liarozole-fumarate exerts its effects by inhibiting ATRA catabolism and thereby accumulating the endogenous ATRA levels.

Aim of this study

ATRA is well known to inhibit the proliferation of cancer cells. This antiproliferative activity of ATRA could be enhanced by liarozole-fumarate, an inhibitor of ATRA catabolism. Besides, ATRA is also known to induce differentiation in cancer cells. The first objective was to test the possibility that liarozole-fumarate could also enhance the differentiation-inducing capacity of ATRA. To test this hypothesis, the structural remodelling induced by ATRA was studied by immunocytochemistry in the presence of liarozole-fumarate.

The second objective was to develop an *in vitro* model system in which cells could be grown in the absence of endogenous retinoids in order to study the effect of catabolism on the antiproliferative activity of ATRA and its metabolites. In addition, the mechanism of action of liarozole-fumarate was further investigated in light of its potential role in cancer therapy.

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Liarozole potentiates the all-*trans*-retinoic acid-induced structural remodelling in human breast carcinoma MCF-7 cells *in vitro*

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Abstract

Liarozole inhibits cytochrome P450-dependent enzymes that play a key role in all-*trans*-retinoic acid (ATRA) catabolism. In MCF-7 cells, liarozole potentiates the antiproliferative effects of ATRA. The present study demonstrates this synergistic effect on cell differentiation of MCF-7 cell cultures as measured by immunocytochemistry for cytokeratins 8, 18, and 19, actin, E-cadherin, desmoglein and desmoplakins I & II. ATRA concentration-dependently (10^{-8} M to 10^{-6} M) induced changes in actin stress

fibers and cytokeratin intermediate filaments. These changes were accompanied by a more obvious interaction of these filaments with junctional complexes. Surface area and volume of the MCF-7 cells increased markedly after ATRA exposure, with extensive filopodia formation. Liarozole (10^{-6} M) alone had no effect on cell morphology, cytokeratin or actin organization, or on cellular junctions. In combination with ATRA (10^{-9} M and 10^{-8} M), liarozole potentiated the ATRA-induced effects. The MCF-7 cell cultures used showed morphological heterogeneity, consisting of at least two cellular subpopulations. This was reflected in the staining for E-cadherin, desmoglein and desmoplakins I & II. ATRA increased E-cadherin staining at cell-cell contact sites, but had no influence on the staining patterns of desmoglein and desmoplakins I & II. Similar to what has been observed for the cytoskeletal differentiation parameters, liarozole alone had no influence on E-cadherin, desmoglein or desmoplakins I & II expression, but in combination with ATRA again intensified the effects on E-cadherin distribution. These effects on MCF-7 cells agree with previously obtained observations concerning the inhibition of ATRA catabolism by liarozole. Furthermore, our data support the hypothesis that the antiproliferative properties of the drug are accompanied by induction of differentiation.

Introduction

Liariozole is currently in phase III clinical trials for the treatment of relapsed prostate cancer. Liariozole exerts retinoid-mimetic effects in both animals and patients (Van Wauwe *et al.*, 1992; Mahler *et al.*, 1993). Recently it has been shown that liarozole acts as a very potent inhibitor of cytochrome P450-dependent all-*trans*-retinoic acid (ATRA) catabolism, inhibiting both the 4-hydroxylation of ATRA and the monohydroxylation reactions of 4-oxo-ATRA (Van Wauwe *et al.*, 1990, 1994). The drug reduces tumor growth of androgen-dependent and androgen-independent Dunning R3327 rat prostate adenocarcinoma cells (Van Ginckel *et al.*, 1990; De Coster *et al.*, 1992; Dijkman *et al.*, 1994; Smets *et al.*, 1995) and of the androgen-independent PC-3ML-B2 human prostate carcinoma xenografts (Stearns *et al.*, 1993). Together, these biochemical and clinical results have given rise to the hypothesis that liarozole may exert its antitumoral effects by inhibition of the cytochrome P450-dependent ATRA catabolism.

In MCF-7 human breast carcinoma cell cultures, liarozole has been shown to enhance the antiproliferative effects of ATRA (Wouters *et al.*, 1992). This enhancement was dependent on the concentration of liarozole (between 10^{-7} M and 10^{-5} M) and was more than 10-fold. Under basal growth conditions, MCF-7 cells are unable to metabolize ATRA, as shown by high-performance liquid chromatography. However, pretreatment with ATRA induced a very active conversion of ATRA to more polar metabolites. This conversion was almost completely inhibited by liarozole at a concentration of 10^{-5} M (Wouters *et al.*, 1992). Also in F9 mouse teratocarcinoma cells, liarozole enhanced the effect of ATRA on the induction of plasminogen activator more than 2-fold (De Coster *et al.*, 1992). In both cell lines, liarozole alone, showed no intrinsic retinoid activity and did not inhibit proliferation (De Coster *et al.*, 1992; Wouters *et al.*, 1992).

It is now well established that ATRA exerts antiproliferative and differentiation-inducing effects on malignant cells (Lotan, 1980). These effects are believed to be directly exerted at the level of gene expression by nuclear retinoic acid receptors and retinoid X receptors that are structurally and functionally related to the steroid/thyroid hormone receptor superfamily (Giguère, 1994; Mangelsdorf *et al.*, 1994). In this respect, retinoic acid has repeatedly been shown to be an important regulator of epithelial differentiation (Chytil, 1984; Wolf, 1984). Such processes are often characterized by the reorganization of the cytoskeleton (Lehtonen *et al.*, 1983; Ng *et al.*, 1985; Rutka *et al.*, 1988; Helige *et al.*, 1993; Plateroti *et al.*, 1993). These filamentous structures associate with a diversity of adhesion molecules at cell-cell contact sites. For example, the cytokeratin intermediate filaments are anchored to desmosomes, containing desmoplakin and desmoglein (Schmidt *et al.*, 1994), and actin microfilaments are linked to adherence type junctions, comprising cadherins (Takeichi, 1991). This interaction of the cytoskeleton with adhesion molecules in the cellular membrane is crucial for maintenance of the proper integrity of tissues (Fleming, 1991; Gumbiner, 1993). The present study explores the current hypothesis according to which liarozole exerts its antitumoral effects by inhibiting ATRA catabolism. MCF-7 human breast cancer cells have an inducible ATRA metabolism and were used to confirm the potentiation of the antiproliferative ATRA effect by liarozole, under the same culture conditions as previously described (Wouters *et al.*, 1992). Furthermore, the differentiation-inducing effects of ATRA on MCF-7 cells were investigated, and tests were carried out to determine whether liarozole was able to potentiate these effects. To monitor the process of differentiation, cytoskeletal proteins (cytokeratins and actin) and cellular junctions (E-cadherin, desmoglein and desmoplakins I & II) were visualized by immunocytochemistry.

Materials and Methods

Drug and chemicals

The fumarate salt of liarozole, $\{\pm\}$ 5-[(3-chlorophenyl)(1*H*-imidazole-1-yl)methyl]-1*H*-benzimidazole (E)-2-butenedioate (2:3) (R085246), was synthesized by the Janssen Research Foundation (Beerse, Belgium). Stock solutions of liarozole (10 mM) and ATRA (4 mM) (Serva, Heidelberg, Germany) were prepared in ethanol and appropriately diluted in culture medium. ATRA was used in a dark room with yellow illumination and was regularly checked for purity using HPLC analysis.

Cell culture

MCF-7 human mammary carcinoma cells, purchased from the American Type Culture Collection (Rockville, MD, U.S.A.), were cultured in Dulbecco's modified Eagle's medium with 4.5 g/liter glucose supplemented by 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µg/ml gentamicin and 10% fetal bovine serum (all reagents from Life Technologies, Ghent, Belgium). The MCF-7 subclone used in this study was estrogen receptor positive and vimentin negative. Cells were grown in a humidified incubator (5% CO₂, 95% air) at 37°C and regularly checked for Mycoplasma contamination. For the experiments described in this study late passage (over 150) MCF-7 cells were used. Cell viability was determined by trypan-blue exclusion using a hemocytometer.

For the experiments described below, MCF-7 cells were plated onto Chamber Slides™ (Nunc Inc., Naperville, IL, U.S.A.) at a concentration of 5000 cells/chamber. Cells were allowed to attach for 24 h before the addition of the drugs.

Drug incubations

Cells plated onto slides were incubated for 7 days with the following test conditions : ATRA at concentrations of 10⁻¹¹ M, 10⁻¹⁰ M, 10⁻⁹ M, 10⁻⁸ M, 10⁻⁷ M and 10⁻⁶ M, liarozole at concentrations of 10⁻⁷ M, 10⁻⁶ M and 10⁻⁵ M and liarozole (10⁻⁶ M) in combination with ATRA at concentrations of 10⁻¹⁰ M, 10⁻⁹ M and 10⁻⁸ M. Medium was changed on days 2 and 5. Under control conditions, cells reached confluence after 7 days.

Scanning electron microscopy (S.E.M.)

Cells grown with or without test compounds for 7 days were washed twice for 15 min in serum-free culture medium. Cells were fixed in 2% paraformaldehyde (Merck, Darmstadt, Germany) and 2.5% glutaraldehyde (Fluka, Buchs, Switzerland) buffered in 0.1 M phosphate buffer (pH 7.4) for 24 h. After being washed in 7.5% sucrose (Merck) in 0.1 M phosphate buffer (pH 7.4), cells were postfixated for 1 h at 4°C in 2% osmium tetroxide (Acros Chimica, Geel, Belgium) in 0.05 M veronal acetate (pH 7.4). The slides were washed twice for 5 min in 7% sucrose in 0.05 M veronal acetate and dehydrated through a graded series of ethanol and acetone. The samples were mounted and shadowed with 30 nm gold. They were examined and photographed in a Philips XL20 scanning electron microscope.

Transmission electron microscopy

Cells grown with or without test compounds for 7 days were fixed and postfixed as described for scanning electron microscopy. Thereafter, they were stained with 0.5% uranyl acetate (Merck) in 0.05 M veronal acetate (pH 5.2) for 40 min at room temperature. After the cells were washed twice for 5 min with 0.05 M veronal acetate (pH 5.2), they were dehydrated by ethanol and embedded in Epon. Sections 110 nm thick were cut with a diamond knife and collected on Formvar-coated grids (300 mesh). They were examined and photographed in a Philips EM410 electron microscope.

Bromodeoxyuridine (BrdU) incorporation and immunodetection

Cells grown with or without test compounds for 7 days were incubated for 2.5 h with an excess (100 μ M) of 5-bromo-2'-deoxyuridine (BrdU) (Acros Chimica) in serum-free culture medium. Then cells were washed three times for 15 min with a mixture of culture medium and 0.1 M phosphate buffer (pH 7.4) (8:2 v/v). Cells were subsequently fixed in methanol (5 min, -20°C), acetone (10 s, -20°C) and air-dried. Denaturation of the DNA was performed with 1% periodic acid (Merck) for 30 min at 60°C , followed by Schiff's reagent (Sigma Diagnostics, St. Louis, MO, U.S.A.) for 30 min at room temperature. Cells were washed three times with distilled water and treated with 0.01% trypsin (Life Technologies, Ghent, Belgium) in 0.05 M Tris-HCl (pH 7.6). After the cells were washed in 0.05 M Tris-HCl (pH 7.6) and 0.1 M phosphate buffer, the slides were subsequently blocked in 0.5% lysozyme in 0.1 M phosphate buffer for 1 h, incubated overnight at room temperature in a moist chamber with a primary monoclonal antibody against BrdU (1:50) (Janssen Biochimica, Beerse, Belgium) and antibody binding visualized with a secondary gold-labeled (5 nm) goat anti-mouse IgG (1:50) (Amersham International, Little Chalfont, Buckinghamshire, United Kingdom). The signal was enhanced for light microscopy with a silver enhancement kit (Amersham International).

The BrdU labeling index was defined as the proportion of BrdU positive cells, representing cells in S-phase. It was calculated by counting 700 cells per incubation per test condition (see drug conditions). Average results of six experiments are presented.

Immunocytochemistry

Cells grown with or without test compounds for 7 days were washed twice with serum-free culture medium. Cells were fixed in methanol (5 min, -20°C) and acetone (10 s, -20°C) and air-dried. Cells were subsequently blocked in 5% normal goat serum in 10 mM phosphate buffered saline (PBS) (pH 7.4) for 1 h, incubated for 1 h at room temperature in a dark moist chamber with the primary monoclonal antibody and visualized with the immunofluorescence technique using a secondary fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL, U.S.A.). Slides were air-dried and mounted in Mowiol (Hoechst, Frankfurt a.M., Germany). Nuclei were stained with 4'-6-diamidine 2-o-phenylindole (DAPI; diluted 1:40000) (Sigma Chemicals). The characteristics and references of the primary antibodies used in this study are summarized in Table I.

Table 1: Primary monoclonal antibodies used for immunocytochemistry

Antibody	Ig subclass	Dilution	Antigen recognized	Source	Reference
AC-15	Ig G ₁	1:1000	β -actin	Sigma	North <i>et al.</i> , 1994
DG 3.10	Ig G ₁	1:10	desmoglein	Progen	Schmelz <i>et al.</i> , 1986
DP 1&2	Ig G ₁	1:10	desmoplakins I&II	Progen	Franke <i>et al.</i> , 1983
HECD-1	Ig G ₁	1:400	E-cadherin	TaKaRa	Shimoyama <i>et al.</i> , 1989
LP2K	Ig G _{2b}	1:10	cytokeratin 19	F. Ramaekers	Broers <i>et al.</i> , 1986
M 20	Ig G ₁	1:3	cytokeratin 8	F. Ramaekers	Schaafsma <i>et al.</i> , 1989
NCC-CAD-299	Ig G ₁	1:200	P-cadherin	TaKaRa	Shimoyama <i>et al.</i> , 1989
RCK 106	Ig G ₁	1:5	cytokeratin 18	F. Ramaekers	Ramaekers <i>et al.</i> , 1987
RV203	Ig G ₁	1:5	vimentin	F. Ramaekers	Schaart <i>et al.</i> , 1991

One- and two-dimensional gel electrophoresis

Equal numbers of control and ATRA-treated cells (4.5×10^6) were washed with 10 mM PBS (pH 7.4) and scraped into sodium dodecyl sulfate (SDS)-electrophoresis sample buffer (Laemmli, 1970) and boiled for 10 min. For one-dimensional gel electrophoresis, samples were diluted 1:4, and proteins were electrophoretically separated on 12% SDS-containing polyacrylamide gels (Mini Protean II Cell; Bio-Rad Laboratories, Nazareth, Belgium). Gels were stained with Coomassie Brilliant Blue R250.

For two-dimensional gel electrophoresis the samples were prepared as described above. Before loading onto isoelectric focusing (IEF)-gels, an equal volume of buffer containing 2% Nonidet P-40 (Fluka), 5% β -mercaptoethanol (Merck), 5% Bio-Lyte carrier ampholytes (pH 3-10) (Bio-Rad Laboratories) and 9 M urea (Merck) was added, followed by addition of 1 M 3-[(3-cholamidopropyl)-dimethylammonio]-1 propane sulfonate (CHAPS) (Sigma) (1:4 v/v) and urea until saturation (Cupo *et al.*, 1990). Two-dimensional gel electrophoresis (Mini-Protean II 2-D system, Bio-Rad Laboratories) was performed as described by O'Farrell (1975), with the following modifications.

In the first dimension, IEF was performed in 4% polyacrylamide rod gels containing 8 M urea, 1.3% Bio-Lyte carrier ampholytes (pH 3-10) and 12.5 mM CHAPS. For IEF no preelectrophoresis was performed. Gels were run at increasing voltages starting at 500 V for 10 min, followed by 1000 V for 20 min and 1500 V for 100 min. For the second dimension, the rod gels were applied directly onto the stacking gel of a 10% SDS-containing polyacrylamide gel. The gels were stained with Coomassie Brilliant Blue R250. The stained protein bands were quantitated by digitizing with a RX5 videocamera attached to a Macintosh II_{FX} computer. Integration of the optical density was performed with the Image 1.44 software (NIH, Bethesda, MD, U.S.A.). Signals were corrected for background.

Statistical analysis

Where appropriate, data were analyzed using the two-tailed Mann-Whitney *U*-test using the Stat View II software (Abacus Concepts, Inc., Berkeley, CA, U.S.A.). Significance was defined at the level of $p < 0.05$.

Results

Liarozole enhances ATRA effects on MCF-7 cell morphology

MCF-7 cell monolayers showed morphological heterogeneity. As illustrated in the scanning electron micrographs, about 80 to 90% of the cells in MCF-7 cultures exhibited a small, round and tightly packed morphology (Fig. 1a), occasionally forming glandular structures (Fig. 1c). About 10 to 20% of the cell population consisted of large cells (Fig. 1b), mostly single growing. They were obviously more voluminous, exhibited a lower ratio of nuclear to cytoplasmic area and formed sparse loose intercellular junctions.

Increasing ATRA concentrations were tested for their influence on cell morphology. Both cell populations had an increased surface area and became clearly more voluminous at ATRA concentrations of 10^{-8} M to 10^{-6} M (Fig. 1d), with the induction of membrane ruffling.

Figure 1: Effect of 10^{-6} M ATRA on the heterogeneous MCF-7 cell population, analyzed by S.E.M. A majority of small cells (a) and a minority of large cells (b) is observed under control conditions. Occasionally, glandular structures (c) could be detected. 10^{-6} M ATRA increased the size of both cell populations (d). It induced membrane ruffling in the small cells and the formation of filopodia in the large cells. - Bars 0.5 mm (a, b, d), 0.1 mm (c).

especially in the population of small cells, and the formation of extensive filopodia, especially in the large cells. Furthermore, all cells appeared to have lower ratios of nuclear to cytoplasmic area. At 10^{-6} M ATRA, virtually no small cells were noted. The morphological distinction between small and large cells diminished to a large extent, but was still evaluable by scoring membrane ruffling and filopodia, respectively. Below 10^{-7} M ATRA, again small cells were observed as under control conditions. Liarozole alone at 10^{-6} M did not induce morphological changes. However, simultaneous addition of 10^{-6} M liarozole and 10^{-10} M to 10^{-8} M ATRA resulted in a clear potentiation of the ATRA-induced effect (data not shown).

Synergistic effects of liarozole and ATRA on inhibition of MCF-7 cell proliferation

The concentration-dependent effects of ATRA and liarozole on proliferative activity of MCF-7 cells are shown in Figure 2. As presented in Figure 2a, the BrdU labeling index for the subpopulation of small cells under control conditions was $36.8 \pm 1.3\%$ ($n = 6$). The BrdU labeling index decreased only slightly when going from 10^{-11} M to 10^{-7} M ATRA. Liarozole alone up to a concentration of 10^{-6} M had no effect on the BrdU labeling index. At 10^{-5} M, liarozole lowered the BrdU labeling index to $27.8 \pm 0.5\%$ ($n = 6$). Simultaneous application of 10^{-6} M liarozole and ATRA however, resulted in a significant 10-fold potentiation of the antiproliferative effect of ATRA. Although ATRA (10^{-8} M) alone had only a minor effect on the BrdU labeling index, ATRA in combination with liarozole (10^{-6} M) resulted in a 2-fold decrease of the number of cells in S-phase. At concentrations of 10^{-6} M ATRA alone and 10^{-7} M and 10^{-6} M ATRA in combination with 10^{-6} M liarozole, the BrdU labeling index could not be evaluated because there were virtually no small cells noted.

The antiproliferative effects of ATRA could be established in both subpopulations of MCF-7 cells. As presented in Figure 2b, the BrdU labeling index for the large MCF-7 cells under control conditions was $42.2 \pm 0.8\%$ ($n = 6$). Cells responded in a concentration-dependent manner to increasing ATRA concentrations. The BrdU labeling index of the large MCF-7 cells decreased 2-fold when going from 10^{-11} M to 10^{-7} M ATRA. Under these conditions, the drug concentration necessary to attain 50% growth inhibition was 5.6×10^{-8} M. Again, liarozole alone up to 10^{-6} M had no effect. At 10^{-5} M, liarozole lowered the BrdU labeling index to $29.5 \pm 1.1\%$ ($n = 6$). In large cells too, liarozole at 10^{-6} M significantly potentiated the antiproliferative effect of ATRA by 10-fold. Note that liarozole is unable to potentiate the effect of 10^{-6} M ATRA.

Figure 2: Concentration-response curves showing the effects of ATRA and liarozole on BrdU incorporation in small (a) and large (b) MCF-7 cells. Cells were seeded at 5000 cells/well and treated with various test compounds one day thereafter. On day 7 after plating, cells were labeled with BrdU. After immunodetection, cells were counted to obtain the BrdU labeling index. Results are presented as mean $\pm$ SEM ($n = 6$). (O) - ATRA; (□) - liarozole; (Δ) - ATRA + 10^{-6} M liarozole. Significance: * $p < 0.05$ versus control; # $p < 0.05$ versus ATRA treatment.

Liarozole potentiates the ATRA modulation of cell adhesion molecules in MCF-7 cells

The presence of desmosomes and adherens junctions in MCF-7 cells under control conditions is illustrated in the transmission electron micrographs in Figure 3. Intermediate filaments were associated with desmosomes (Fig. 3b). Typical adherens junctions were only present at the apical zone of neighboring cells (Fig. 3c), consistent with the polarization of the MCF-7 cells (Fig. 3a).

Figure 3: MCF-7 cells, as analyzed by transmission electron microscopy, showed a polarized organization (a) and were connected by a few well-developed cell-cell junctions. Intermediate filaments were associated with desmosomes (b). Adherens junctions were present at the apical site of neighboring cells (c). - Bars 10 µm (a), 1 µm (b, c).

The effect of ATRA on the expression of cell adhesion molecules was investigated. MCF-7 cells were negative for P-cadherin under all conditions tested (data not shown). The staining results of E-cadherin showed a clear relationship with the morphological heterogeneity of the cell population. The subpopulation of small cells (Fig. 4a) showed a clear E-cadherin staining along the cell membranes, accompanied by some punctate cytoplasmic staining. In large cells (Fig. 4f) E-cadherin staining was predominantly faint, solely localized in the cytoplasm, while at the cell-cell contacts E-cadherin staining was completely negative. With 10^{-8} M (Fig. 4c) to 10^{-6} M ATRA (Fig. 4b), the ruffled membrane of the small cells showed an intense E-cadherin signal, indicative of an up-regulation of this constituent. Upon treatment with 10^{-8} M (Fig. 4h) and 10^{-6} M ATRA (Fig. 4g), the cell junctions at the filopodia of the large cells now showed E-cadherin staining. 10^{-6} M liarozole alone (Figs. 4d, i) had no effect on E-cadherin staining, but in combination with 10^{-9} M and 10^{-8} M (Figs. 4e, j) ATRA, the drug intensified the ATRA-induced changes at the level of the cell-cell contacts, as scored by an increased ruffling of the membrane, indicative for an up-regulation of E-cadherin.

Similar to E-cadherin staining, staining for desmoplakins I & II and desmoglein showed a clear relationship with the different cell types of the culture (data not shown). Under control conditions, the subpopulation of small cells presented a clear punctate staining along the cell membranes. The subpopulation of large cells was either completely negative or showed some dot-like staining at cell-cell contacts. The small cells retained a dot-like staining at 10^{-8} M to 10^{-6} M ATRA (data not shown). Similarly, after treatment with 10^{-8} M to 10^{-6} M ATRA, the large cells showed no difference in the adhesion molecule staining patterns as compared to control cells (data not shown). They remained either completely negative or showed a dot-like reactivity at cell-cell contacts. Liarozole alone or in combination with 10^{-9} M and 10^{-8} M ATRA had no effect on the staining patterns for desmoglein and desmoplakins I & II (data not shown).

Figure 4: Effects of ATRA and liarozole on the distribution patterns of E-cadherin in small (a-e) and large (f-j) MCF-7 cells. Conditions tested: control (a, f), 10^{-6} M ATRA (b, g), 10^{-8} M ATRA (c, h), 10^{-6} M liarozole (d, i), 10^{-6} M liarozole in combination with 10^{-8} M ATRA (e, j). - Bar 0.1 mm.

Figure 5: Effects of ATRA and liarozole on the organization of the cytoskeleton, comprising cyokeratins (a-e) and actin (f-o) in small (a-j) and large (k-o) MCF-7 cells. Conditions tested: control (a, f, k), 10^{-6} M ATRA (b, g, l), 10^{-8} M ATRA (c, h, m), 10^{-6} M liarozole (d, i, n), 10^{-6} M liarozole in combination with 10^{-8} M ATRA (e, j, o). - Bars 0.5 mm (a-e), 0.5 mm (f-o).

Enhanced effects of ATRA in combination with liarozole on the cytoskeletal organization of MCF-7 cells

In parallel with the changes in cell shape, a reorganization of cytoskeletal filaments was observed. The effect of ATRA (ranging from 10^{-11} M - 10^{-6} M) alone or in combination with liarozole (10^{-6} M) on the organization of the cytoskeleton was tested by immunocytochemical staining for cytokeratins 8, 18, 19, actin and vimentin.

The MCF-7 cell clone used in this study was negative for vimentin under all conditions tested (data not shown). The results for the cytokeratin intermediate filaments are essentially the same for the different cytokeratin subtypes. Under control conditions, the staining pattern for cytokeratins in both MCF-7 cell subpopulations was characterized by a compact network of filaments (Fig. 5a). 10^{-7} M and 10^{-6} M (Fig. 5b) ATRA changed the organization to such an extent that a clear cytokeratin filamentous network running across the whole cell became visible. Also at a concentration of 10^{-8} M ATRA (Fig. 5c), this effect was still visible, although less pronounced as compared to 10^{-6} M ATRA. With 10^{-6} M liarozole alone (Fig. 5d), the immunocytochemical staining results resembled those for control conditions. However, 10^{-6} M liarozole in combination with 10^{-9} M and 10^{-8} M (Fig. 5e) ATRA potentiated the ATRA-induced effect, as illustrated by the presence of clear cytokeratin filaments and numerous cell-cell contact sites. At numerous foci of cell-cell adhesion points, cytokeratin filaments could be seen to interact with desmosomal junctions (Figs. 5b, e).

Gel electrophoretic analysis of the ATRA-treated cells showed a decrease in total cytokeratin content, as compared to the histone content (Fig. 6). Two-dimensional gel electrophoresis (Fig. 7) showed a clear decrease of all three cytokeratins 8, 18 and 19 at 10^{-6} M ATRA, in relation to several other proteins which remained present at a constant level. Densitometrical analysis showed a decrease of cytokeratins 8, 18 and 19 of approximately 50% after treatment with 10^{-6} M ATRA.

Similar to the changes described for cytokeratins, ATRA treatment resulted in profound effects on the actin-containing stress fibers. Under control conditions, actin was seen as stress fibers along the cell membrane and as a more diffusely staining meshwork in the cytoplasm (Figs. 5f, k). Treatment with 10^{-7} M and 10^{-6} M (Figs. 5g, l) ATRA induced numerous actin-containing stress fibers throughout the cell. In addition, numerous focal adhesions became apparent. After treatment with 10^{-8} M ATRA (Figs. 5h, m), actin staining predominantly resembled what was observed under control conditions. 10^{-6} M liarozole alone induced no actin reorganization (Figs. 5i, n), but when the drug was given

Figure 6: One-dimensional gel electrophoresis of total protein from equal numbers of control cells and cells treated with 10⁻⁶ M ATRA. - CK Cytokeratin. - H Histones.

simultaneously with 10⁻⁹ M and 10⁻⁸ M (Figs. 5j, o) ATRA, it clearly enhanced the formation of stress fibers and focal adhesions.

Two-dimensional gel electrophoresis (Fig. 7) showed an increase of approximately 40% in actin content after treatment with 10⁻⁶ M ATRA.

Discussion

The present study confirms and extends previous results (Wouters *et al.*, 1992) showing that liarazole potentiates the effects of ATRA. To study this synergism we used MCF-7 cells. MCF-7 cell cultures have been shown to exhibit a profound heterogeneity (Yang *et al.*, 1977; Barkley Butler *et al.*, 1986). For example, Resnicoff *et al.* (1987) showed that MCF-7 cells consist of different subpopulations, which originate from a single clone. Moreover, there exist several clones of this cell line which exhibit biological and phenotypical differences. The MCF-7 cell line used in this study consists of a subpopulation of small cells and a subpopulation of large cells. The phenotype of these MCF-7 cells was evaluated by immunocytochemistry and revealed a lack of vimentin filaments and estrogen receptor expression, albeit heterogeneously.

Since its establishment the MCF-7 cell line (Soule *et al.*, 1973) consists of several size variants. The relative numbers of small and large cells depends on the passages used (Yang *et al.*, 1977). The majority of early passages consist mainly of large cells, as described by Bani *et al.* (1994). In later passages (over 150), as used in this study, small cells have outnumbered the large cells. The heterogeneity within the cell line implies different responsiveness to nutrients, mitogenic and differentiation-inducing signals. At high concentration, ATRA changed the balance between large and small cells. At 10^{-6} M ATRA, large cells by far outnumbered the small cells, but the effect was concentration-related. At concentrations of 10^{-8} M and 10^{-7} M ATRA, small cells outnumbered the large cells, comparable to control cultures.

Figure 7: Two-dimensional gel electrophoresis of total protein from equal numbers of control cells (a) and cells treated with 10^{-6} M ATRA (b). - A Actin; cytokeratins 8, 18 and 19 are indicated by their number according to the catalog of Moll *et al.* (1982).

As shown by BrdU incorporation studies, the antiproliferative effects of ATRA on MCF-7 cells were potentiated by liarozole, as indicated previously by means of an MTT assay (Wouters *et al.*, 1992). The immunocytochemical detection of BrdU enabled the quantification of the differential ATRA-sensitivity in the subpopulations of small and large cells. The combination of liarozole (10^{-6} M) with 10^{-9} M and 10^{-8} M ATRA showed similar BrdU uptake rates for both cell populations. Thus, liarozole is able to potentiate the effects of ATRA to the same extent in both cell populations. Liarozole was unable to potentiate the effect of 10^{-6} M ATRA in large cells. This favours our hypothesis that liarozole potentiates the antiproliferative effects of ATRA via inhibition of its catabolism.

The synergism between liarozole and ATRA was observed on their differentiation-inducing effect on the heterogeneous MCF-7 cell population. As recently described by Bani Sacchi *et al.* (1994), E-cadherin staining is closely related to this morphological heterogeneity. The subpopulation of small cells showed a clear E-cadherin staining along the cell membranes. In contrast, in the subpopulation of large cells, E-cadherin staining was faint and solely localized in the cytoplasm. However, upon treatment with ATRA, E-cadherin staining was increased at the cellular membrane of both cell populations. These results are similar to those recently described for the SK-BR-3 human breast cancer cell line (Anzano *et al.*, 1994) and suggest induction of differentiation. The increased E-cadherin staining at the junctional complex was concomitant with the observed cytoskeletal changes. This is not surprising in light of the association of the cytoskeleton with cell-cell junctions (Takeichi, 1991; Gumbiner, 1993). Liarozole potentiated these ATRA-induced effects. The increased E-cadherin staining at cell-cell contacts might also have functional effects on the cells, as for example increased cell aggregation as seen for the invasive MCF-7/6 cell line variant after ATRA treatment (Bracke *et al.*, 1994). Given that E-cadherin expression is correlated with a differentiated non-invasive phenotype (Takeichi, 1991) and that ATRA enhances the functionality of E-cadherin (Bracke *et al.*, 1994), liarozole might exert anti-metastatic effects *in vivo* (Stearns *et al.*, 1993), where the drug has been shown to have retinoid activity (Van Wauwe *et al.*, 1992; Mahler *et al.*, 1993). This would have important clinical implications for the use of liarozole in the treatment of prostate cancer, in which decreased expression of E-cadherin has been correlated with tumor progression (Umbas *et al.*, 1992).

The expression of desmosomal proteins (desmoglein and desmoplakins I & II) was related to both MCF-7 cellular subtypes, but ATRA treatment had no influence on their staining. Also in FRIC B fetal rat intestine cells the expression of desmoplakins I & II did not change upon retinoid treatment (Plateroti *et al.*, 1993).

The changes in cell morphology and at the level of cell-cell junctions were accompanied by a profound reorganization of the cytoskeleton. ATRA treatment changed the organization of cytokeratin filaments so much that a clear network of cytokeratin filaments became visible, accompanied by a 50% down-regulation of cytokeratin expression. Liarozole potentiated this ATRA-induced reorganization of cytokeratin filaments. These effects were also seen when MCF-7 cells were treated with the neurohypophyseal peptide oxytocin (Cassoni *et al.*, 1994). Cytokeratin modulation by liarozole was also observed concomitant with a shift in the differentiation status in prostate tumors (Smets *et al.*, 1995). Down-regulation of cytokeratins in cancer cells is considered as an induction of differentiation by some authors (Kopan and Fuchs, 1989; Tomic *et al.*, 1990).

Analogous to the changes described for cytokeratins, ATRA had a profound effect on actin organization. ATRA induced numerous actin-containing stress fibers and focal adhesions. This effect, which also suggests induction of differentiation (Lehtonen *et al.*, 1983; Ng *et al.*, 1985), was enhanced by liarozole. Furthermore, the occurrence of actin-containing stress fibers has been correlated with anti-metastatic properties (Volk *et al.*, 1984). Similar changes in the organization of actin fibers have been described in connection with ATRA-treated F9 testicular teratocarcinoma cells (Lehtonen *et al.*, 1983), FRIC B fetal rat intestine cells (Plateroti *et al.*, 1993) and UMR 106-06 osteogenic sarcoma cells (Ng *et al.*, 1985). ATRA has been found to stimulate actin polymerization (Murali Krishna Rao, 1985). In contrast to down-regulation of cytokeratins, actin content was up-regulated upon ATRA treatment.

In conclusion, liarozole not only potentiated the antiproliferative effects of ATRA on MCF-7 cells, but also ATRA-induced differentiation. This potentiation is most probably due to inhibition of ATRA catabolism and agrees with previously obtained biochemical findings on the synergistic interaction of ATRA and liarozole in MCF-7 cells.

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3 **Liarozole-fumarate potentiates the all-*trans*-retinoic acid-induced differentiation of F9 teratocarcinoma cells by inhibition of the all-*trans*-retinoic acid catabolism**

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Abstract

F9 teratocarcinoma cells are nullipotent embryonic carcinoma cells that differentiate upon treatment with all-*trans*-retinoic acid (ATRA). To evaluate the potentiating effects of liarozole-fumarate on ATRA treatment, the present study was focused on its potentiation of ATRA-induced differentiation and on the inhibition of ATRA catabolism by liarozole-fumarate in F9 monolayer cell cultures. As markers for differentiation, the expression of E-cadherin, N-CAM, vimentin, cytokeratins 8, 18 and 19, actin as well as laminin and

collagen IV was analyzed by immunocytochemistry. Treatment with ATRA at concentrations ranging from 10^{-11} M to 10^{-6} M was compared to treatment with liarozole-fumarate at 10^{-6} M or 10^{-5} M, or to the combined treatment with ATRA and liarozole-fumarate. Liarozole-fumarate alone did not induce any phenotypic shift. ATRA between 10^{-7} M and 10^{-6} M changed expression of all markers, while lower concentrations only influenced laminin and collagen IV expression. Whereas 10^{-8} M ATRA was not capable of inducing or enhancing the expression of most markers, combination of 10^{-8} M ATRA with 10^{-6} M or 10^{-5} M liarozole-fumarate induced a F9 phenotype shift comparable to that obtained with 10^{-7} M ATRA alone. Liarozole-fumarate inhibited ATRA catabolism with an IC_{50} of 3 μ M. These results supports the hypothesis that liarozole-fumarate potentiates ATRA-induced differentiation in F9 through inhibition of the ATRA catabolism. The 10-fold higher IC_{50} value in F9 as compared to the previously obtained value in MCF-7 ($IC_{50} = 0.3 \mu$ M, Krekels *et al.*, 1997), could be explained by differences in uptake of both ATRA and liarozole-fumarate. About 5-fold less ATRA and 5-fold less liarozole-fumarate was taken up by F9 cells as compared to MCF-7 cells.

Introduction

Liarozole-fumarate is currently in phase III clinical trials for the treatment of relapsed prostate cancer. Pre-clinical animal models showed that liarozole-fumarate reduced tumor growth of androgen-dependent and androgen-independent Dunning R3327 rat prostate adenocarcinoma cells (Van Ginckel *et al.*, 1990; De Coster *et al.*, 1992; Dijkman *et al.*, 1994; Smets *et al.*, 1995). In an *in vitro* model with MCF-7 human breast carcinoma monolayers, both the antiproliferative and the differentiation-inducing effect of all-*trans*-retinoic acid (ATRA) were potentiated by liarozole-fumarate (Van heusden *et al.*, 1996) with a concomitant decrease of ATRA metabolites (Wouters *et al.*, 1992; Krekels *et al.*, 1997).

Both in biological models as well as in patients, the antitumoral effects of liarozole-fumarate are supposed to be linked to its retinoid-mimetic effects (Van Wauwe *et al.*, 1992; Mahler *et al.*, 1993). Liarozole-fumarate blocks the catabolic pathway of ATRA, by inhibition of the cytochrome P450-dependent 4-hydroxylation of ATRA and by inhibition of mono-

hydroxylation reactions of 4-oxo-ATRA (Van Wauwe *et al.*, 1990, 1994). This might be the mechanism of action through which liarozole-fumarate enhances endogenous plasma and tumor ATRA levels and also increases the plasma half-life of exogenously administered ATRA (Van Ginckel *et al.*, 1990; De Coster *et al.*, 1992; Smets *et al.*, 1995). As ATRA is a well-known inducer of differentiation, the observed induction of differentiation in tumors and tumor cell lines upon treatment with liarozole-fumarate (Smets *et al.*, 1995; Van heusden *et al.*, 1996) might thus be linked to the inhibition of ATRA catabolism by liarozole-fumarate. To further illustrate the differentiation-enhancing capacities of the liarozole-fumarate-ATRA chain, the present study was undertaken to evaluate the ATRA-induced differentiation in combination with liarozole-fumarate in F9 teratocarcinoma cells, as yet the best documented *in vitro* model for biochemical as well as morphological ATRA-linked differentiation. F9 is a nullipotent embryonic carcinoma cell line incapable to differentiate spontaneously *in vitro*. However, after treatment of F9 with ATRA, a process of differentiation is induced resembling the segregation of cell layers into visceral or parietal endoderm during gastrulation. In normal embryogenesis the visceral endoderm is the cell mass remaining associated with the embryoid body in the blastocyst, whereas the parietal endoderm is formed by migratory cells colonizing the inner surface of normal trophectoderm. The type of cell culture system used, influences the patterns of gene expression (Martin and Evans, 1975; Strickland *et al.*, 1976, 1978) and consequently, determines the development of cells with typical visceral endoderm- or parietal endoderm-like characteristics (Hogan *et al.*, 1983). When F9 cells are cultured as small aggregates, treatment with ATRA will induce differentiation towards embryoid bodies consisting of a core of undifferentiated stem cells surrounded by a polarized epithelium of visceral endoderm, typically secreting α -fetoprotein and the urokinase form of plasminogen activator (Hogan *et al.*, 1981). On the contrary, in F9 monolayer cell cultures, ATRA treatment induces differentiation in a fraction of the cells towards fibroblast-like cells in a sequence that is considered to model the mammalian equivalent of the differentiation from stem cells of the inner cell mass to parietal endoderm (Burdsal *et al.*, 1994). As such, a typical extraembryonic or parietal endoderm-type of differentiation is seen, characterized by the secretion of tissue plasminogen activator, the expression of cytokeratins or components of the thick basement membrane (Reichert's membrane) like laminin and collagen IV. Expression of cell adhesion molecules is embryologically and developmentally regulated and therefore, might serve as a morphogenetic marker in F9 differentiation upon ATRA treatment (Takeichi, 1991). The present study focused on ATRA-induced differentiation of F9 cells, grown as monolayers on gelatinized culture plates and investigated the inhibition of ATRA catabolism by liarozole-

fumarate. The potentiating effect of liarozole-fumarate on ATRA was monitored by evaluating the expression levels of E-cadherin, N-CAM, vimentin, cytokeratins 8, 18 and 19, actin as well as laminin and collagen IV. The F9 data are discussed in view of data previously obtained in MCF-7 monolayers.

Materials and Methods

Drugs and chemicals

The fumarate salt of liarozole [$\pm$ -5-[(3-chlorophenyl)(1*H*-imidazole-1-yl)methyl]-1*H*-benzimidazole (E)-2-butenedioate (2:3)] (R085246), and [14 C]liarozole-fumarate with a specific radioactivity of 882 MBq/mmol were synthesized at the Janssen Research Foundation (Beerse, Belgium). ATRA (Serva, Heidelberg, Germany) and [11, 12- 3 H(N)]ATRA (specific radioactivity of 1894.4 MBq/mmol) (DuPont NEN, Boston, MA, U.S.A.) were used in a dark room with yellow illumination. Stock solutions of liarozole-fumarate (10 mM) and ATRA (4 mM) were prepared in ethanol and appropriately diluted in culture medium. Final solvent concentration in the cell cultures was always less than 0.5%. All chemicals and solvents were of the highest purity available.

Cell lines

F9 mouse teratocarcinoma cells and MCF-7 human mammary carcinoma cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 4.5 g/liter glucose supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 50 μ g/ml gentamycin and with 15% fetal bovine serum for F9, while 10% fetal bovine serum was added for MCF-7 (all cell culture reagents from Life Technologies, Ghent, Belgium). Cells were grown in a humidified incubator (5% CO₂, 95% air) at 37°C and were *Mycoplasma* free as tested by the *Mycoplasma* T.C. kit (Gen-Probe Incorporated, CA, U.S.A.). For the experiments described in this study, F9 and MCF-7 cells between 10 and 15 passages were used.

Induction of differentiation (according to induction of differentiation in MCF-7 cells: Van heusden *et al.*, Eur. J. Cell Biol., 71: 89-98, 1996)

CELL CULTURE SYSTEM

F9 cells were plated onto Chamber Slides™ (Nunc Inc., Naperville, IL, U.S.A.) at a concentration of 500 cells/chamber. The Chamber Slides™ were pretreated with 20 μ g/ml poly-L-lysine and subsequently coated with 0.1% gelatin. Cells were allowed to attach for 24 h before the addition of the drugs and, then, incubated for 3 days under the following test conditions: ATRA at concentrations from 10⁻¹¹ M to 10⁻⁶ M, liarozole-fumarate at concentrations from 10⁻⁸ M to 10⁻⁵ M and liarozole-fumarate (10⁻⁶ M or 10⁻⁵ M) in combination with ATRA from 10⁻¹¹ M to 10⁻⁷ M.

IMMUNOCYTOCHEMISTRY

Cells grown with or without test compounds for 3 days were washed twice with serum-free culture medium. Cells were fixed in methanol (5 min, -20°C) and acetone (10 s, -20°C) and subsequently, air-dried. Aspecific antibody binding was prevented by blocking in 5% normal goat serum in 10 mM PBS (pH 7.4) for 1 h. Then cells were incubated for 1 h at room temperature in a dark moist chamber with the primary monoclonal antibody. After a series of washing steps in PBS, bound antibodies were visualized with the immunofluorescence technique using a secondary fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL, U.S.A.). Slides were air-dried and mounted in Mowiol (Hoechst, Frankfurt, Germany) containing 1% propidium iodide. The characteristics and references of the primary antibodies used in this study are summarized in Table I.

Table I: Primary antibodies used for immunocytochemistry on F9 cells

Antigen recognized	Antibody	Dilution	Ig subclass	Source : Primary
cytokeratin 8	LE41	1/100	IgG ₁ (monoclonal)	E.B. Lane, Dundee, U.K.
cytokeratin 18	RGE53	1/100	IgG ₁ (monoclonal)	F. Ramaekers, Maastricht, The Netherlands
cytokeratin 19	LP2K	1/100	IgG ₁ (monoclonal)	E.B. Lane, Dundee, U.K.
vimentin	RV202	undiluted	IgG ₁ (monoclonal)	F. Ramaekers, Maastricht, The Netherlands
actin	β -actin	1/1000	IgG ₁ (monoclonal)	Sigma Immunochemicals, St. Louis, MO, U.S.A.
collagen IV	M3F7	undiluted	IgG ₁ (monoclonal)	DSHB, Iowa City, IA, U.S.A.
laminin	186103	1/200	Ig (polyclonal)	DSHB, Iowa City, IA, U.S.A.
E-cadherin	ECCD-2	10 $\mu\text{g/ml}$	IgG ₁ (monoclonal)	Zymed, San Francisco, U.S.A.
N-CAM	Cl10008	10 $\mu\text{g/ml}$	IgG ₁ (monoclonal)	Cederlane, Ontario, U.S.A.

Inhibition of retinoic acid catabolism (according to IC₅₀ determination for MCF-7: Krekels *et al.*, Br. J. Cancer, 75: 1098-1104, 1997)

CELL CULTURE

F9 cultures were treated for 24 h with 10^{-6} M ATRA. Cells were then washed twice with 25 ml culture medium and trypsinized. Cells were resuspended at 4×10^6 cells/ml in DMEM without phenol red, containing 1 g/liter glucose and supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 50 $\mu\text{g/ml}$ gentamycin and 10% heat-inactivated fetal bovine serum (DMEM*) (all reagents from Life Technologies). Aliquots (450 μl) of this cell suspension were pre-incubated for 5 min at 37°C with liarozole-fumarate or the solvent in glass test tubes. Incubation was then continued for 90 min in the presence of 25 μl [11, 12- $^3\text{H}(\text{N})$]ATRA. At the end of the respective incubation, the reaction mixture was analyzed for retinoic acid metabolites using a quantitative microcolumn assay.

MICROCOLUMN ASSAY

The reaction mixture was mixed with 2 ml 100% acetonitrile. After centrifugation for 10 min at 780g the resulting protein-free supernatant was acidified with 2.5 ml 40 mM acetic acid. The acidified liquid was applied to a 3 ml C18 Bond Elut LRC column (Varian, Harbor City, CA, U.S.A.) under a vacuum of 127 mmHg using a VAC ELUT SPS-24 and the effluent (fraction 1) was collected. The column was then eluted with 1 ml of 40% acetonitrile in water (fraction 2). Fractions 1 and 2 were combined and the collected effluent was counted for radioactivity in a Tri-carb 4530 liquid scintillation analyzer. Optiphase "HiSafeII" (Wallac, Milton Keynes, United Kingdom) was used as a scintillator. Experiments were performed three times and duplicate values were obtained for each data point. IC_{50} values were calculated by linear regression analysis.

QUANTIFICATION OF ATRA AND LIARAZOLE-FUMARATE UPTAKE IN MCF-7 AND IN F9 CELLS

F9 and MCF-7 cell cultures were treated for 24 h with 10^{-6} M ATRA. Cells were then washed twice with 25 ml culture medium, harvested and resuspended at 4×10^6 cells/ml in DMEM*. Aliquots (2 ml) of this cell suspension were incubated for 2 h at 37°C with [14 C]liarozole-fumarate at 10^{-5} M and 10^{-6} M or with [11 , 12 - 3 H(N)]ATRA at 10^{-7} M and 10^{-8} M. Cells were washed twice with 2 ml PBS by centrifugation at 4°C. The final cell pellet was solubilized for 30 min at 20°C with 1 ml 5% Triton X-100; 10 ml optiphase "Hi Safe II" scintillation liquid (Wallac, Milton Keynes, United Kingdom) was added and the total fraction counted in a Packard Tri-carb 4530 liquid scintillation analyzer.

The ratio for uptake in MCF-7 versus uptake in F9 was then calculated. Experiments were performed three times and triplicate values were obtained for each data point.

Results

Liarozole-fumarate enhances ATRA-induced differentiation in F9 cells

GENERAL MORPHOLOGY

The differentiation-inducing effect of ATRA was tested in a concentration range from 10^{-11} M to 10^{-6} M. Liarozole-fumarate was studied at 10^{-6} M and at 10^{-5} M, as a single treatment or in combination with the respective ATRA concentrations. Upon treatment with ATRA at 10^{-7} M and at 10^{-6} M, all cells developed a flattened morphology but only a minority of cells ($\pm 20\%$) acquired a phenotype that resembled neuronal cells characterized by long cellular branched axon-like processes (Figs. 3, 4, 5). From 10^{-8} M on, the induction of the neuronal-like differentiation was less than 3%. Liarozole-fumarate alone did not induce any change in the basal F9 morphology. However, the combined treatment of 10^{-6} M or 10^{-5} M liarozole-fumarate with ATRA at 10^{-9} M, 10^{-8} M and 10^{-7} M potentiated the ATRA effect. As an example, the combined treatment of 10^{-6} M liarozole-fumarate with ATRA at 10^{-8} M resulted in a cell

culture composed of about 20% neuronal-like cells mixed with otherwise undifferentiated cells, comparable to the image after treatment with single ATRA treatment at 10^{-7} M.

EXPRESSION OF LAMININ AND COLLAGEN IV

Treatment with 10^{-8} M to 10^{-6} M ATRA induced expression of the extracellular matrix proteins, laminin (Fig. 1) and collagen IV (Fig. 2), in all cells at 10^{-6} M to about 50% of the cells at 10^{-8} M. However, at 10^{-9} M the number of laminin and collagen IV immunopositive cells sharply decreased to about 20%. At 10^{-10} M ATRA, only very few cells ($<1\%$) did immunostain, whereas at 10^{-11} M no cells were found immunopositive anymore. Cells treated with vehicle or with 10^{-6} M or 10^{-5} M liarozole-fumarate were only weakly immunostained at their cell border comparable to control conditions. However, treatment with the combination of 10^{-6} M or 10^{-5} M liarozole-fumarate and ATRA at 10^{-9} M, 10^{-8} M and 10^{-7} M potentiated the ATRA effect. As an example, the treatment with 10^{-9} M ATRA together with 10^{-6} M or 10^{-5} M liarozole-fumarate increased the matrix protein expression in about 50% cells, resembling the image obtained with 10^{-8} M ATRA alone. There was no differential immunostaining between the cells with the neuronal phenotype and the other cells.

Figure 1: Expression of laminin in F9 monolayer cell cultures in vehicle treated cells (a), in 10^{-6} M liarozole-fumarate treated cells (b), in 10^{-7} M ATRA treated cells (c), in 10^{-8} M ATRA + 10^{-6} M liarozole-fumarate treated cells (d). - Bar 20 μ m.

Figure 2: Expression of collagen IV in F9 monolayer cell cultures in vehicle treated cells (a), in 10^{-6} M liarozole-fumarate treated cells (b), in 10^{-7} M ATRA treated cells (c), in 10^{-8} M ATRA + 10^{-6} M liarozole-fumarate treated cells (d). - Bar 20 μ m.

EXPRESSION OF CYTOSKELETAL PROTEINS (CYTOKERATINS 8, 18, 19; VIMENTIN; β -ACTIN)

The cytoskeletal proteins cytokeratins, vimentin and β -actin responded differentially upon ATRA treatment. Cytokeratins 8, 18 and 19 were absent in control conditions and became intensely expressed after 10^{-7} M or 10^{-6} M ATRA treatment, but only in a low percentage of the cells ($\pm 20\%$). As an example, Figure 3 represents the expression obtained for cytokeratin 18 in the different treatment conditions. Strikingly, the neuronal-like cell type was the major cell phenotype staining for cytokeratin. Concentrations of ATRA lower than 10^{-7} M were not effective in inducing cytokeratin expression. Single liarozole-fumarate treatment at 10^{-6} M or 10^{-5} M resulted in a F9 phenotype which was identical to control condition. However, the combination of 10^{-6} M or 10^{-5} M liarozole-fumarate with ATRA at 10^{-8} M and 10^{-7} M potentiated the ATRA effect. As an example, the same number and appearance of cytokeratin immunopositive F9 cells was visible after the combined 10^{-8} M ATRA and 10^{-6} M liarozole-fumarate treatment as compared to treatment with 10^{-7} M ATRA alone. In contrast to cytokeratins, both vimentin (Fig. 4) and β -actin were expressed under all conditions. Treatment with either ATRA alone or liarozole-fumarate in combination with ATRA -at any concentration tested- did not induce any changes in the expression of vimentin or β -actin.

EXPRESSION OF CELL ADHESION MOLECULES (E-CADHERIN, N-CAM)

The cell adhesion molecule E-cadherin (Fig. 5) was expressed in all F9 cells at the cell-cell boundary in control condition. Liarozole-fumarate treatment (10^{-6} M or 10^{-5} M) resulted in a F9 phenotype which was identical to that of the control condition. However, treatment with ATRA at 10^{-7} M or 10^{-6} M downregulated E-cadherin expression in all cells. The neuronal-like cells, appearing by ATRA treatment, also showed E-cadherin expression (Fig. 5). Concentrations of ATRA below 10^{-7} M were ineffective in changing E-cadherin expression. However, the combination of 10^{-6} M or 10^{-5} M liarozole-fumarate with ATRA at 10^{-8} M and 10^{-7} M potentiated the ATRA effect. As an example, 10^{-8} M ATRA combined with 10^{-6} M or 10^{-5} M liarozole-fumarate downregulated E-cadherin expression to the same extent as 10^{-7} M ATRA alone.

N-CAM was expressed in all F9 cells at control conditions. Liarozole-fumarate treatment (10^{-6} M or 10^{-5} M) resulted in a F9 phenotype which was identical to that of the control condition. Treatment with ATRA at concentrations ranging from 10^{-7} M to 10^{-6} M increased N-CAM immunostaining in a subpopulation of cells (30%) without any specific phenotypical appearance. At the same time, N-CAM was downregulated in most other cells. There was no link between the appearance of the neuronal-like cell type and N-CAM expression. The

Figure 3: Expression of cytokeratin 18 in F9 monolayer cell cultures in vehicle treated cells (a), in 10^{-6} M liarozole-fumarate treated cells (b), in 10^{-6} M ATRA treated cells (c), in 10^{-7} M ATRA + 10^{-6} M liarozole-fumarate treated cells (d). - Bar 10 μ m.

combination of 10^{-6} M or 10^{-5} M liarozole-fumarate with ATRA at 10^{-8} M and 10^{-7} M potentiated the ATRA effect. As an example, 10^{-8} M ATRA combined with 10^{-6} M or 10^{-5} M liarozole-fumarate increased N-CAM immunostaining to the same extent as 10^{-7} M ATRA alone.

COMPARISON OF POTENTIATION OF LIAROZOLE-FUMARATE AT 10^{-6} M AND 10^{-5} M

For all the previously described markers, the number of immunopositive cells was the same after the combined treatment of ATRA with liarozole-fumarate at 10^{-6} M or 10^{-5} M. Thus, no differences in potentiation by the two liarozole-fumarate concentrations of the ATRA-induced differentiation were observed.

Liarozole-fumarate inhibits ATRA catabolism in F9 cells

Using 10^{-7} M [3 H]ATRA as the substrate, liarozole-fumarate was shown to inhibit ATRA catabolism with an IC_{50} of 3 μ M (Fig. 6). This IC_{50} value was 10 times higher than the IC_{50} value previously measured in MCF-7 cells (0.3 μ M) (Krekels *et al.*, 1997).

Figure 4: Expression of vimentin in F9 monolayer cell cultures in vehicle treated cells (a), in 10^{-6} M liarozole-fumarate treated cells (b), in 10^{-6} M ATRA treated cells (c), in 10^{-7} M ATRA + 10^{-6} M liarozole-fumarate treated cells (d). - Bar 20 μ m.

Figure 5: Expression of E-cadherin in F9 monolayer cell cultures in vehicle treated cells (a), in 10^{-6} M liarozole-fumarate treated cells (b), in 10^{-6} M ATRA treated cells (c), in 10^{-7} M ATRA + 10^{-6} M liarozole-fumarate treated cells (d). - Bar 10 μ m.

Figure 6: Inhibition of ATRA catabolism by liarozole-fumarate.

Uptake of [^3H]ATRA and [^{14}C]liarozole-fumarate in MCF-7 cells versus F9 cells

The uptake of both ATRA and liarozole-fumarate was quantified in the two different cell lines and the respective ratio calculated. For [^3H]ATRA at 10^{-8} M and 10^{-7} M, the mean ratio of uptake in MCF-7 versus uptake in F9 was 3.4 ± 1.6 . For [^{14}C]liarozole-fumarate at 10^{-6} M and 10^{-5} M, the mean ratio of uptake in MCF-7 versus uptake in F9 was 6.9 ± 2.1 . The respective ratios at each of the two concentrations of the respective compounds are given in Table II. Considerably lower uptake of both ATRA and liarozole-fumarate was thus seen in F9 cells as compared to MCF-7 cells.

Table II: Ratio for uptake of ATRA and of liarozole-fumarate in MCF-7 versus F9

[^3H]ATRA	uptake MCF-7/uptake F9
10^{-8} M	5.0 ± 1.9
10^{-7} M	3.9 ± 1.5
	mean = 3.4 ± 1.6
[^{14}C]liarozole-fumarate	uptake MCF-7/uptake F9
10^{-6} M	5.6 ± 1.5
10^{-5} M	8.1 ± 1.9
	mean = 6.9 ± 2.1

Discussion

The present study confirms and extends the knowledge about the differentiation-inducing effects of ATRA in F9 monolayer cell cultures and, in addition, demonstrates the potentiation of this ATRA-induced differentiation process by liarozole-fumarate. ATRA at concentrations of 10^{-7} M and 10^{-6} M was mostly effective in inducing differentiation. Moreover, the potentiation by liarozole-fumarate was due to inhibition of the ATRA catabolism (IC_{50} of $3 \mu\text{M}$).

ATRA-induced differentiation in F9 has been extensively described with ATRA at 10^{-6} M, but not yet in a concentration-responsive way. The present study demonstrated that only 10^{-7} M

and 10^{-6} M ATRA-induced differentiation while lower concentrations were not active, except for the induction of laminin and collagen IV expression. These effects were noticed for both general phenotypic cellular appearance and for specific markers such as cytokeratins, E-cadherin and N-CAM. ATRA concentrations ranging from 10^{-7} M to 10^{-6} M induced two phenotypes: although all cells apparently flattened and got larger, about 20% of the cells differentiated into a neuronal-like cell type with long slender multiple branched outgrowths comparable to axons. Whereas most authors assume that ATRA treatment converts the epithelioid-like colonies of rounded undifferentiated F9 cells into triangular and flattened fibroblasts (for an overview: Burdsal *et al.*, 1994), some authors described such an induction of a neuronal-like phenotype, but appearing in all cells of a F9 monolayer (Linder *et al.*, 1981). Whether or not these discrepancies are related to the passage number of F9 cells cultures remains to be established.

Between 10^{-8} M and 10^{-6} M, ATRA enhanced the expression of laminin and collagen IV. These data confirm the previously described upregulation of these extracellular matrix proteins in F9 monolayers upon ATRA treatment (Strickland and Mahdavi, 1978; Vasios *et al.*, 1991; Maillet and Shur, 1994). Potentiation of these ATRA-linked effects was seen after liarozole-fumarate treatment: the combination of 10^{-9} M ATRA with liarozole-fumarate enhanced the expression of both markers to the same extent as treatment with 10^{-8} M ATRA alone. In contrast to the other markers, the expression of laminin and collagen IV was still upregulated by 10^{-8} M ATRA and potentiated at 10^{-9} M ATRA in combination with liarozole-fumarate treatment. Apparently, laminin and collagen IV are more sensitive to ATRA differentiation than the other investigated markers, the latter being induced only at 10^{-7} M and at 10^{-6} M. The induction of laminin and collagen IV expression appears to be directly regulated through ligated RAR binding on the corresponding RARE of their gene promoters. E.g. laminin B1 gene transcription occurred in F9 cells in function of ATRA treatment (Vasios *et al.*, 1991). Chronologically, first RAR β was expressed 3 h after ATRA addition followed by the transcription of the laminin B1 gene. The induction of laminin B1 appeared to be mediated by direct binding of ligated RAR to three direct repeat TGACC-related motifs (RARE). There are several reports on the induction of cytokeratin expression in F9 cells treated with ATRA at 1 μ M (Ramaekers *et al.*, 1984; Trevor *et al.*, 1985, 1987, 1992; Kurki *et al.*, 1989; Pankov *et al.*, 1994). The present study confirms these previous reports and illustrates the 10-fold potentiating effect of liarozole-fumarate on cytokeratin expression. Liarozole-fumarate potentiated the differentiation effect of ATRA at 10^{-8} M and induced a F9 phenotype comparable to that obtained with ATRA alone at 10^{-7} M. Strikingly, the cells with the neuronal phenotype were the only ones which showed expression of cytokeratin upon ATRA treatment.

Their concomitant expression of vimentin is characteristic for an as yet rather low-grade neuronal differentiation.

On the other hand, the differentiation of a small fraction of F9 cells towards a phenotype with such neuronal-like characteristics might explain why the expression of E-cadherin declined upon ATRA treatment with a concomitant upregulation of N-CAM. The absence of N-CAM from the neuronal-like cell type appearing after ATRA treatment is an as yet unexplained phenomenon. The cadherins and CAM's are spatiotemporally regulated during development and associated with a variety of morphogenetic events (Takeichi, 1988). E.g., during chicken embryogenesis, the ectoderm of the neural tube originally expresses E-cadherin in the entire region, but the part of the ectoderm that differentiates into the neuronal tube only gradually turns off expression of E-cadherin, to be replaced by N-cadherin.

Uptake of both ATRA and liarozole-fumarate was about 5-fold lower in F9 than in MCF-7. These differences in uptake at least partially explain the 10 times higher IC_{50} value for inhibition of the retinoic acid catabolism by liarozole-fumarate in F9 ($IC_{50} = 3 \mu M$) as compared to MCF-7 ($IC_{50} = 0.3 \mu M$, Krekels *et al.*, 1997). There was no difference in the number of immunopositive cells between the two tested liarozole-fumarate concentrations of $10^{-6} M$ (close to IC_{50}) and $10^{-5} M$ ($> IC_{50}$). The intensity of immunostaining is not sufficiently analytical, however, to evaluate whether eventually a higher amount of protein within the immunopositive cells was present at $10^{-5} M$ liarozole-fumarate as compared to $10^{-6} M$.

Not unexpectedly, the shifts in the expression of the same markers by ATRA and their concomitant potentiation by liarozole-fumarate were not comparable in F9 and MCF-7. F9 is an embryonic cell line differentiating towards predominant fibroblastoid endoderm, whereas MCF-7 is a typical epithelial tumor cell line with other phenotypical characteristics.

In conclusion, ATRA concentration dependently modulated expression in F9 of laminin, collagen IV, cytokeratins 8, 18, 19, vimentin, actin, E-cadherin and N-CAM. Liarozole-fumarate potentiated the ATRA effects by inhibition of catabolism.

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Fluorescein-labeled tyramide strongly enhances the detection of low bromodeoxyuridine incorporation levels

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Abstract

Immunocytochemical detection of bromodeoxyuridine (BrdU) labeling can be hampered by low BrdU incorporation levels. We describe here an amplification method for weak BrdU immunosignals.

The tyramide signal amplification method, based on catalyzed reporter deposition (CARD) uses fluorescein-labeled tyramide as a substrate for horseradish peroxidase. The enzyme catalyzes the formation of highly reactive tyramide radicals with a very short half-life, resulting in the binding

of fluorescein-conjugated tyramide only at the site of the enzymatic reaction. MCF-7 cells were grown *in vitro* in medium containing charcoal-stripped fetal bovine serum supplemented by growth factors. Under these culture conditions, the BrdU immunosignal was hard to detect, but could be enhanced specifically by the tyramide signal amplification system, resulting in clear-cut differences between BrdU-negative and BrdU-positive cells. This enabled a rapid and objective quantification of the BrdU labeling index without the risk of underestimating the number of cells in S-phase. Therefore, this amplification of BrdU immunosignals might also prove valuable for *in vivo* cancer prognosis, cell kinetics studies, and computer-assisted image analyses.

Introduction

Assessment of cell proliferation is considered a basic parameter in cancer studies. A range of techniques have evolved to quantify this process (Boulton and Hodgson, 1995). Bromodeoxyuridine (BrdU) labeling is an accurate method to measure cell proliferation because it is a direct assay of DNA synthesis (Dolbeare, 1995*a*, 1995*b*). BrdU is a halogenated derivative of thymidine that is incorporated into DNA only during S-phase of the cell cycle. It can be detected specifically by monoclonal antibodies with immunocytochemical techniques (Gratzner, 1982). Pulse labeling and a subsequent chase of BrdU allows the determination of dynamic proliferation parameters (Tinnemans *et al.*, 1993).

However, the immunocytochemical detection of BrdU labeling can be hampered by low BrdU incorporation levels. Low incorporation levels of labeled nucleotides can occur as a consequence of altered cell growth rate (Aakvaag *et al.*, 1990), which can be the result of the culture conditions used, such as the use of charcoal-stripped serum instead of untreated serum (Roman *et al.*, 1993). This would result in weakly immunostained nuclei. In such cases, amplification of the BrdU signal is necessary to eliminate the considerable risk of underestimating the number of cells in S-phase and to ensure an objective quantification of the BrdU labeling index.

Catalyzed reporter deposition (CARD) is a signal amplification method that has been described by Bobrow *et al.* (1989, 1991). The amplification is achieved by biotin- or fluorescein-labeled tyramides that act as substrates for horseradish peroxidase. The horseradish peroxidase catalyzes the formation of highly reactive tyramide radicals that bind covalently to tyrosine

residues (Bobrow *et al.*, 1989, 1991). Because of the extremely short half-life of the tyramide radicals, only tyrosine residues in the direct surroundings of the horseradish peroxidase will bind tyramide (Bobrow *et al.*, 1989, 1991). The biotin-labeled tyramide can then be visualized by fluorochrome- or enzyme-labeled streptavidin.

Although originally developed for ELISA, this technique has been adapted for immunohistochemistry on tissue sections (Adams, 1992; Berghorn *et al.*, 1994; Merz *et al.*, 1995) and for *in situ* hybridization (Kerstens *et al.*, 1995; Raap *et al.*, 1995).

In this study, MCF-7 human breast cancer cells were used, grown *in vitro* in medium containing charcoal-stripped fetal bovine serum supplemented by growth factors. Under these culture conditions, these cells are known to have an increased cell doubling time (Berthois *et al.*, 1986; Aakvaag *et al.*, 1990; Roman *et al.*, 1993), resulting in low BrdU incorporation levels and thus weak BrdU immunosignals. Fluorescein-labeled tyramide (Bobrow *et al.*, 1991) was used as a substrate for horseradish peroxidase to amplify specifically the weak immunocytochemical signal of BrdU labeling. The tyramide signal amplification method enabled a rapid and objective quantification of the BrdU labeling index without the risk of underestimating the number of cells in S-phase.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter glucose, phenol red-free DMEM with 1 g/liter glucose, and 1 mM sodium pyruvate, fetal bovine serum (FBS), L-glutamine, gentamicin, bovine insulin, basic fibroblast growth factor, sodium selenite, and transferrin were purchased from Life Technologies (Ghent, Belgium).

Dextran T500 was obtained from Pharmacia (Uppsala, Sweden) and activated charcoal from Sigma (St. Louis, MO, U.S.A.). Charcoal-stripped FBS was prepared by treating FBS with dextran-coated charcoal (DCC-FBS), according to the protocol of Horwitz and McGuire (1978).

Monoclonal antibody to BrdU (clone BU-1) containing nuclease activity was purchased from Amersham (Poole, United Kingdom). Secondary biotinylated goat anti-mouse IgG was obtained from DAKO A/S (Prosan; Ghent, Belgium) and secondary fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG from Southern Biotechnology Associates (Birmingham, AL, U.S.A.). The Tyramide Signal Amplification (TSA)-Direct kit (Green) was obtained from DuPont NEN Products (Brussels, Belgium) and contained blocking reagent, horseradish peroxidase-conjugated streptavidin, 2X concentrated amplification diluent, and fluorescein-labeled tyramide.

Cell Culture

Routinely, MCF-7 human mammary carcinoma cells, purchased from the American Type Culture Collection (Rockville, MD, U.S.A.), were cultured in DMEM with 4.5 g/liter glucose supplemented by 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 µg/ml gentamicin. The MCF-7 subclone used in this study has been described previously (Van heusden *et al.*, 1996). Cells were grown in a humidified incubator (5% CO₂, 95% air) at 37°C and were *Mycoplasma*-free.

For the growth experiments, cells were cultured for 6 days in phenol red-free DMEM containing 5% DCC-FBS, 4.5 g/liter glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µg/ml gentamicin, 30 nM sodium selenite, and 10 µg/ml transferrin. Then cells were seeded onto Chamber Slides™ (Nunc, Naperville, IL, U.S.A.), coated with 50 µg/ml poly-L-lysine (PLL) 1 day before use, at a concentration of 15,000 cells/chamber. Cells were allowed to attach for 24 h and thereafter growth factors were added (10 µg/ml final concentration insulin and 5 ng/ml final concentration basic fibroblast growth factor). Cells were grown under these conditions for 7 days, with medium changes 3 and 6 days after seeding. Control cells, growing in medium containing 10% untreated FBS, were seeded from stock cultures onto PLL-coated Chamber Slides™ at a concentration of 2500 cells/chamber. Cells were grown under these conditions for 7 days, with medium changes 3 and 6 days after seeding.

BrdU labeling and immunodetection

Cells grown for 7 days under the different culture conditions described above, were incubated for 2.5 h with an excess (100 µM) of BrdU at 37°C in an incubator. Thereafter, cells were washed twice with serum-free culture medium and fixed in methanol (5 min, -20°C) and acetone (10 s, -20°C), and air-dried. Cells were then ready to use for BrdU detection, according to the following Protocols A or B.

PROTOCOL A: BRDU DETECTION BY CONVENTIONAL IMMUNOFLUORESCENCE STAINING

1. Slides were blocked in 5% normal goat serum in 10 mM phosphate buffered saline (PBS) (pH 7.4) for 1 h in a dark, moist chamber at room temperature (RT).
2. Slides were rinsed three times for 10 min with PBS at RT.
3. Slides were incubated with monoclonal antibody against BrdU containing nuclease (diluted 1:5 in PBS) for 5 h at RT in a dark, moist chamber. Omission of the primary antibody did not reveal any signal. Different incubation times (1 h, 3 h, 4 h, 5 h, and 16 h) and antibody dilutions (1:2, 1:5, 1:8, 1:10) were tested. The best results were obtained with an incubation time of 5 h and an antibody dilution of 1:5.
4. Slides were rinsed three times for 10 min with PBS at RT and stored overnight.
5. Slides were incubated with FITC-labeled goat anti-mouse IgG (diluted 1:60 in PBS) for 1 h at RT in a dark, moist chamber.
6. Slides were rinsed three times for 10 min with PBS at RT.
7. Slides were postfixed for 10 min in methanol at RT.
8. Slides were postfixed for 10 min in 4% paraformaldehyde in PBS at RT.
9. Slides were rinsed three times for 10 min with PBS at RT.

10. Slides were rinsed three times for 10 min with distilled water at RT.
11. Slides were air-dried and mounted in Mowiol containing propidium iodide (PI, 2 µg/ml final concentration) and RNase (20 µg/ml final concentration) to stain nuclei.

PROTOCOL B: BRdU DETECTION BY TYRAMIDE SIGNAL AMPLIFICATION

1. Endogenous peroxidase activity was blocked by incubating slides in 0.03% H₂O₂ diluted in PBS for 30 min at room temperature (RT).
2. Slides were washed twice for 5 min in PBS at RT.
3. Slides were blocked in 5% normal goat serum in 10 mM PBS (pH 7.4) for 1 h at RT in a dark, moist chamber.
4. Slides were rinsed three times for 10 min with PBS at RT.
5. Slides were incubated with monoclonal antibody against BrdU containing nuclease (diluted 1:8 in PBS) for 5 h at RT in a dark, moist chamber. Omission of the primary antibody did not reveal any signal. Different incubation times (1 h, 3 h, 4 h, and 5 h) and antibody dilutions (1:2, 1:5, 1:8, 1:10) were tested. The best results were obtained with an incubation time of 5 h and an antibody dilution of 1:8.
6. Slides were rinsed three times for 10 min with PBS at RT and stored overnight.
7. Slides were incubated with biotinylated goat anti-mouse IgG (diluted 1:300 in PBS) for 30 min at RT in a dark, moist chamber.
8. Slides were rinsed three times for 5 min with PBS at RT.
9. Slides were blocked with 0.5% DuPont Blocking Reagent in 0.1 M Tris-HCl (pH 7.5) containing 0.15 M NaCl (TBS) for 30 min at RT in a dark, moist chamber.
10. Slides were incubated for 30 min with streptavidin-HRP diluted 1:500 in TBS containing 0.5% DuPont Blocking Reagent at RT in a dark, moist chamber.
11. Slides were rinsed three times for 5 min with TBS containing 0.05% Tween 20 (TBST) by shaking at RT.
12. Slides were incubated with fluorescein-labeled tyramide diluted 1:50 in 1X amplification buffer for 10 min at RT in a dark, moist chamber. Different incubation times (1 min, 5 min, and 10 min) and dilutions (1:25 and 1:50) were tested. An incubation of 10 min and a dilution of 1:50 gave the best results.
13. Slides were rinsed three times for 5 min with TBST by shaking.
14. Slides were rinsed three times for 5 min with distilled water at RT.
15. Slides were postfixed in methanol for 5 min at RT.
16. Slides were air-dried and mounted in Mowiol containing PI (2 µg/ml final concentration) and RNase (20 µg/ml final concentration) to stain nuclei.

The BrdU labeling index, i.e. the percentage of cells in S-phase of the cell cycle, was determined by counting cells under a fluorescence microscope (Axiophot; Zeiss, Oberkochen, Germany) with a dual filter set for simultaneous visualization of both fluorescein and PI. About 800 cells were counted twice for each test condition per experiment. Results are presented as mean $\pm$ SEM ($n = 3$).

Statistical analysis

Data were analyzed with the two-tailed Mann-Whitney U-test using Stat View II software (Abacus Concepts; Berkeley, CA, U.S.A.). Significance was defined at the level of $p < 0.05$.

Results

Control MCF-7 cells, grown in medium containing untreated FBS, showed a BrdU labeling index (Table I) of $36.8 \pm 1.3\%$, as described previously (Van heusden *et al.*, 1996). On BrdU immunodetection, normal BrdU incorporation levels were observed, which resulted in strongly immunoreactive nuclei.

In contrast, cells grown in medium containing charcoal-stripped FBS supplemented with growth factors showed low BrdU incorporation levels. This was reflected by weak BrdU immunosignals, which were hard to detect, using the conventional immunofluorescence staining procedure (Fig. 1a). BrdU-positive nuclei were stained faintly and showed a heterogeneous punctate staining pattern (Fig. 1a, inset). The BrdU labeling index (Table I) was $27.9 \pm 0.6\%$, which was statistically different ($p < 0.05$) from that of cells grown in medium containing untreated serum.

The tyramide signal amplification method strongly enhanced the BrdU immunosignal (Fig. 1b) of cells grown in medium containing charcoal-stripped serum. The nuclei were now stained intensely and the majority of the cells were homogeneously BrdU-positive (Fig. 1b, inset), whereas some cells partially retained their original punctate staining (Fig. 1b, inset).

Table I: BrdU labeling index for MCF-7 cells

culture conditions	BrdU labeling index ^a	
	conventional immunofluorescence	TSA amplification
10% untreated FBS	$36.8 \pm 1.3\%$	ND ^b
5% DCC-FBS + growth factors	$27.9 \pm 0.6\%$ ^c	$26.9 \pm 0.9\%$

^a Mean $\pm$ SEM ($n = 3$).

^b ND = not done.

^c Hardly detectable.

Figure 1: BrdU immunodetection in MCF-7 human breast cancer cells grown in medium containing 5% charcoal-stripped fetal bovine serum supplemented with growth factors. Visualization was achieved by (a) conventional immunofluorescence staining or (b) tyramide signal amplification. - Bars 20 μm , insets 10 μm .

On immunofluorescence microscopy, no significant fading of the fluorescein signal was observed, in contrast to the conventional immunofluorescence staining preparations, most probably owing to the intense amplification of the immunosignal.

After visualization with the tyramide signal amplification method, a BrdU labeling index (Table I) of $26.9 \pm 0.9\%$ was obtained which was not statistically different ($p = 0.14$) from the labeling index (Table I) of $27.9 \pm 0.6\%$ that was obtained with normal immunofluorescence staining. This means that no additional cells became labeled after the tyramide signal amplification.

Discussion

MCF-7 cells, grown in medium containing charcoal-stripped FBS supplemented by growth factors, showed a decreased BrdU labeling index compared to that of cells grown in medium containing untreated fetal bovine serum. This was not surprising because it is known that MCF-7 cells are estrogen-dependent (Brooks *et al.*, 1973; Aakvaag *et al.*, 1990) and the MCF-7 cells used in this study were cultured essentially in the complete absence of estrogens by the use of phenol red-free medium and charcoal-stripped serum (Horwitz and McGuire, 1978; Berthois *et al.*, 1986). Furthermore, MCF-7 cells grown in medium containing charcoal-stripped serum and insulin showed an increased cell doubling time and lowered thymidine incorporation in the absence of 17 β -estradiol (Aakvaag *et al.*, 1990).

As a consequence of these culture conditions low BrdU incorporation levels were observed, most probably due to an increased cell doubling time (Berthois *et al.*, 1986; Aakvaag *et al.*, 1990; Roman *et al.*, 1993). This resulted in weak BrdU immunosignals that made quantification of the BrdU labeling index very labor-intensive and time-consuming and, more importantly, a considerable risk remained for underestimating the number of cells in S-phase.

Therefore, an immunofluorescence amplification procedure using fluorescein-labeled tyramide, based on the principle of CARD, was developed for cell cultures to enhance specifically the BrdU immunosignals. In contrast to the weak, heterogeneous BrdU staining using the conventional immunofluorescence protocol, the tyramide signal amplification method resulted in a much clearer and homogeneous BrdU immunosignal in the majority of the cells. The difference between BrdU-positive and BrdU-negative cells became clear-cut, which made quantification rapid, easy, and objective without the risk of underestimating the number of cells in S-phase. Some cells stained intensely but partially retained their punctate staining pattern, which is indicative of the specificity of the tyramide signal amplification in that it is restricted to the immediate surroundings of the HRP deposits. The BrdU labeling index determined after tyramide signal amplification was the same as that determined after conventional immunofluorescence staining. This suggests that the tyramide signal amplification method is specific and that no additional cells became positive. The tyramide signal amplification for low BrdU levels might also lend itself to providing more accurate results for prognosis after *in vivo* BrdU administration.

Another application of the tyramide signal amplification might be in cell kinetics studies. For that purpose, it would be necessary to adapt the current fluorescent amplification procedure so that cells could be stained in suspension for flow cytometric analysis.

Furthermore, the tyramide signal amplification method might help in the development of computer-assisted image analysis. For this application, it is necessary to obtain consistently clear and stable immunostaining, resulting in high-contrast images that facilitate the identification of labeled and unlabeled nuclei.

In conclusion, we have described an amplification method using CARD to amplify specifically weak BrdU immunosignals due to low BrdU incorporation levels. Amplification was achieved by the use of fluorescein-labeled tyramide as a substrate for horseradish peroxidase. The tyramide signal amplification method holds the promise to be of great value for *in vivo* cancer prognosis, cell kinetics studies, flow cytometry, and computer-assisted image analysis.

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All-*trans*-retinoic acid metabolites significantly inhibit the proliferation of MCF-7 human breast cancer cells *in vitro*

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Abstract

All-*trans*-retinoic acid (ATRA) is well known to inhibit the proliferation of human breast cancer cells. Much less is known about the antiproliferative activity of the naturally occurring metabolites of ATRA. In the present study, we investigated the antiproliferative activity of ATRA, its physiological catabolites 4-oxo-ATRA and 5,6-epoxy-ATRA and isomers 9-*cis*-RA and 13-*cis*-RA in MCF-7 human breast cancer cells by bromodeoxyuridine incorporation.

MCF-7 cells were grown in steroid- and retinoid-free medium supplemented

with growth factors. Under these culture conditions, ATRA and its naturally occurring catabolites and isomers showed significant antiproliferative activity in MCF-7 cells in a concentration-dependent manner (10^{-11} M to 10^{-6} M). The antiproliferative activity of ATRA catabolites and isomers was equal to that of the parent compound ATRA at concentrations of 10^{-8} M and 10^{-7} M. Only at 10^{-6} M, the catabolites and the stereoisomer 13-*cis*-RA were less potent. The stereoisomer 9-*cis*-RA was as potent as ATRA at all concentrations tested (10^{-11} M to 10^{-6} M). In addition, we show that the catabolites and isomers were formed from ATRA to only a limited extent.

Together, our findings suggest that in spite of their high antiproliferative activity the catabolites and isomers of ATRA cannot be responsible for the observed growth inhibition induced by ATRA.

Introduction

Retinoic acid (RA) has been shown to exert antiproliferative and differentiation-inducing effects on cancer cells both *in vitro* and *in vivo* (Gudas *et al.*, 1994; Moon *et al.*, 1994). These effects are mediated through binding to nuclear retinoid receptors, namely the RA receptors (RARs) and the retinoid X receptors (RXRs). Both receptors are members of the nuclear receptor superfamily and function as ligand-dependent transcription factors (Chambon, 1996). The RAR family is activated both by ATRA and by 9-*cis*-RA, whereas the RXR family is activated exclusively by 9-*cis*-RA (Chambon, 1996). The ability of the natural stereoisomer 13-*cis*-RA to bind to RARs is controversial.

Diversity in the control of gene expression by RA exists because of the complexity at different levels of the signalling pathway. An important level in this complex retinoid signalling pathway is represented by the existence of natural metabolites of all-*trans*-RA (ATRA), whose synthesis may be modulated cell-specifically (Napoli, 1996). ATRA metabolism has been studied in a variety of tissues and a number of metabolites have been identified (Fig. 1). One important catabolic pathway for ATRA is initiated by hydroxylation at the 4-position of the β -ionone ring of ATRA to yield 4-hydroxy-ATRA (Frolik *et al.*, 1979; Roberts *et al.*, 1980). This step is catalyzed by a cytochrome P450-dependent ATRA 4-hydroxylase (Roberts *et al.*, 1980; White *et al.*, 1996). 4-Hydroxy-ATRA is further oxidized via 4-oxo-ATRA to more polar metabolites (Roberts *et al.*, 1980). This latter step involves at least one other, presently unknown, cytochrome P450-dependent enzyme (Roberts *et al.*, 1980; Van Wauwe *et al.*, 1994). Epoxidation of ATRA yields 5,6-epoxy-ATRA (McCormick *et al.*, 1978). ATRA can also

Figure 1: Chemical structures of ATRA, its naturally occurring isomers and catabolites arising from two different catabolic pathways.

isomerize to 9-*cis*-RA and 13-*cis*-RA, an obviously non-enzymatic process (Urbach and Rando, 1994).

Cancer cells have been shown to possess RA catabolic activity. Oxidative catabolism of RA to more polar metabolites was observed in *N*-methyl-*N*-nitrosourea-induced mammary tumors in the rat (Bhat and Lacroix, 1989), in rat Dunning R3327G prostate tumors (Krekels *et al.*, 1996), F9 mouse teratocarcinoma cells (Williams and Napoli, 1985), LLC-PK₁ pig kidney cancer cells (Napoli, 1986), BA-HAN-1C rat rhabdomyosarcoma cells (Biesalski *et al.*, 1990), as well as in the human breast cancer cells MCF-7 (Wouters *et al.*, 1992; Krekels *et al.*, 1997) and T47D (Han and Choi, 1996).

Recently, Takatsuka *et al.* (1996) showed a positive relationship between metabolism of ATRA in human breast cancer cells and the antiproliferative activity of ATRA, suggesting that an undefined metabolite of ATRA, rather than the parent compound itself, could be responsible for the observed growth inhibition induced by ATRA. Up to now, only limited and conflicting results have been published concerning the activity of the naturally occurring

ATRA metabolites in tumors. To clarify these points, we studied the antiproliferative activity of ATRA, its physiological catabolites 4-oxo-ATRA and 5,6-epoxy-ATRA and isomers 9-*cis*-RA and 13-*cis*-RA in MCF-7 cells using a bromodeoxyuridine incorporation assay. Bromodeoxyuridine labeling is considered an accurate method to measure cell proliferation because it is a direct assay of DNA synthesis (Dolbeare, 1995). Furthermore, the antiproliferative activities could only be properly studied in a steroid- and retinoid-free medium, because of the presence of endogenous retinoids in serum. Therefore, MCF-7 cells were grown in a phenol red-free medium supplemented with charcoal-treated fetal bovine serum and growth factors.

Materials and Methods

Drugs and chemicals

ATRA was obtained from Serva (Heidelberg, Germany) and 13-*cis*-RA was purchased from Eastman Kodak (Rochester, New York, U.S.A.). 9-*cis*-RA, 4-oxo-ATRA and 5,6-epoxy-ATRA were a generous gift from Dr. M. Klaus (Hoffmann-La-Roche, Basel, Switzerland). All retinoids were dissolved in ethanol to an initial concentration of 4 mM and appropriately diluted in culture medium. The final solvent concentration during the proliferation studies was always < 0.5% (v/v). The retinoid stock solutions were checked for purity using HPLC analysis. Experiments with retinoids were always performed in a dark room with yellow illumination.

Preparation of dextran-coated charcoal (DCC)-treated fetal bovine serum (FBS)

DCC-treated FBS was prepared as described by Horwitz and McGuire (1978). Briefly, FBS (Life Technologies, Paisley, United Kingdom) was heat-inactivated by a 30 min incubation at 56°C. Activated charcoal (0.25% (w/v); Sigma, St. Louis, U.S.A.) was coated overnight at 4°C with dextran (0.025% (w/v); Pharmacia, Uppsala, Sweden) in 0.01 M Tris/HCl (pH 8.0). Then, 100 ml of this suspension was pelleted by centrifugation and 50 ml heat-inactivated FBS was incubated with the resulting DCC pellet for 45 min at 45°C. This procedure was repeated and finally the activated charcoal was removed from the FBS by centrifugation. DCC-treated FBS was sterilized by passage through a 0.22 µm Millipore filter (low protein binding) and stored at -20°C until use. The efficiency of this procedure was assessed by the addition of a trace amount of [6, 7-³H(N)]estradiol (Dupont NEN, Boston, MA, U.S.A.), [11, 12-³H(N)]retinol (Dupont NEN) and [11, 12-³H(N)]ATRA (Dupont NEN).

Cell culture

Stock cultures of MCF-7 human breast cancer cells, purchased from the American Type Culture Collection (Rockville, MD, U.S.A.), were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter glucose supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 µg/ml gentamicin (all reagents from Life Technologies). The MCF-7 subclone used in this study has been characterized previously (Van heusden *et al.*, 1996). Cells were grown in a humidified incubator (5% CO₂, 95% air) at 37°C and were *Mycoplasma*-free as tested by the *Mycoplasma* T.C. kit (Gen-Probe Incorporated, CA, U.S.A.).

For the proliferation studies, the MCF-7 cells were cultured for 6 days in phenol red-free DMEM containing 5% (v/v) DCC-treated FBS, 4.5 g/liter glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µg/ml gentamicin, 30 nM sodium selenite and 10 µg/ml transferrin (all reagents from Life Technologies). Then, cells were seeded onto Chamber Slides™ (Nunc Inc., Naperville, IL, U.S.A.) at a concentration of 15,000 cells/chamber. Chamber Slides™ had been coated with 50 µg/ml poly-L-lysine one day before use. Cells were allowed to attach for 24 h and thereafter the medium was supplemented with growth factors (10 µg/ml final concentration insulin (Life Technologies) and 5 ng/ml final concentration basic fibroblast growth factor (Life Technologies)) and retinoids (concentration ranging from 10⁻¹¹ M to 10⁻⁶ M). Cells were grown under these conditions for 7 days, with medium changes 3 and 6 days after seeding.

Bromodeoxyuridine (BrdU)-detection

After 7 days of culture with retinoids as described above, MCF-7 cells were labeled with 100 µM BrdU for 2.5 h and fixed. Incorporated BrdU was visualized by immunofluorescence staining using the Tyramide Signal Amplification (TSA)-Direct kit (Green) (DuPont NEN Life Science Products, Boston, MA, U.S.A) as described in detail previously (Van heusden *et al.*, 1997).

The BrdU labeling index was defined as the proportion of BrdU-positive cells, representing cells in S-phase and was calculated by counting cells under a fluorescence microscope (Axiophot, Zeiss, Germany) with a dual filter set for simultaneous visualization of fluorescein and propidium iodide signals. About 800 cells were counted twice for each test compound per experiment. Average results are presented as mean ± SD of three or five experiments.

HPLC analysis

Confluent MCF-7 cells, cultured in medium containing 5% DCC-treated FBS, were treated for 24 h with 10⁻⁶ M ATRA to induce ATRA catabolism (Wouters *et al.*, 1992; Krekels *et al.*, 1997). Cells were then washed twice with 25 ml culture medium, trypsinized and harvested. Cells were resuspended at a concentration of 4 × 10⁶ cells/ml. This cell suspension (450 µl) was incubated with 10⁻⁷ M [11, 12-³H(N)]ATRA for different times. After centrifugation for 10 min at 780 g, the supernatant was analyzed for the presence of ATRA metabolites and isomers.

Analysis of ATRA metabolites: Reverse-phase HPLC analysis was carried out on a Varian HPLC system consisting of a HPLC pump 9010, an autosampler 9095 and a diode array detector (Polyview, 9065). The Star 4.0 data software (Varian, Harbor City, CA, U.S.A.) was used to analyze the chromatograms. Radioactivity in the eluate was monitored on-line by β -counting (Packard Radiomatic radioactivity monitor) using Ultima-flo M (Packard, Meriden, CT, U.S.A.) as the scintillation solvent. The samples (150 μ l) were analyzed on a Zorbax 5C8 column (4.6 mm i.d. x 250 mm, 5 μ m; Chrompack). The mobile phase was methanol/2% acetic acid/acetonitrile (1.5:93:5.5) containing 40 mM ammonium acetate (solvent A). Solvent B consisted of methanol/2% acetic acid/acetonitrile (15:30:55) containing 40 mM ammonium acetate and solvent C was 100% methanol. A linear gradient at a flow rate of 1 ml/min was performed in 25 min from 24% A - 76% B to 15% A - 85% B. The solvent was then changed to 50% B - 50% C in 15 min. To elute unchanged ATRA the solvent was changed to 100% C after 40 min.

Analysis of ATRA isomers: For the separation of the isomers of ATRA the same HPLC equipment was used. Samples (150 μ l) were analyzed on a Novapak column (3.9 mm i.d. x 300 mm). Solvent D was methanol/2% acetic acid/acetonitrile (15:30:55) containing 40 mM ammonium acetate. Solvent E consisted of methanol/2% acetic acid/acetonitrile (20:20:60) containing 40 mM ammonium acetate and solvent F was 100% methanol. The mobile phase was 50% D - 50% E for 30 min at a flow rate of 1 ml/min. Then a linear gradient was performed to 100% F.

Statistical analysis

Data were analyzed using the two-tailed Mann-Whitney *U*-test using the Stat View II software (Abacus Concepts, Inc., Berkeley, CA, U.S.A.). Significance was defined at the level of * $p < .01$ and ** $p < .001$.

Results

Dextran-coated charcoal treatment of FBS efficiently removed estrogens and retinoids

The efficiency of heat inactivation and subsequent DCC-treatment to remove steroids and retinoids from FBS was assessed by the addition of trace amounts of tritiated 17 β -estradiol, ATRA and retinol. As shown in Table I, this procedure efficiently removed 17 β -estradiol and ATRA, and retinol to a lesser extent. 17 β -Estradiol was removed for more than 99%, ATRA for more than 93%, and retinol for 64%. When the tritiated compounds were preincubated with FBS for 24 h prior to DCC-treatment, similar results were obtained (data not shown).

Table 1: Efficiency of estrogen and retinoid extraction from fetal bovine serum by dextran-coated charcoal treatment

	amount of tritiated label after DCC-treatment (%)
17 β -estradiol	0.64 $\pm$ 0.17
ATRA	6.43 $\pm$ 0.52
retinol	35.78 $\pm$ 6.78
mean $\pm$ SD ($n = 3$)	

ATRA and its naturally occurring catabolites and isomers significantly inhibit MCF-7 cell proliferation

MCF-7 cells, grown in medium containing 5% DCC-treated FBS, showed a BrdU labeling index of $8.0 \pm 1.0\%$ ($n = 3$). Cells were stimulated to proliferate by the addition of 10 $\mu\text{g/ml}$ insulin and 5 ng/ml basic fibroblast growth factor, resulting in a BrdU labeling index of $25.2 \pm 1.4\%$ ($n = 5$).

Under these culture conditions, ATRA inhibited the proliferation of MCF-7 cells in a concentration-dependent manner, at concentrations from 10^{-8} M to 10^{-6} M (Fig. 2A), which was reflected by a decrease in the BrdU labeling index. At a concentration of 10^{-6} M, ATRA inhibited MCF-7 cell proliferation by $61 \pm 4\%$ ($n = 3$).

Figure 2B shows that the ATRA catabolite 4-oxo-ATRA decreased the labeling index at concentrations ranging from 10^{-8} M to 10^{-6} M. At a concentration of 10^{-6} M, cell proliferation was inhibited by $37 \pm 11\%$ ($n = 5$).

5,6-epoxy-ATRA, another catabolite of ATRA, decreased the BrdU labeling index at 10^{-8} M to 10^{-6} M to the same extent as 4-oxo-ATRA (Fig. 2C).

The stereoisomer 9-*cis*-RA (Fig. 2D) also inhibited MCF-7 cell proliferation concentration-dependently (10^{-8} M to 10^{-6} M). Note that 9-*cis*-RA was equipotent as compared to ATRA at all concentrations tested.

Figure 2E shows the antiproliferative activity of the stereoisomer 13-*cis*-RA. At concentrations ranging from 10^{-8} M to 10^{-6} M, 13-*cis*-RA decreased the BrdU labeling index to the same extent as the ATRA catabolites 4-oxo-ATRA and 5,6-epoxy-ATRA.

Figure 2: Concentration-response curves showing the antiproliferative activity of ATRA (A), its catabolites 4-oxo-ATRA (B) and 5,6-epoxy-ATRA (C) and its isomers 9-*cis*-RA (D) and 13-*cis*-RA (E) in MCF-7 cells. Cells were cultured for 7 days in the presence of test compounds. Cell proliferation was measured by BrdU incorporation, as described in "Materials and Methods". Results are presented as mean $\pm$ SD of three (A, D, E) or five (B, C) experiments. * $p < .01$ and ** $p < .001$ versus control cells (Mann-Whitney *U*-test). # $p < .01$ versus ATRA-treated cells (Mann-Whitney *U*-test).

In vitro metabolism and isomerization of ATRA in MCF-7 cells

MCF-7 cells, grown in medium containing 5% DCC-treated FBS, were pretreated for 24 h with 10^{-6} M ATRA to induce ATRA catabolism. Cells were then incubated with 10^{-7} M [3 H]ATRA for 4 h and the supernatant was analyzed for the presence of labeled ATRA catabolites and isomers by HPLC analysis. As shown in Figure 3A, MCF-7 cells converted ATRA into very polar metabolites (retention time: 2-11 min) and several metabolites of intermediate polarity (retention time: 16-25 min). In the absence of cells no polar metabolites could be detected (data not shown). Combined, the intermediate and very polar metabolites accounted for about 60% of total recovered radioactivity. Peaks were detected (Fig. 3A) co-eluting with authentic 4-hydroxy-ATRA (retention time: 19 min) and 4-oxo-ATRA (retention time: 20.3 min). Figure 4 shows that the intermediate polar metabolites were formed to only a limited extent (less than 10%) and their amount did not increase as a function of time.

Figure 3: Chromatograms illustrating the metabolism of ATRA in MCF-7 cells grown in medium containing 5% DCC-treated FBS. Cells were incubated for 24 h with 10^{-6} M ATRA to induce ATRA catabolism, washed twice and collected. Cells were then incubated for 4 h with 10^{-7} M [3 H]ATRA and the supernatant was analyzed by reverse phase HPLC for the presence of catabolites (A) and isomers (B). I: very polar metabolites. II: intermediate polar metabolites. III: unchanged RA. IV: apolar peak.

Figure 4: Metabolism and isomerization of ATRA in MCF-7 cells grown in medium containing 5% DCC-treated FBS. Cells were incubated for 24 h with 10^{-6} M ATRA to induce ATRA catabolism, washed twice and collected. Cells were then incubated for various times with 10^{-7} M [^3H]ATRA and the supernatant was analyzed by reverse phase HPLC for the presence of catabolites and isomers.

Increasing amounts of very polar metabolites were formed from ATRA as a function of time (Fig. 4). No peak co-eluting with authentic 5,6-epoxy-ATRA (retention time: 37 min) could be detected in the culture medium of cells that were pretreated to induce ATRA catabolism (Fig. 3A). In the culture medium of cells that were not pretreated with ATRA or in the absence of cells, 5,6-epoxy-ATRA could not be detected at time points ranging from 30 min to 24 h (data not shown). Other polar metabolites were present in the cell culture medium (Fig. 3A) but their nature remains to be identified. MCF-7 cells also converted ATRA into an apolar metabolite, that was not detected in the absence of cells.

Figure 3B shows the isomers of ATRA that were formed when MCF-7 cells, pretreated for 24 h with 10^{-6} M ATRA, were incubated with 10^{-7} M [^3H]ATRA. After 4 h, only about 30% of total radioactivity was recovered in the RA peak, i.e. ATRA and its stereoisomers 9-*cis*-RA and 13-*cis*-RA. This RA peak consisted for about 83% of ATRA, 8% of 13-*cis*-RA and 7% of 9-*cis*-RA. Similar isomer composition of the RA peak was obtained with cells that were not pretreated with ATRA and in the absence of cells (data not shown). After 24 h, only about 11% of total radioactivity was recovered in the RA peak of MCF-7 cells that were pretreated with ATRA (Fig. 4). In the absence of cells about 80% of total radioactivity was recovered in the RA peak (data not shown). Under both culture conditions, the relative isomer composition was about 71% ATRA, 12% 13-*cis*-RA and 8% 9-*cis*-RA. An additional peak (peak i, Fig. 3B) could be detected but its nature remains unknown.

Discussion

The present study demonstrates that not only ATRA itself, but also its naturally occurring catabolites 4-oxo-ATRA and 5,6-epoxy-ATRA as well as its isomers 9-*cis*-RA and 13-*cis*-RA possess significant antiproliferative activity in MCF-7 human breast cancer cells, grown in a steroid- and retinoid-free medium. A significant antiproliferative activity equal to that of ATRA, was observed with these catabolites and isomers at concentrations of 10^{-8} M and 10^{-7} M. At a concentration of 10^{-6} M, the ATRA catabolites and the stereoisomer 13-*cis*-RA were less potent than ATRA. The stereoisomer 9-*cis*-RA was as potent as ATRA at all concentrations tested (10^{-11} M to 10^{-6} M). Since these catabolites and isomers were formed to only a limited extent, our findings suggest that they cannot be responsible for the observed growth inhibition induced by ATRA.

The antiproliferative activity of ATRA and its naturally occurring catabolites and isomers could only be properly studied in a steroid- and retinoid-free culture medium. For this purpose, phenol red-free medium (Berthois *et al.*, 1986) was used supplemented with DCC-treated FBS (Horwitz and McGuire, 1978). DCC-treatment of FBS is well-known to remove endogenous steroids present in the serum (Horwitz and McGuire, 1978). In this study, we showed that also ATRA was efficiently removed by DCC-treatment. This would result in a final concentration of 1.3×10^{-11} M to 4.5×10^{-11} M ATRA in the culture medium, calculated from ATRA levels present in human plasma, i.e. 4-14 nM. Up to now, only indirect evidence suggested that retinoids could be removed from the serum by DCC-treatment (Mummery *et al.*, 1991). Retinol, a possible source of RA, was harder to extract and about 40% could not be removed. We assumed that the remaining amount of retinol did not interfere with our results since MCF-7 cells are unable to convert retinol into ATRA (Krekels *et al.*, 1997).

The antineoplastic activity of synthetic retinoids, such as for example *N*-(4-hydroxyphenyl)retinamide, has been amply described. In contrast, limited and conflicting results have been reported about the activity of naturally occurring ATRA metabolites in tumor cells. The catabolite 4-oxo-ATRA has been shown to bind to RARs (Pijnappel *et al.*, 1993). Furthermore, both 4-oxo-ATRA and 5,6-epoxy-ATRA can activate RAR-dependent gene transcription in cotransfected CV-1 cells (Duell *et al.*, 1992). However, their activity in our study was higher than would have been expected from previous reports where these catabolites were found to be less potent than ATRA in *i*) the inhibition of growth and the inhibition of hormone-isobutylmethylxanthine-inducible tyrosinase activity

of Cloudman S-91 mouse melanoma cells (Lotan *et al.*, 1980; Reynolds *et al.*, 1993), ii) the induction of differentiation of F9 mouse teratocarcinoma cells as measured by an ELISA for laminin (Williams *et al.*, 1987) and iii) the induction of differentiation and inhibition of proliferation of BA-HAN-1C rat rhabdomyosarcoma cells (Ramp *et al.*, 1994). On the other hand, both catabolites have been reported to be virtually inactive (Frolik *et al.*, 1979; Silva *et al.*, 1987). In our MCF-7 model system, the catabolites 4-oxo-ATRA and 5,6-epoxy-ATRA were as potent as ATRA at concentrations of 10^{-8} M and 10^{-7} M, but were twofold less potent at 10^{-6} M. It is clear that a complex phenomenon is occurring for which there is no obvious explanation. It is very unlikely that the observed antiproliferative activity of 4-oxo-ATRA and 5,6-epoxy-ATRA was due to conversion back to ATRA, since the reactions involved in their formation are irreversible (Roberts *et al.*, 1980; Napoli *et al.*, 1982). Although conversion to other active retinoids is conceivable, such compounds have yet to be identified (Reynolds *et al.*, 1993). Up to now only in non-tumor models, namely in a model of positional specification (Pijnappel *et al.*, 1993) and in spermatogonia (Gaemers *et al.*, 1996), 4-oxo-ATRA has been shown to be as potent as ATRA.

ATRA catabolism, under the present culture conditions, was induced to the same extent as previously described (Wouters *et al.*, 1992; Krekels *et al.*, 1997). Only the supernatant was analyzed for the presence of ATRA metabolites and isomers. We have previously shown that in the cell extract the same overall metabolite profile can be found (Wouters *et al.*, 1992). By HPLC analysis peaks were identified that co-eluted with authentic 4-hydroxy-ATRA and 4-oxo-ATRA. No 5,6-epoxy-ATRA could be detected under basal culture conditions nor after induction of ATRA catabolism, indicating that 5,6-epoxy-ATRA was either not formed *in vitro* or formed in quantities below the detection limit. Therefore, it is rather unlikely that 5,6-epoxidation is a major catabolic pathway of ATRA in MCF-7 cells. In addition, other polar metabolites could be detected but their nature remains to be elucidated. We cannot exclude the possibility that these metabolites possess antiproliferative activity. In this context it is interesting to remark, however, that we have shown previously that the antiproliferative activity of ATRA can be enhanced by the addition of an inhibitor of ATRA catabolism (Wouters *et al.*, 1992; Van heusden *et al.*, 1996), suggesting that catabolism of ATRA is not necessary to inhibit MCF-7 cell proliferation. The exact nature of the apolar peak, which has been described previously (Wouters *et al.*, 1992), remains to be determined.

Recently, Takatsuka *et al.* (1996) showed a positive relationship between ATRA-induced growth inhibition of human breast cancer cells and intracellular ATRA metabolism, suggesting

that a metabolite of ATRA, rather than the parent compound itself, could be responsible for the observed growth inhibition. It is important to note, however, that these experiments were conducted under serum-free conditions, resulting in an altered bioavailability of RA to cells (Hodam and Creek, 1996). Our data showed that 4-oxo-ATRA and 5,6-epoxy-ATRA are strong inhibitors of MCF-7 cell proliferation. However, since 5,6-epoxy-ATRA could not be identified in our cell culture system and 4-oxo-ATRA was formed to only a limited extent, our results do not favour the hypothesis that these catabolites are responsible for the observed growth inhibition when given ATRA.

The ability of ATRA to isomerize in cell culture (Urbach and Rando, 1994) further complicates the interpretation of its antiproliferative activity. In the present study, isomerization of ATRA to 9-*cis*-RA and 13-*cis*-RA was also observed, but only to a limited extent. This process was not enzymatic since it also occurred in the absence of cells, in agreement with previous results (Urbach and Rando, 1994). The major isomer detected was the all-*trans* form of RA. The naturally occurring stereoisomer 9-*cis*-RA acts as a *pan* agonist that can bind and activate both RARs and RXRs (Chambon, 1996). 9-*cis*-RA was equipotent to ATRA in inhibiting MCF-7 cell proliferation at all concentrations tested, in agreement with previous reports (Rubin *et al.*, 1994). The ability of the naturally occurring stereoisomer 13-*cis*-RA to bind and transactivate RARs is controversial. The antiproliferative activity of 13-*cis*-RA has been described to be less (Redfern *et al.*, 1990) or equal (Frey *et al.*, 1991) to that of ATRA. In our experiments, 13-*cis*-RA, at concentrations of 10^{-8} M and 10^{-7} M, was equipotent to ATRA in inhibiting the growth of MCF-7 cells, in agreement with previous reports (Frey *et al.*, 1991) and with its therapeutic effect in some forms of cancer.

In conclusion, we can state that the naturally occurring catabolites and isomers of ATRA show significant antiproliferative activity in MCF-7 human breast cancer cells. However, since these catabolites and isomers were formed from ATRA to only a limited extent, our findings suggest that they cannot be responsible for the observed growth inhibition induced by ATRA.

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6 **The antiproliferative activity of all-*trans*-retinoic acid catabolites and isomers is differentially modulated by liarozole-fumarate in MCF-7 human breast cancer cells**

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Abstract

The clinical use of all-*trans*-retinoic acid (ATRA) in the treatment of cancer is significantly hampered by the prompt emergence of resistance, believed to be caused by increased ATRA catabolism. Inhibitors of ATRA catabolism may therefore prove valuable for cancer therapy. Liarozole-fumarate is an antitumor drug that inhibits the cytochrome P450-dependent catabolism of ATRA. ATRA, but also its naturally occurring catabolites, 4-oxo-ATRA and 5,6-epoxy-ATRA, as well as its stereoisomers, 9-*cis*-RA and 13-*cis*-RA, show significant antiproliferative activity in MCF-7 human breast cancer cells. In

order to further elucidate its mechanism of action, we investigated whether liarozole-fumarate was able to enhance the antiproliferative activity of ATRA catabolites and isomers. Liarozole-fumarate alone up to a concentration of 10^{-6} M had no effect on MCF-7 cell proliferation. However, in combination with ATRA or the ATRA catabolites, liarozole-fumarate (10^{-6} M) significantly enhanced their antiproliferative activity. On the contrary, liarozole-fumarate (10^{-6} M) was not able to potentiate the antiproliferative activity of the ATRA stereoisomers, most probably because of the absence of cytochrome P450-dependent catabolism. Together, these findings show that liarozole-fumarate acts as a versatile inhibitor of retinoid catabolism in that it not only blocks the breakdown of ATRA, but also inhibits the catabolic pathway of 4-oxo-ATRA and 5,6-epoxy-ATRA, thereby enhancing their antiproliferative activity.

Introduction

The therapeutic potential of all-*trans*-retinoic acid (ATRA) in cancer is obvious from a vast number of publications (for review see Hong and Itri, 1994; Moon *et al.*, 1994). However, a major drawback to the clinical application of ATRA is the prompt emergence of resistance, most probably because of the induction of oxidative catabolism through cytochrome P450-dependent enzymes (Muindi *et al.*, 1992; Smith *et al.*, 1992; Warrell *et al.*, 1993; Warrell, 1993; Kizaki *et al.*, 1996).

Liarozole-fumarate, an imidazole-containing antitumor drug, was recently identified as an inhibitor of cytochrome P450-dependent RA catabolism (Van Wauwe *et al.*, 1990, 1994; Wouters *et al.*, 1992; Krekels *et al.*, 1996). *In vivo*, liarozole-fumarate showed retinoid-mimetic effects (Van Wauwe *et al.*, 1991; Smets *et al.*, 1995; Mahler *et al.*, 1993). In animals, the drug reduced the growth of both androgen-dependent and androgen-independent Dunning R3327 rat prostate adenocarcinoma cells (Van Ginckel *et al.*, 1990; Dijkman *et al.*, 1994; Smets *et al.*, 1995), and of androgen-independent PC-3ML-B2 human prostate carcinoma xenografts (Stearns *et al.*, 1993). In the Dunning AT-6sq, an androgen-independent rat prostate carcinoma, liarozole-fumarate reduced tumor weight, and concomitantly increased ATRA levels both in plasma and tumors, resulting in a shift of their differentiation status (Smets *et al.*, 1995). In patients, the drug is currently being tested in phase III clinical trials for the treatment of relapsed prostate cancer (Mahler *et al.*, 1993; Wouters, 1994).

In vitro, liarozole-fumarate has no intrinsic retinoid-like activity, but enhances the beneficial

effects of ATRA, most probably by inhibiting catabolism. The drug has been shown to potentiate the antiproliferative activity of ATRA in MCF-7 human breast cancer cells (Wouters *et al.*, 1992; Van heusden *et al.*, 1996) and to enhance the differentiation-inducing capacity of ATRA in F9 mouse teratocarcinoma cells (De Coster *et al.*, 1992) and MCF-7 cells (Van heusden *et al.*, 1996). The cancer chemopreventive activity of ATRA and β -carotene was potentiated in 10T1/2 mouse embryonal fibroblasts (Acevedo and Bertram, 1995) and retinoid-induced apoptosis was enhanced in DU145 human prostate cancer cells (Hall, 1996).

ATRA is well known to inhibit proliferation and to induce differentiation of malignant cells (Lotan, 1980). ATRA is rapidly metabolized in a catabolic pathway that includes the 4-hydroxylation of the β -ionone ring to yield 4-hydroxy-ATRA (Frolik *et al.*, 1979; Roberts *et al.*, 1980). This step is catalyzed by a cytochrome P450-dependent ATRA 4-hydroxylase (Roberts *et al.*, 1980; White *et al.*, 1996). 4-Hydroxy-ATRA is then converted to more polar metabolites via 4-oxo-ATRA (Roberts *et al.*, 1980), involving at least one, presently unknown, cytochrome P450-dependent enzyme (Roberts *et al.*, 1980; Van Wauwe *et al.*, 1994). Other catabolic pathways of ATRA have been described including epoxidation to yield 5,6-epoxy-ATRA (McCormick *et al.*, 1978; Barua *et al.*, 1991). Also, ATRA can isomerize in cell culture to 9-*cis*-RA and 13-*cis*-RA, an obviously non-enzymatic process (Urbach and Rando, 1994). ATRA and its naturally occurring catabolites and isomers possess significant antiproliferative activity in MCF-7 cells (Van heusden *et al.*, 1997b). To further explore the mechanism of action, we studied whether liarozole-fumarate was able to enhance the antiproliferative activity of these naturally occurring ATRA catabolites and isomers.

Materials and Methods

Drugs and chemicals

The fumarate salt of liarozole, $\{\pm 5-[(3\text{-chlorophenyl})(1H\text{-imidazole-1-yl)methyl}]-1H\text{-benzimidazole (E)-2-butenedioate (2:3)}\}$ (R085246), was synthesized at the Janssen Research Foundation (Beerse, Belgium). ATRA was obtained from Serva (Heidelberg, Germany) and 13-*cis*-RA was purchased from Eastman Kodak (Rochester, NY, U.S.A.). 9-*cis*-RA, 4-oxo-ATRA and 5,6-epoxy-ATRA were generous gifts from Dr. M. Klaus (Hoffmann-La-Roche, Basel, Switzerland). Stock solutions of liarozole-fumarate (10 mM) and retinoids (4 mM) were prepared in ethanol and appropriately diluted in culture medium. The final ethanol concentration did not exceed 0.5% (v/v). The retinoid stock solutions were checked for purity using HPLC analysis. Experimental studies with retinoids were always performed in a dark room with yellow illumination.

Preparation of dextran-coated charcoal (DCC)-treated fetal bovine serum (FBS)

DCC-treated FBS was prepared as described by Horwitz and McGuire (1978). Briefly, FBS (Life Technologies, Paisley, United Kingdom) was heat-inactivated by a 30 min incubation at 56°C. Activated charcoal (0.25% (w/v); Sigma Chemical Co., St. Louis, MO, U.S.A.) was coated overnight at 4°C with dextran (0.025% (w/v); Pharmacia, Uppsala, Sweden) in 0.01 M Tris/HCl (pH 8.0). Then, 100 ml of this suspension was pelleted by centrifugation and 50 ml heat-inactivated FBS was incubated for 45 min at 45°C with the resulting DCC pellet. This procedure was repeated and finally the activated charcoal was removed from the FBS by centrifugation. DCC-treated FBS was sterilized by passage through a 0.22 μ m Millipore filter (low protein binding) and stored at -20°C until use. The efficiency of this procedure was assessed by the addition of a trace amount of [6, 7-³H(N)]estradiol (DuPont NEN, Boston, MA, U.S.A.), [11, 12-³H(N)]retinol (DuPont NEN) and [11, 12-³H(N)]ATRA (DuPont NEN). The DCC-treatment efficiently removed estradiol and ATRA, and retinol to a lesser extent (Van heusden *et al.*, 1997b).

Cell culture

Stock cultures of MCF-7 human breast cancer cells, purchased from the American Type Culture Collection (Rockville, MD, U.S.A.), were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter glucose supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μ g/ml gentamicin (all reagents from Life Technologies). The MCF-7 subclone used in this study has been characterized previously (Van heusden *et al.*, 1996). Cells were grown in a humidified incubator (5% CO₂, 95% air) at 37°C and were *Mycoplasma*-free as tested by the *Mycoplasma* T.C. kit (Gen-Probe Incorporated, CA, U.S.A.).

For the proliferation studies, MCF-7 cells were cultured for 6 days in phenol red-free DMEM containing 5% (v/v) DCC-treated FBS, 4.5 g/liter glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μ g/ml gentamicin, 30 nM sodium selenite and 10 μ g/ml transferrin (all reagents from Life Technologies). Cells were seeded onto Chamber Slides™ (Nunc Inc., Naperville, IL, U.S.A.) at a concentration of 15,000 cells/chamber. Chamber Slides™ had been coated with 50 μ g/ml poly-L-lysine one day before use. Cells were allowed to attach for 24 h and thereafter the medium was supplemented with growth factors, i.e. 10 μ g/ml final concentration insulin (Life Technologies) and 5 ng/ml final concentration basic fibroblast growth factor (Life Technologies), retinoids (concentration ranging from 10⁻¹¹ M to 10⁻⁶ M) and liarozole-fumarate (concentration ranging from 10⁻⁷ M to 10⁻⁵ M). Cells were grown under these conditions for 7 days with medium changes 3 and 6 days after seeding.

Bromodeoxyuridine (BrdU) detection

Cell proliferation was measured using BrdU incorporation, which is considered an accurate method because it is a direct assay of DNA synthesis (Dolbeare, 1995).

After 7 days of culture with retinoids as described above, MCF-7 cells were labeled with 100 μ M BrdU for 2.5 h and fixed. Incorporated BrdU was visualized by immunofluorescence staining using the

Tyramide Signal Amplification (TSA)-Direct kit (Green) (DuPont NEN) as described in detail previously (Van heusden *et al.*, 1997a).

The BrdU labeling index was defined as the proportion of BrdU-positive cells, representing cells in S-phase, and was estimated by counting cells under a fluorescence microscope (Axiophot, Zeiss, Germany) with a dual filter set for simultaneous visualization of fluorescein and propidium iodide signals. About 800 cells were counted twice for each test compound per experiment. Average results are presented as mean $\pm$ SD of three to five experiments.

Microcolumn assay for ATRA catabolism

ATRA catabolism was quantitatively determined using the microcolumn assay as described previously (Krekels *et al.*, 1997). Briefly, cells cultured in medium containing 5% DCC-treated FBS, were pretreated for 24h with 10^{-6} M ATRA to induce the ATRA catabolic pathway (Wouters *et al.*, 1992; Krekels *et al.*, 1997). Cells were then washed with culture medium, harvested and resuspended at 4×10^6 cells/ml. This cell suspension (450 μ l) was incubated for 90 min in the presence of 10^{-7} M [11, 12- 3 H(N)]ATRA or 10^{-7} M [3 H]9-*cis*-RA (Amersham Life Science, Buckinghamshire, United Kingdom), and thereafter 2 ml 100% acetonitrile was added. After centrifugation for 10 min at 780g the resulting deproteinized supernatant was acidified with 2.5 ml 40 mM acetic acid and applied to a 3 ml C18 Bond Elut LRC column (Varian, Harbor City, CA, U.S.A.; pretreated with 4 ml distilled water) under a vacuum of 127 mm Hg using VAC ELUT SPS-24 and the effluent was collected. The column was eluted with 1 ml 40% acetonitrile and the effluent was collected in the same vial. The collected effluent, containing the polar metabolites, was counted for radioactivity in a Packard Tri-carb 4530 liquid scintillation analyzer. Optiphase "Hi Safe II" (Wallac, Milton Keynes, United Kingdom) was used as a scintillator.

HPLC analysis

The MCF-7 cell suspension was prepared as described above and 450 μ l was incubated with 10^{-7} M [11, 12- 3 H(N)]ATRA or 10^{-7} M [3 H]9-*cis*-RA for 4 h. After centrifugation for 10 min at 780g, the supernatant was analyzed for the presence of ATRA metabolites and isomers.

Analysis of ATRA metabolites: Reverse-phase HPLC analysis was carried out on a Varian HPLC system consisting of a HPLC pump 9010, an autosampler 9095 and a diode array detector (Polyview, 9065). The Star 4.0 data software (Varian) was used to analyze the chromatograms. Radioactivity in the eluate was monitored on-line by β -counting (Packard Radiomatic radioactivity monitor) using Ultima-flo M (Packard, Meriden, CT, U.S.A.) as the scintillation solvent. The samples (150 μ l) were analyzed on a Zorbax 5C8 column (4.6 mm i.d. x 250 mm, 5 μ m; Chrompack). The mobile phase was methanol/2% acetic acid/acetonitrile (1.5:93:5.5) containing 40 mM ammonium acetate (solvent A). Solvent B consisted of methanol/2% acetic acid/acetonitrile (15:30:55) containing 40 mM ammonium acetate and solvent C was 100% methanol. A linear gradient at a flow rate of 1 ml/min was performed in 25 min from 24% A - 76% B to 15% A - 85% B. The solvent was then changed to 50% B - 50 % C in 15 min. To elute unchanged ATRA the solvent was changed to 100% C after 40 min.

Analysis of ATRA isomers: For the separation of the isomers of ATRA the same HPLC equipment was used. Samples (150 μ l) were analyzed on a Novapak column (3.9 mm i.d. x 300 mm). Solvent D was methanol/2% acetic acid/acetonitrile (15:30:55) containing 40 mM ammonium acetate. Solvent E consisted of methanol/2% acetic acid/acetonitrile (20:20:60) containing 40 mM ammonium acetate and solvent F was 100% methanol. The mobile phase was 50% D - 50% E for 30 min at a flow rate of 1 ml/min. Then a linear gradient was performed to 100% F.

Statistical analysis

Data were analyzed using the two-tailed Mann-Whitney *U*-test using the Stat View II software (Abacus Concepts, Inc., Berkeley, CA, U.S.A.). Significance was defined at the level of **p* < .01 and ***p* < .001.

Results

Liarozole-fumarate potentiates the antiproliferative activity of ATRA and its catabolites, but not of its stereoisomers

MCF-7 cells were cultured in steroid- and retinoid-free medium supplemented with growth factors. Under these culture conditions, MCF-7 cells showed a BrdU labeling index of $25.2 \pm 1.4\%$ (*n* = 5). Liarozole-fumarate alone had no effect on MCF-7 cell proliferation up to a concentration of 10^{-6} M (Fig. 1a). At 10^{-5} M, liarozole-fumarate decreased the BrdU labeling index to $21.5 \pm 2.8\%$ (*n* = 4). In all following experiments liarozole-fumarate was therefore used at a concentration of 10^{-6} M.

Liarozole-fumarate (10^{-6} M) significantly enhanced the antiproliferative activity of ATRA (Fig. 1b) and its naturally occurring catabolites 4-oxo-ATRA (Fig. 1c) and 5,6-epoxy-ATRA (Fig. 1d) as reflected by a further decrease in the BrdU labeling index. This potentiation was more pronounced at lower retinoid concentrations. Note that liarozole-fumarate was not able to enhance the antiproliferative activity at retinoid concentrations of 10^{-6} M (Figs. 1b, c, d). In the case of 5,6-epoxy-ATRA, liarozole-fumarate was not able to enhance the antiproliferative activity at concentrations of 10^{-7} M and 10^{-6} M (Fig. 1d).

Liarozole-fumarate (10^{-6} M) did not enhance the antiproliferative activity of the stereoisomers 9-*cis*-RA (Fig. 1e) and 13-*cis*-RA (Fig. 1f).

5,6-Epoxy-ATRA competes with ATRA for ATRA catabolism

MCF-7 cells, cultured in medium containing 5% DCC-treated FBS, were pretreated for 24h with 10^{-6} M ATRA to induce ATRA catabolism (Wouters *et al.*, 1992; Krekels *et al.*, 1997). ATRA catabolism was measured using 10^{-7} M [3 H]ATRA as substrate. As shown in Figure 2,

Figure 1: Concentration-response curves showing the effect of liarozole-fumarate alone (a) or in combination with ATRA (b), 4-oxo-ATRA (c), 5,6-epoxy-ATRA (d), 9-*cis*-RA (e) and 13-*cis*-RA (f) in MCF-7 cells. Cells were cultured for 7 days in the presence of test compounds. Cell proliferation was measured by BrdU incorporation, as described in "Materials and Methods". Results are presented as mean $\pm$ SD of three (b, e, f), four (a) or five (c, d) experiments. * $p < .01$ and ** $p < .001$ versus control cells (Mann-Whitney *U*-test). # $p < .05$ and ## $p < .01$ versus retinoid-treated cells (Mann-Whitney *U*-test).

Figure 2: Competition between radioactive [^3H]ATRA and nonradioactive ATRA ($\text{IC}_{50} = 129 \pm 40 \text{ nM}$) and 5,6-epoxy-ATRA ($\text{IC}_{50} = 50 \pm 28 \text{ nM}$). MCF-7 cells were preincubated for 24 h with 10^{-6} M ATRA to induce ATRA catabolism. Concentration-response curves for ATRA catabolism were determined using 10^{-7} M [^3H]ATRA as substrate. Results are presented as mean $\pm$ SD of five experiments. * $p < .01$ versus control (Mann-Whitney U -test). # $p < .05$ versus ATRA treatment (Mann-Whitney U -test).

nonradioactive ATRA competed with [^3H]ATRA with an IC_{50} value of $129 \pm 40 \text{ nM}$ ($n = 5$). 5,6-epoxy-ATRA was a better competitor than ATRA and decreased the amount of polar [^3H]ATRA metabolites with an IC_{50} value of $50 \pm 28 \text{ nM}$ ($n = 5$).

Liarozole-fumarate inhibits the catabolism of ATRA

Liarozole-fumarate concentration-dependently increased the level of unmetabolized [^3H]ATRA and concomitantly inhibited the formation of polar [^3H]ATRA metabolites (Fig. 3, Table I). The unidentified apolar peak was concentration-dependently increased by liarozole-fumarate. The isomerization process of ATRA to 9-*cis*-RA and 13-*cis*-RA was not affected by liarozole-fumarate (Table I). A peak co-eluting with 4-oxo-ATRA was formed to only a limited extent (data not shown). No peak co-eluting with authentic 5,6-epoxy-ATRA could be detected with or without treatment with liarozole-fumarate at concentrations from 10^{-6} M to 10^{-5} M .

Figure 3: Chromatograms illustrating the metabolism of $[^3\text{H}]\text{ATRA}$ in the absence (a) or presence of liarozole-fumarate at a concentration of 10^{-6} M (b) or 10^{-5} M (c). Cells were incubated for 24 h with 10^{-6} M ATRA to induce ATRA catabolism, washed twice and collected. Cells were then incubated for 4 h with 10^{-7} M $[^3\text{H}]\text{ATRA}$ and the supernatant was analyzed by reverse phase HPLC. I: very polar metabolites. II: intermediate polar metabolites. III: apolar metabolite.

9-*cis*-RA is not metabolized in MCF-7 cells

[^3H]9-*cis*-RA was not converted to polar metabolites in MCF-7 cells as measured by HPLC analysis. Similar results were obtained by the microcolumn assay (data not shown). The metabolism of 13-*cis*-RA, 4-*oxo*-ATRA and 5,6-*epoxy*-ATRA could not be studied because these retinoids are not available as tritiated compounds and unlabeled retinoids could not be used for these purposes (data not shown; Gubler and Sherman, 1990)

Table 1: Quantitative analysis of [^3H]ATRA metabolism in MCF-7 cells

HPLC peaks	Radioactivity (%)		
	control	+ 10^{-6} M liarozole	+ 10^{-5} M liarozole
RA(%ATRA, 9- <i>cis</i> -RA and 13- <i>cis</i> -RA)	43 (78, 13, 10)	52 (78, 13, 10)	73 (82, 10, 9)
Metabolites			
very polar	36	28	10
intermediately polar	15	12	5
apolar	6	8	12

Discussion

The present study further elucidates the mechanism of action of liarozole-fumarate by showing its enhancing effect on the antiproliferative activity of naturally occurring ATRA catabolites. These effects could only be properly studied in a steroid- and retinoid-free culture medium supplemented with growth factors. We have previously demonstrated that not only ATRA itself, but also its naturally occurring catabolites, 4-*oxo*-ATRA and 5,6-*epoxy*-ATRA, as well as its stereoisomers, 9-*cis*-RA and 13-*cis*-RA, significantly inhibit the proliferation of MCF-7 human breast cancer cells (Van heusden *et al.*, 1997b). Liarozole-fumarate, at a concentration of 10^{-6} M, enhanced the antiproliferative activity of ATRA and its catabolites, but not that of the stereoisomers of ATRA. As such liarozole-fumarate acts as a versatile inhibitor of the cytochrome P450-dependent ATRA catabolic pathway (Fig. 4).

Liarozole-fumarate (10^{-6} M) significantly enhanced the antiproliferative activity of ATRA in MCF-7 cells. This potentiating effect was more than 100-fold at low retinoid-concentrations (10^{-10} M to 10^{-9} M) in contrast to the 10-fold enhancement as previously demonstrated under growth conditions with culture medium containing untreated FBS (Wouters *et al.*, 1992; Van heusden *et al.*, 1996). The effect of liarozole-fumarate on ATRA metabolism was analyzed

by HPLC. Only the supernatant was studied. We have previously shown that in the cell extract the same overall metabolite profile can be found (Wouters *et al.*, 1992). Liarozole-fumarate increased the level of RA and concomitantly inhibited the formation of more polar metabolites in a concentration-dependent manner. Together, these findings suggest that liarozole-fumarate enhances the antiproliferative activity of ATRA by inhibiting its catabolism, in agreement with our previous findings (Wouters *et al.*, 1992).

Liarozole-fumarate (10^{-6} M) enhanced the antiproliferative activity of the naturally occurring catabolite 4-oxo-ATRA, confirming that its catabolism is inhibited by liarozole-fumarate.

Figure 4: Proposed mechanism of action of liarozole-fumarate. The drug acts as a versatile inhibitor at different steps of the ATRA catabolic pathway. Liarozole-fumarate not only inhibits the catabolism of ATRA, but also of its catabolites 4-oxo-ATRA and 5,6-epoxy-ATRA, thereby enhancing their antiproliferative activity.

Indeed, Van Wauwe *et al.* (1994) have shown that liarozole-fumarate inhibited the formation of more polar metabolites from 4-oxo-ATRA in hamster liver microsomes. In addition, liarozole-fumarate enhanced the plasma half-life of 4-oxo-ATRA in rats (Van Wauwe *et al.*, 1994). Taken together, these findings suggest that catabolism of 4-oxo-ATRA involves at least one cytochrome P450-dependent enzyme (Roberts *et al.*, 1980; Van Wauwe *et al.*, 1994) that is inhibited by liarozole-fumarate.

The antiproliferative activity of 5,6-epoxy-ATRA was also potentiated by liarozole-fumarate (10^{-6} M). One possible explanation for this synergism is that liarozole-fumarate inhibits the cytochrome P450-dependent catabolism of 5,6-epoxy-ATRA. Although such enzyme(s) are unknown, it is not unlikely that 5,6-epoxy-ATRA is metabolized by the same enzyme(s) as ATRA, since 5,6-epoxy-ATRA was a better competitor than ATRA itself in our assay for measuring ATRA catabolism. Although liarozole-fumarate was present at a tenfold higher concentration, i.e. 10^{-6} M, the drug was not able to enhance the antiproliferative activity of a higher concentration (10^{-7} M) of 5,6-epoxy-ATRA when tested at a concentration of 10^{-7} M. These data support the hypothesis that both liarozole-fumarate and 5,6-epoxy-ATRA compete for the same enzyme(s) and that the higher affinity of 5,6-epoxy-ATRA for these enzyme(s) does not allow enhancement.

The ability of ATRA to isomerize in cell culture has been described before (Urbach and Rando, 1994). Liarozole-fumarate (10^{-6} M) had no effect on isomerization of ATRA. The antiproliferative activity of 9-*cis*-RA and 13-*cis*-RA could not be enhanced by liarozole-fumarate (10^{-6} M). In contrast, *in vivo* liarozole-fumarate has been shown to enhance plasma levels of 9-*cis*-RA (Achkar *et al.*, 1994) and 13-*cis*-RA (Westarp *et al.*, 1994). Since RA catabolism may be cell-specifically regulated, the most likely explanation for this discrepancy is the absence of cytochrome P450-dependent enzymes that catabolize 9-*cis*-RA and 13-*cis*-RA in MCF-7 cells. In favour of this hypothesis is our finding that 9-*cis*-RA was not converted to polar metabolites in these cultures. Although, we have previously shown that 9-*cis*-RA and 13-*cis*-RA can isomerize to ATRA in MCF-7 cells (Krekels *et al.*, 1997), the fact that their antiproliferative activity was not potentiated by liarozole-fumarate, indicates that their effect on MCF-7 cell proliferation was not due to conversion to ATRA.

We have shown that both 9-*cis*-RA and 13-*cis*-RA compete with ATRA in our assay for measuring ATRA catabolism in MCF-7 cells (Krekels *et al.*, 1997). In this study, we show that liarozole-fumarate was not able to enhance the antiproliferative activity of these stereoisomers. Taken together, these findings suggest that although both stereoisomers are able to bind to ATRA catabolizing enzyme(s), they are not used as substrates by these enzyme(s).

because then an enhancing effect by liarozole-fumarate would be expected. These latter data are in agreement with the observations of Duell *et al.* (1996) in mouse skin.

In conclusion, we can state that liarozole-fumarate is not only able to inhibit the catabolism of ATRA, but also that of 4-oxo-ATRA and 5,6-epoxy-ATRA, thereby enhancing their antiproliferative activity. Liarozole-fumarate does not enhance the antiproliferative activity of the stereoisomers 9-*cis*-RA and 13-*cis*-RA in MCF-7 human breast cancer cells, most probably due to the absence of their oxidative catabolism in these cells. Together, these findings extend and confirm the hypothesis that liarozole-fumarate acts as a versatile inhibitor in the ATRA catabolic pathway (Fig. 4), and may show potential in circumvention of RA resistance in cancer.

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7

General discussion

Retinoids are well-known to inhibit cell proliferation and to induce differentiation of cancer cells. The most active naturally occurring retinoid is believed to be all-*trans*-retinoic acid (ATRA). Cancer therapy with ATRA so far has met with limited success and has been significantly hampered by toxicity and the prompt emergence of ATRA resistance. Inhibitors of ATRA catabolism might therefore prove valuable to circumvent these problems.

Catabolism of ATRA represents an inactivation pathway.

ATRA catabolism was originally believed to convert ATRA into degradation products without biological activity (Frolik *et al.*, 1979; Sietsema and De Luca, 1982; Silva *et al.*, 1987). More recently it was shown that ATRA catabolites, including 4-oxo-ATRA and 5,6-epoxy-ATRA, have significant biological activity in a number of growth and differentiation assays, but their activity was always less than that of ATRA (Williams *et al.*, 1987; Pijnappel *et al.*, 1993; Reynolds *et al.*, 1993; Ramp *et al.*, 1994). Furthermore, in another system 4-oxo-ATRA was shown to be more potent than ATRA, suggesting that ATRA catabolites can fulfill a specific role in ATRA signalling. Indeed, in early *Xenopus laevis* embryos 4-oxo-ATRA was shown to be a highly active modulator of positional specification and to be a potent inducer of the expression of Hox genes in these embryos (Pijnappel *et al.*, 1993). In addition, Takatsuka *et al.* (1996) suggested that a metabolite of ATRA rather than ATRA itself was responsible for the observed growth inhibition in human breast cancer cells. We investigated the latter hypothesis and our data suggest that ATRA itself, and not one of its metabolites, is responsible for the observed growth inhibition in MCF-7 (Chapter 5). This hypothesis was further

supported by showing that the antiproliferative activity of ATRA could be enhanced while its catabolism was blocked by an inhibitor (Chapter 6). In agreement with our findings, van der Leede *et al.* (1997) clearly demonstrated that catabolism of ATRA is not required to inhibit growth of human breast cancer cells.

In the context of this present knowledge, we hypothesize that ATRA catabolism is essentially an inactivation pathway, and that the intermediates formed in this pathway have a significant biological activity, but less than that of ATRA. This hypothesis does not exclude the possibility that in some models ATRA catabolites can contribute to the observed effects of ATRA, depending on the balance between their formation and further breakdown. The apparent contradiction that in some models ATRA catabolites are as active (Chapter 5; Gratas *et al.*, 1993; Nikawa *et al.*, 1995; Gaemers *et al.*, 1996) or even more active (Pijnappel *et al.*, 1993) than the parent compound ATRA, are most likely to be explained by possible differences in uptake, metabolism and transport to the nucleus.

Liarozole-fumarate: a versatile inhibitor of ATRA catabolism

Since ATRA is inactivated via catabolism, compounds that are able to inhibit this breakdown would be expected to enhance the biological activity of ATRA. Indeed, liarozole-fumarate has been shown to enhance the antiproliferative activity of ATRA (Wouters *et al.*, 1992; Chapter 2 and 6). Besides, we showed that liarozole-fumarate also enhances the differentiation-inducing activity of ATRA (Chapter 2 and 3). In MCF-7 human breast cancer cells, liarozole-fumarate enhanced the ATRA-induced structural remodelling (Chapter 2). However, since differentiation of MCF-7 cells is not well defined, F9 mouse teratocarcinoma cells, as yet one of the best documented *in vitro* models for biochemical and morphological ATRA-linked differentiation, were used to study the effect of liarozole-fumarate. In Chapter 3 it is shown that liarozole-fumarate potentiates ATRA-induced differentiation in F9 cells, as monitored by well-defined differentiation parameters. Whether these *in vitro* findings can be extrapolated to the *in vivo* situation remains to be established.

Although liarozole-fumarate has been shown to inhibit ATRA catabolism (Van Wauwe *et al.*, 1990; Wouters *et al.*, 1992; Chapter 3 and 6), its molecular target(s) is/are unknown. Cytochrome P450-dependent enzymes are believed to play a key role in ATRA catabolism (Chapter 1). The finding that imidazole-containing compounds, such as for example liarozole-fumarate, inhibit ATRA catabolism (Van Wauwe *et al.*, 1990; Wouters *et al.*, 1992), is consistent with this idea because imidazoles are well-known inhibitors of cytochrome P450-dependent

enzymes (Halpert, 1995). The recent cloning of a zebrafish cytochrome P450-dependent enzyme, that catalyzed the 4-hydroxylation of ATRA, was the first molecular proof that cytochrome P450-dependent enzymes are indeed involved in ATRA catabolism (White *et al.*, 1996). It would be interesting to investigate whether liarozole-fumarate is able to bind and inhibit the human counterpart of this ATRA 4-hydroxylase. Furthermore, the fact that liarozole-fumarate acts as a versatile inhibitor of ATRA catabolism (Chapter 6), suggests that cytochrome P450-dependent enzymes are also involved at other steps in the ATRA catabolic pathway. It remains to be established if these different steps are catalyzed by different enzymes.

Inhibitors of ATRA catabolism as anticancer drugs

ATRA has been proven to be active in the prevention and therapy of certain preneoplastic and neoplastic lesions (Bollag, 1995). However, clinical success with ATRA treatment has been limited. The main reasons that hamper the successful clinical use of ATRA are its limited efficacy, toxicity, and the prompt emergence of ATRA resistance. These clinical drawbacks could be circumvented by the use of inhibitors of ATRA catabolism.

Limited efficacy

Limited efficacy of exogenous administered ATRA in a number of cancers can be explained by an insufficient uptake of ATRA into the tumor. This hypothesis is sustained by the finding that uptake of ATRA from plasma is strongly tissue-dependent. The underlying molecular mechanisms of ATRA uptake are unknown (Pfahl and Chytil, 1996), but some tissues, e.g. testis and kidney, have indeed been shown to take up very little ATRA from plasma (Kurlandsky *et al.*, 1995). On the other hand, insensitivity to ATRA therapy can also be explained by an impairment at the level of nuclear receptors (van der Leede *et al.*, 1995; Li *et al.*, 1995; Seewaldt *et al.*, 1995; Liu *et al.*, 1996) or the presence of a constitutive tumor ATRA catabolism (Krekels *et al.*, 1996). In the latter case, inhibitors of ATRA catabolism might prove valuable by increasing the level of ATRA inside the tumor itself. That this hypothesis is not merely speculative is shown by the finding that liarozole-fumarate enhances the level of ATRA both in plasma and in tissue (Van Wauwe *et al.*, 1992; Smets *et al.*, 1995).

Toxicity

For therapy, high doses of exogenous administered ATRA are required, that give rise to toxic side effects. The limited success of ATRA therapy might thus in part be explained by the

fact that the toxicity of high doses of ATRA limits the maximal achievable dose *in vivo* that is needed to exert a therapeutic effect. The use of inhibitors of ATRA catabolism, that are able to enhance endogenous levels of ATRA, cannot totally circumvent this problem as shown by signs of hypervitaminosis A in prostate cancer patients. However, it is possible that by increasing local, i.e. tumor, ATRA levels, systemic ATRA levels remain lower as compared to that after exogenous ATRA administration, thereby minimizing the toxic side effects.

ATRA resistance

Another problem that has hampered the clinical use of ATRA is the prompt emergence of ATRA resistance. This phenomenon has not only been observed in APL patients (Castaigne *et al.*, 1990; Warrell *et al.*, 1993; Lazzarino *et al.*, 1996), but also in patients with solid tumors (Lee *et al.*, 1993; Muindi *et al.*, 1994).

The exact molecular mechanisms behind the emergence of ATRA resistance remain to be elucidated. Proposed explanations include the increased expression of CRABPs, that can sequester ATRA and reduce its concentration in the nucleus (Kósa *et al.*, 1995; Widschwendter *et al.*, 1995; Warrell, 1993; Boylan and Gudas, 1991; Cornic *et al.*, 1992; Adamson *et al.*, 1993), and the increased expression of multidrug-resistance genes (Kizaki *et al.*, 1996). However, at present the most favoured explanation is that ATRA resistance emerges by the autoinduction of enzymes that increase its catabolism. Numerous findings are consistent with this view and suggest indeed that ATRA induces its own catabolism (McPhillips *et al.*, 1987; Muindi *et al.*, 1992a, 1992b, 1994; Smith *et al.*, 1992; Rigas *et al.*, 1993). If this latter hypothesis is true, inhibitors of ATRA catabolism might circumvent the problem of ATRA resistance.

Conclusions and perspectives

ATRA catabolism plays an important role in the inactivation of ATRA. However, the enzymes that are involved in this catabolic pathway and the molecular mechanisms that control cell-specific catabolism largely remain to be discovered.

Liarozole-fumarate is a versatile inhibitor of ATRA catabolism and enhances the antiproliferative as well as the differentiation-inducing activity of ATRA.

It remains to be established whether inhibitors of ATRA catabolism, such as liarozole-fumarate, will prove valuable for non-palliative treatment, thereby opening a new era in differentiation therapy.

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Summary

Retinoids are a group of natural and synthetic compounds that share structural and/or functional similarity to vitamin A. The most active naturally occurring retinoid is probably all-*trans*-retinoic acid (ATRA). ATRA is known to inhibit proliferation and to induce differentiation of cancer cells. Therefore, the use of ATRA in the treatment of cancer has been explored extensively. Unfortunately, its use as an anticancer drug in differentiation therapy has shown only limited clinical success. One major drawback in the use of ATRA is the prompt emergence of ATRA resistance, believed to be caused by increased ATRA metabolism. One way to tackle this problem is to develop inhibitors of ATRA metabolism that could increase endogenous levels of ATRA, thereby exerting antitumoral responses. Liarozole-fumarate has recently been identified as an inhibitor of ATRA metabolism that might prove valuable in the treatment of cancer (**Chapter 1**).

To find support for the hypothesis that liarozole-fumarate, besides enhancing the antiproliferative activity of ATRA, can also enhance the differentiation-inducing capacity of ATRA its effect was studied in the breast cancer cell line MCF-7 (**Chapter 2**). It is shown that liarozole-fumarate enhances the ATRA-induced structural remodelling of cytoskeletal proteins, cytokeratins and actin, as well as of the cell-cell adhesion molecule E-cadherin. Upregulation of E-cadherin expression by ATRA is enhanced by liarozole-fumarate, suggesting that the drug can also enhance the differentiation-inducing capacities of ATRA.

The effect of liarozole-fumarate was also studied in a well-known and generally accepted model for cellular differentiation, i.e. F9 mouse teratocarcinoma cells (**Chapter 3**). In these cells, liarozole-fumarate enhances the ATRA-induced expression of well-characterized differentiation markers, such as laminin, collagen IV and cytokeratins. Concomitantly, liarozole-fumarate inhibits ATRA metabolism in these cells, linking its enhancing effects on ATRA-induced differentiation to inhibition of ATRA metabolism. Together, these data show that liarozole-fumarate enhances both the antiproliferative and the differentiation-inducing activity of ATRA by inhibiting ATRA metabolism.

In order to further finetune the mechanism of action of liarozole-fumarate, its effect was studied on the metabolites and isomers of ATRA. These studies could only be properly carried out in a steroid- and retinoid-free cell culture medium. Under these culture conditions cells are growth-arrested and can be restimulated to proliferate by the addition of well defined growth factors. Despite the stimulated cell proliferation, bromodeoxyuridine-immunosignals (a measure for cell proliferation) are barely detectable. **Chapter 4** describes an amplification method using fluorescein-labeled tyramide to specifically enhance these weak bromodeoxyuridine-immunosignals.

In **Chapter 5** it is shown that not only ATRA itself but also its catabolites, 4-oxo-ATRA and 5,6-epoxy-ATRA, as well as its stereoisomers, 9-*cis*-RA and 13-*cis*-RA, show significant antiproliferative activity in MCF-7 human breast cancer cells. However, since these catabolites and stereoisomers are formed from ATRA only to a limited extent, these results suggest that the parent compound ATRA itself is mainly responsible for its antiproliferative activity.

The antiproliferative activity of the ATRA catabolites, but not of the ATRA stereoisomers, can be enhanced by liarozole-fumarate (**Chapter 6**). Together, these results suggest that liarozole-fumarate acts as a versatile inhibitor of ATRA metabolism, in that it not only inhibits the breakdown of ATRA but also of the ATRA catabolites.

In **Chapter 7** it is discussed that catabolism of ATRA represents an inactivation pathway and that inhibition of this inactivation by liarozole-fumarate might prove valuable as a new alternative in differentiation therapy of cancer.

Samenvatting

Retinoïden zijn een groep van natuurlijke en synthetische stoffen die structureel en/of functioneel lijken op vitamine A. Het meest actieve retinoïd dat in de natuur voorkomt, is waarschijnlijk all-*trans*-vitamine A zuur (ATRA). ATRA inhibeert de proliferatie van kankercellen en induceert differentiatie in deze cellen. Daarom is het gebruik van ATRA als een antitumorale stof zeer uitgebreid onderzocht. Helaas vertoont ATRA slechts een beperkte werking als antitumorale stof in differentiatietherapie. Eén probleem bij het klinisch gebruik van ATRA, is het snelle optreden van resistentie tegen ATRA zelf. De meest aanvaarde reden voor het ontstaan van deze resistentie is een versnelde afbraak van ATRA, waardoor het zijn gunstige werking niet meer kan uitoefenen. Eén manier om dit probleem aan te pakken, is de ontwikkeling van inhibitoren om de afbraak van ATRA tegen te gaan. Zulke inhibitoren zouden endogene ATRA-spiegels kunnen verhogen en zo een antitumorale werking uitoefenen. Liarozole-fumaraat werd recent geïdentificeerd als een inhibitor van ATRA-metabolisme die beloftevol blijkt voor de behandeling van kanker (Hoofdstuk 1). Om de hypothese te testen dat liarozole-fumaraat ook de differentiatie-inducerende werking van ATRA kan versterken, werd zijn effect bestudeerd in de humane borstkanker cellijn MCF-7 (Hoofdstuk 2). Liarozole-fumaraat versterkt de structurele veranderingen die veroorzaakt worden door ATRA. Niet alleen eiwitten van het cytoskelet, zoals cytokeratines en actine, ondergaan structurele veranderingen, maar ook de cel-cel adhesiemoleculen

E-cadherine. Deze verhoging van E-cadherine expressie onder invloed van ATRA wordt versterkt door liarozole-fumaraat, hetgeen er op duidt dat liarozole-fumaraat ook de differentiatie-inducerende eigenschappen van ATRA kan versterken. Het effect van liarozole-fumaraat werd nagegaan in een goed gekarakteriseerd model voor differentiatie, namelijk F9-teratogene tumorcellen van de muis (**Hoofdstuk 3**). In deze cellen versterkt liarozole-fumaraat de expressie van differentiatie markers zoals laminine, collageen type IV en cytokeratines, die door ATRA geïnduceerd worden. Tegelijkertijd inhibeert liarozole-fumaraat het metabolisme van ATRA. Tezamen duiden deze gegevens erop dat liarozole-fumaraat zowel de antiproliferatieve als differentiatie-inducerende werking van ATRA versterkt door inhibitie van het ATRA-metabolisme.

Om het werkingsmechanisme van liarozole-fumaraat verder te ontrafelen, werd het effect bestudeerd op metabolieten en isomeren van ATRA. Deze studies konden enkel uitgevoerd worden in een celkweekmedium dat geen steroiden of retinoiden bevatte. Onder deze kweekcondities stoppen de cellen echter met delen, maar kunnen wel weer tot proliferatie gestimuleerd worden door groeifactoren toe te voegen. Ondanks de gestimuleerde proliferatie, blijven de bromodeoxyuridine immunosignalen (een maat voor celproliferatie) nauwelijks detecteerbaar. **Hoofdstuk 4** beschrijft een methode om deze zwakke immunosignalen te versterken, waarbij gebruik gemaakt wordt van tyramide dat gelabeld is met een fluorescerende stof.

In **Hoofdstuk 5** wordt aangetoond dat niet alleen ATRA zelf maar ook zijn katabolieten, 4-oxo-ATRA en 5,6-epoxy-ATRA, alsook zijn stereoisomeren, 9-*cis*-RA en 13-*cis*-RA, de proliferatie van MCF-7-cellen kunnen inhiberen. Aangezien echter deze katabolieten en stereoisomeren in beperkte mate gevormd worden vanuit ATRA, suggereren deze resultaten dat vooral ATRA zelf verantwoordelijk is voor zijn antiproliferatieve werking.

Liarozole-fumaraat kan de antiproliferatieve werking van de katabolieten versterken maar niet van de isomeren (**Hoofdstuk 6**). Dit wijst erop dat liarozole-fumaraat een veelzijdige inhibitor is, die niet alleen het metabolisme van ATRA inhibeert maar ook de verdere afbraak van de katabolieten van ATRA.

In **Hoofdstuk 7** wordt bediscussieerd dat het katabolisme van ATRA leidt tot een inactivatie en dat inhibitie van deze inactivatie door liarozole-fumaraat waardevol kan zijn als een nieuw alternatief voor differentiatie therapie van kanker.

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Curriculum vitae

Jimmy Van heusden werd geboren op 27 april 1971 te Wilrijk. Na het behalen van zijn diploma middelbaar onderwijs aan het Xaveriuscollege te Borgerhout in 1989, begon hij aan het Rijksuniversitair Centrum te Antwerpen zijn universitaire opleiding Scheikunde van 1989 tot 1991. Van 1991 tot 1993 vervolgde hij zijn opleiding aan de Universitaire Instelling te Antwerpen met de studie Biochemie en behaalde daar het diploma van licentiaat in de Wetenschappen.

In oktober 1993 werd hij aangesteld tot assistent in opleiding bij de vakgroep Moleculaire Celbiologie & Genetica (promotores: Prof. dr. F.C.S. Ramaekers, Prof. dr. M. Borgers en co-promotor: Dr. G. Smets) aan de Universiteit van Maastricht. Het project werd gefinancierd door de Janssen Research Foundation, waar ook het in dit proefschrift beschreven onderzoek werd uitgevoerd.

Vanaf 1 oktober 1997 is hij werkzaam als onderzoeker op de afdeling Oncologie (Dr. W. Wouters) van de Janssen Research Foundation.