Molecular and Cellular Endocrinology 267 (2007) 89–96

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Expression, protein stability and transcriptional activity of retinoic acid receptors are affected by microtubules interfering agents and all-*trans*-retinoic acid in primary rat hepatocytes

Zdeněk Dvořák [a,*], Radim Vrzal [a], Jitka Ulrichová [a], Dana Macejová [b], Slavomíra Ondková [b], Július Brtko [b]

[a] *Institute of Medical Chemistry and Biochemistry, Faculty of Medicine, Palacký University Olomouc, Hněvotínská 3, 77515 Olomouc, Czech Republic*
[b] *Laboratory of Molecular Endocrinology, Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlárska 3, Bratislava 83306, Slovak Republic*

Received 22 August 2006; received in revised form 16 November 2006; accepted 2 January 2007

Abstract

Cellular signaling by glucocorticoid receptor and aryl hydrocarbon receptor is restricted by microtubules interfering agents (MIAs). This leads to down-regulation of drug metabolizing enzymes and drug interactions. Here we investigated the effects of all-*trans*-retinoic acid (ATRA) and MIAs, i.e. colchicine, nocodazole and taxol on the regulation of retinoic acid receptor (RAR) genes in primary cultures of rat hepatocytes. ATRA (1 μM) down-regulated RARα and RARγ mRNAs (decrease 23% and 41%, respectively) whereas it up-regulated RARβ mRNA (4.3-fold induction). All MIAs diminished the expression of RARs in dose-dependent manner; the potency of MIAs increased in order NOC < COL < TAX and the extent of inhibition increased in order RARα < RARγ < RARβ. The levels of RARα protein were decreased by both MIAs and ATRA. The effects of ATRA were reversed by proteasome inhibitor MG-132, implying ligand-dependent RARα degradation. In contrast, the effects of MIAs were proteasome-independent and decrease in RARα protein content was due to RARα gene down-regulation. We monitored transcriptional activity of RARα. For this purpose, we measured catalytic activity of *trans*-glutaminase—target gene of RARα. *trans*-Glutaminase activity was increased by ATRA (1.23-fold increase) and decreased by colchicine (decrease 51%). Co-treatment with proteasome inhibitor MG-132 partly reversed inhibitory effect of colchicine, and it further augmented the increase of *trans*-glutaminase activity by ATRA. We have also observed decrease of RARα protein level and inhibition of RARs mRNAs expression in HeLa cells by MIAs. In conclusion, our data indicate that microtubules play the role in regulation of RARs activity and expression. Our data are the first report on the effects of ATRA and MIAs on RARs regulation in quiescent cells.

Keywords: Retinoic acid receptors; Cellular signaling; Microtubules; Rat hepatocytes

1. Introduction

Cellular signaling by nuclear receptors, including steroid, thyroid and retinoid receptors, is influenced by variety of endogenous factors as well as exogenous stimuli such as inflammation, infection, stress, drugs, environmental pollutants, UV-light, etc. Perturbation of cytoskeleton can be considered as pathophysiological factor affecting the function of several nuclear receptors with consequences in endogenous metabolism as well as in drug metabolism (Pascussi et al., 2003). For instance, microtubules interfering agents (MIAs) colchicine and nocodazole down-regulated expression of important biotransformation enzymes in primary human hepatocytes via inhibition of glucocorticoid receptor (GR) nuclear import and suppression of its transcriptional activity (Pascussi et al., 2003; Dvorak et al., 2002). Recently, drug–drug interaction between administered colchicine and anti-coagulants was attributed to down-regulation of CYP2C9 by microtubule disruption and subsequent diminution of anti-coagulant metabolism (Gras-

Abbreviations: AhR, aryl hydrocarbon receptor; ATRA, all-*trans*-retinoic acid; CAR, constitutive androstane receptor; COL, colchicine; DEX, dexamethasone; GR, glucocorticoid receptor; HeLa, human cervix carcinoma epithelial cell line; JNK, c-Jun-N-terminal kinase; MG-132, proteasome inhibitor; MIAs, microtubules interfering agents; NOC, nocodazole; PXR, pregnane X receptor; RAR, retinoic acid receptor; RXR, retinoin X receptor; TAX, taxol

* Corresponding author. Tel.: +42 58 5632311; fax: +42 58 5632302.
E-mail address: moulin@email.cz (Z. Dvořák).

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doi:10.1016/j.mce.2007.01.004

Champel et al., 2005; Rojas et al., 2005). Similarly, inhibitory effects of MIAs on aryl hydrocarbon receptor (AhR)-dependent cytochrome P450 CYP1A1 expression were reported (Bonzo et al., 2005; Santini et al., 2001; Scholler et al., 1994; Dvorak et al., 2006). Albeit the role of microtubules network in cellular signaling by GR and AhR receptors is ambiguous, there are apparently differences between effects in proliferating and non-proliferating cells (Dvorak et al., 2005a,b; Dvorak et al., 2004a). Inhibitory effects of MIAs on nuclear receptors in proliferating cells were attributed to the oscillation of the receptors function throughout the cell cycle, implying minimal activities of GR (Abel et al., 2002; Cidlowski and Cidlowski, 1982; Cidlowski and Michaels, 1977; Distelhorst et al., 1984; Hsu and DeFranco, 1995; Hsu et al., 1992; Hu et al., 1994) and AhR (Bonzo et al., 2005; Santini et al., 2001; Scholler et al., 1994) in G2/M phase of the cycle. However, down-regulation of AhR and GR dependent genes was observed in hepatocytes, which are non-proliferating (quiescent) cells. The inhibition of GR function in hepatocytes proceeded probably via activation of c-Jun-N-terminal kinase (JNK) that in turn negatively modulated transcriptional activity of GR, but the mechanism of AhR inhibition was not elucidated yet (Dvorak et al., 2004b).

Retinoic acid receptors (RARs) are typical nuclear receptors that are involved in essential endogenous processes and they are often targets in human pharmacotherapy (Brtko and Thalhamer, 2003). Retinoids have been studied as chemopreventive agents in clinical trials due to their established role in regulating cell growth, differentiation and apoptosis in preclinical models (Zanardi et al., 2006). Based on the knowledge available, we may anticipate that cellular signaling by RARs could be affected by MIAs. Surprisingly, to date, there is only one report on the possible interactions between MIAs and RARs. It describes synergistic cytotoxicity exhibited by combination treatment with selective retinoid ligands and taxol, but the mechanism was not investigated (Vivat-Hannah et al., 2001). Thus, further investigation of the effects of MIAs on cellular signaling by RARs should be carried out because both MIAs (e.g. vincristine, vinblastine, vimentine, taxol, colcemide, colchicine) and retinoids are used in human pharmacotherapy and possible drug interactions may occur. In addition, it is well known that RARs proteins undergo ligand-dependent proteasome-mediated degradation, similarly as other nuclear receptors do. The effects of all-*trans*-retinoic acid (ATRA) on RARs proteins stability were described in numerous cell lines (Andela and Rosier, 2004; Boudjelal et al., 2002; Pratt et al., 2003; Gianni et al., 2002; Tanaka et al., 2001), however the data from non-proliferating cells are scarce.

The aims of present study were to examine the effects of MIAs (colchicine, nocodazole, taxol) and all-*trans*-retinoic acid (ATRA) on cellular signaling by RARs in primary rat hepatocytes as model no-proliferating cells. We evaluated: (i) expression of RARα, RARβ and RARγ mRNAs; (ii) expression and stability of RARα protein; (iii) transcriptional activity of RARs—monitored as catalytic activity of *trans*-glutaminase, the target gene of RARs. In parallel, we have performed pilot examination of MIAs and ATRA effects on the expression of RAR receptors in HeLa cells, as the model of proliferating cells.

2. Materials and methods

2.1. Chemicals

Williams' medium E, bovine serum, foetal calf serum, penicillin, streptomycin, L-glutamine, sodium pyruvate, dexamethasone, all-*trans*-retinoic acid, proteasome inhibitor MG-132, colchicine, nocodazole, taxol, monoclonal anti-β-tubulin antibody and Kodak X-Omat AR photographic film were purchased from Sigma Chemicals (St. Louis, MO, USA). Trizol® Reagents, was purchased from GibcoBRL Life Technologies (Cergy Pontoise, France). Complete™ protease inhibitor cocktail tablets was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Secondary horseradish peroxidase conjugated antibody, and Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor 596-conjugated anti-mouse IgG was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were of the highest grade commercially available.

2.2. Rat hepatocytes cultures

Primary rat hepatocytes were isolated by two-step collagenase perfusion according to a published protocol (Moldeus et al., 1978). Following isolation, the cells were plated on collagen-coated culture dishes using cell density 2×10^5 cells/cm^2. Williams' medium E supplemented with 2 mM L-glutamine, 10 μM streptomycin, 100 U/mL penicillin, 350 nM insulin, and 1 μM dexamethasone, was used for culture maintenance. The medium was enriched for plating with 5% bovine serum (FCS) (v/v). After 4 h, culture medium was replaced by a serum-free one and the cells were ready for the treatments. Cultures were maintained at 37 °C in 5% CO_2 (air:CO_2, 95:5) humidified incubator.

2.3. HeLa cell cultures

Human Negroid cervix epitheloid carcinoma cells HeLa (ECACC no. 93021013) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 U/mL streptomycin, 100 μg/mL penicillin, 4 mM L-glutamine, and non-essential amino acids. Cultures were maintained at 37 °C in 5% CO_2 (air:CO_2, 95:5) humidified incubator. For the treatments, the cells were plated on Petri dishes (100 mm i.d.) in a density of 2×10^6 cells/dish. Cells were allowed to grow up to 70–80% of confluence. Following exchange for a serum-free medium, the culture was stabilized for an additional 4–6 h prior to the treatments.

2.4. mRNA analyses

Rat hepatocytes were plated on collagen-coated Petri dishes (100 mm i.d.) in a density of 1×10^7 cells/well using culture media enriched with bovine serum (5%, v/v). After 4 h, culture medium was replaced by a serum-free one. Thereafter, the cells were treated 24 h with all-*trans*-retinoic acid (ATRA; 1 μM), colchicine (COL; 0.1 μM and 1 μM), nocodazole (NOC; 1 μM, 10 μM and 40 μM), taxol (TAX; 1 μM, 10 μM and 40 μM) and/or with DMSO as vehicle for control. Total RNA was isolated using Trizol reagent according to the manufacturer's instructions. Concentration of RNA was quantified by spectrometry at 260 nm and purity was assessed from the ratio of absorbances $A_{260\,nm}/A_{280\,nm}$. Reverse transcription (RT) was performed with 2 μg of total RNA and the Ready-to-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech Inc., USA) according to the manufacturer's protocol. PCR was performed in a 25 μl total volume consisting of 4 μl RT mixture, 2.5 μl of 10× PCR buffer, 0.75 μl of 50 mM $MgCl_2$, 0.5 μl 10 mM dNTP, 25 pmol of each specific gene primer set and 0.6 U of DyNAzyme II DNA polymerase (Finnzymes OY, Finland) in buffer provided by the manufacturer. After treatment of samples at 94 °C for 3 min to inactivate reverse transcriptase, PCR consisted of 35 cycles of denaturing (95 °C, 45 s), annealing (57 °C, 30 s), extension (72 °C, 90 s), and a final extension at 72 °C for 10 min. The oligonucleotide sequences of primers used in PCR are rat RARα: sense 5′-CAGATGCACAACGCTGGC-3′, antisense 5′-CCGACTGTCCGCTTAGAG-3′; rat RARβ: sense 5′-ATGC-TGGCTTCGGTCCTC-3′, antisense 5′-CTGCAGCAGTGGTGACTG-3′; rat RARγ: sense 5′-GTGGAGACCGAATGGACC-3′, antisense 5′-GACAGG-GATGAACACAGG-3′ and GAPDH: sense 5′-TGAACGGGAAGCTCA-

CTGG-3′, antisense 5′-TCCACCACCCTGTTGCTGTA-3′. These conditions were proven to be in the log phase for each amplified sequence by us or were already described elsewhere (Ohata et al., 2000). The PCR products were separated on 2% agarose gel and stained with ethidium bromide. The band intensities were measured using the STS 6220I Documentation System (Ultralum, USA) and normalized to the band intensity of PCR product corresponding to the house keeping gene GAPDH. Analogical experiments were performed in HeLa cells. PCR was performed in a 25 μl total volume consisting of 4 μl (RARα and γ, GAPDH) and 8 μl (RARβ) RT mixture, 2.5 μl of 10× PCR buffer, 0.75 μl of 50 mM $MgCl_2$, 0.5 μl 10 mM dNTP, 25 pmol of each specific gene primer set and 0.6 U of DyNAzyme II DNA polymerase (Finnzymes OY, Finland) in buffer provided by the manufacturer. After treatment of samples at 94 °C for 3 min to inactivate reverse transcriptase, PCR consisted of 35 cycles of denaturing (95 °C, 1 min), annealing (60 °C, 1 min—RARα and γ, GAPDH) and (62 °C, 1 min—RARβ), extension (72 °C, 1 min), and a final extension at 72 °C for 10 min. The oligonucleotide sequences of primers used in PCR are: human RARα sense 5′-ACCCCCTCTACCCCGCATCTACAAG-3′, antisense 5′-CATGCCCACTTCAAAGCACTTCTGC-3′; human RARβ sense 5′-ATTCCAGTGCTGACCATCGAGTCC-3′, antisense 5′-cctgtttctgtgtcatccatttcc-3′; human RARγ sense 5′-TACCACTATGGGGTCAGC-3′, antisense 5′-CCGGTCATTTCGCACAGCT-3′ (Kimura et al., 2002).

2.5. Protein analyses

Rat hepatocytes were seeded on six-well dishes in a density of 2×10^6 cells/well using culture media enriched with bovine serum (5%, v/v). After 4 h, culture medium was replaced by a serum-free one. Thereafter, the cells were treated 24 h with all-*trans*-retinoic acid (ATRA; 1 μM), proteasome inhibitor MG-132 (1 μM), colchicine (COL; 0.1 μM and 1 μM), nocodazole (NOC; 1 μM, 10 μM and 40 μM), taxol (TAX; 1 μM, 10 μM and 40 μM) and/or with DMSO as vehicle for control. Total protein extracts were prepared as follows: cells were washed twice with 1 mL of ice-cold PBS and scraped into 1 mL of PBS. The suspension was centrifuged (1500 × *g*/5 min/4 °C) and the pellet was vigorously re-suspended in 150 μl of ice-cold lysis buffer (10 mM Hepes pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.5 mM DTT; 0.1% (v/v) NP-40; anti-protease cocktail, 0.2% (w/v) sodium dodecylsulfate). The mixture was incubated for 20 min on ice and then centrifuged (12,000 × *g*/10 min/4 °C). Supernatant was collected and the protein content in extracts was determined by the biscinchoninic acid method.

The extracts were analyzed on SDS-PAGE gels (7.5%) according to the general procedure (Laemmli, 1970). Protein transfer onto nitrocellulose membrane was carried out as described (Towbin et al., 1979). The membrane was stained with Ponceau S red dye for control of transfer and then saturated with 8% non-fat dried milk overnight. Blots were probed with primary antibodies against: retinoic acid receptor alpha (sc-551; RARα(C-20) rabbit polyclonal; dilution 1/1000) and α-actin (sc-1616; Actin (I-19) goat polyclonal; dilution 1/1000); both purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Chemiluminescence detection using horseradish peroxidase conjugated secondary antibodies and an Amersham ECL kit was performed. Analogical experiments were performed in HeLa cells.

2.6. Histochemical detection of microtubules disruption

Rat hepatocytes were plated on collagen-coated glass slides at density 5×10^5 cells/cm^2. Cells were treated for 24 h with colchicine (COL; 0.1 μM, 1 μM), nocodazole (NOC; 1 μM, 10 μM, 40 μM), taxol (TAX; 1 μM, 10 μM, 40 μM) and DMSO as vehicle for control. Cells were then washed twice with ice-cold PBS, fixed with methanol, and washed twice with PBS. After blocking for 60 min in 5% (w/v) bovine serum albumin, cells were incubated with monoclonal anti-β-tubulin antibody for 60 min. Secondary antibody used was Alexa Fluor 596-conjugated anti-mouse IgG. Microscopy (400× magnification) was performed on Olympus microscopes and digital images of all cultures were taken and stored as JPG files.

2.7. Cytotoxicity assays

Rat hepatocytes were seeded on 96-well collagen-coated dishes using culture media enriched with bovine serum (5%, v/v). Following 4 h of stabilization, the cells were treated for 24 h with tested compounds. In parallel, cultures were treated with DMSO (vehicle) and 1% (v/v) Triton X-100 to assess the minimal and maximal cell damage, respectively. MTT assay was measured spectrophotometrically at 540 nm (TECAN, Schoeller Instruments s.r.o.). The data were expressed as % of viability, when 100% and 0% represent the treatments with DMSO and Triton X-100, respectively.

2.8. Tissue trans-glutaminase activity assay

Rat hepatocytes were plated on collagen-coated Petri dishes (100 mm i.d.) in a density of 1×10^7 cells/well using culture media enriched with foetal calf serum (5%, v/v). After 4 h, culture medium was replaced by a serum-free one. Thereafter, the cells were treated 24 h with all-*trans*-retinoic acid (ATRA; 1 μM), colchicine (COL; 1 μM) and with DMSO as vehicle for control. Cells were washed twice with 1 ml of ice-cold PBS and scraped into 1 ml of PBS. The suspension was centrifuged (1500 × *g*/5 min/4 °C) and resulting pellet was stored at −80 °C. Tissue *trans*-glutaminase activity assay was measured by detecting the incorporation of [^{3}H] putrescine into *N*,*N*′-dimethylcaseine (Piacentini et al., 1992). One of the functions of tissue transglutaminase is to form a protein polymer by ε(γ-glutamyl)lysine and, specifically γ-glutamylpolyamine cross-links. A slight modification of the method commonly used for tissue transglutaminase activity in liver tissue (Pallet et al., 1997) enabled us to adapt that assay in which the protein concentration either in PBMC or cell culture samples was reduced six times (Dvorcakova et al., 2001).

3. Results

3.1. Effects of microtubule interfering agents on viability and microtubular network of primary rat hepatocytes

Prior to the principal experiments, we have examined the effects of MIAs on the viability and microtubular network integrity in primary rat hepatocytes. Viability of the cells was assessed as MTT test (Fig. 1). The effects of MIAs on microtubules network were monitored by immune-histochemical detection of β-tubulin (Fig. 2). Based on these results, we used following concentrations of MIAs in ongoing experiments: colchicine (COL; 0.1 μM, 1 μM), nocodazole (NOC; 1 μM, 10 μM, 40 μM), and taxol (TAX; 1 μM, 10 μM, 40 μM). The effects of MIAs in HeLa cells we described elsewhere (Dvorak et al., 2005b).

3.2. Effects of microtubule interfering agents and ATRA on the expression of retinoic acid receptors mRNAs

In first series of experiments, we have examined the effects of all-*trans*-retinoic acid (ATRA; 1 μM) and MIAs, i.e. colchicine

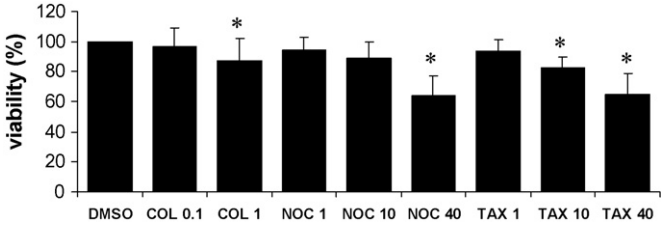

Fig. 1. Effects of MIAs on viability of primary rat hepatocytes. Cells were treated for 24 h with colchicine (COL; 0.1 μM, 1 μM), nocodazole (NOC; 1 μM, 10 μM, 40 μM), taxol (TAX; 1 μM, 10 μM, 40 μM), and DMSO as vehicle for control. MTT assay was measured as the indicator viability. Cells treated with DMSO represented 100% metabolic activity in the MTT assay. Bar plots represent means ± S.D. from three independent experiments. *Significantly different from that of DMSO-treated cells at $p < 0.05$.

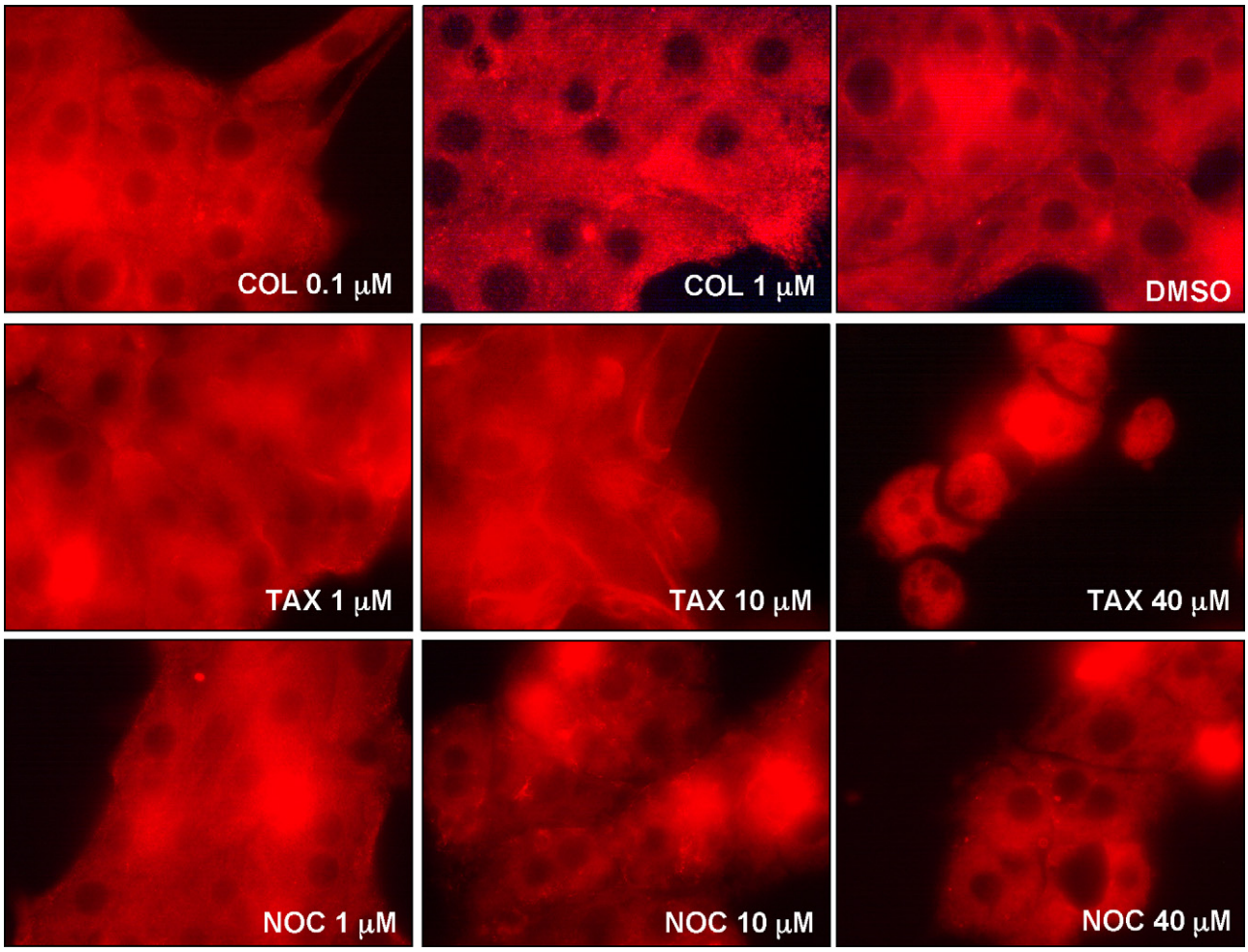

Fig. 2. Effects of MIAs on microtubules integrity in primary rat hepatocytes. Cells were treated for 24 h with colchicine (COL; 0.1 μM, 1 μM), nocodazole (NOC; 1 μM, 10 μM, 40 μM), taxol (TAX; 1 μM, 10 μM, 40 μM), and DMSO as vehicle for control. Microtubules visualization was performed with monoclonal anti-β-tubulin antibody followed by Alexa Fluor 596 conjugated anti-mouse IgG. Microscopy (400× magnification) was performed on Olympus microscopes.

(COL; 0.1 μM and 1 μM), nocodazole (NOC; 1 μM, 10 μM and 40 μM), taxol (TAX; 1 μM, 10 μM and 40 μM) on the expression of retinoic acid receptors RARα, RARβ and RARγ mRNAs. Primary cultures of rat hepatocytes were treated 24 h with tested compounds, total RNA was isolated and the levels of RARα, RARβ, RARγ and GAPDH mRNAs were determined by RT-PCR. ATRA selectively modulated the expression of RAR genes (Fig. 3a); it down-regulated RARα and RARγ mRNAs (decrease 23% and 41%, respectively) whereas it up-regulated RARβ mRNA (4.3-fold induction). All tested MIAs inhibited the expression of RARs in dose-dependent manner. The potency of tested MIAs increased in order NOC < COL < TAX and the extent of inhibition increased in order RARα < RARβ < RARβ (Fig. 3a).

In parallel, we have examined the effects of MIAs and ATRA on the expression of RARα, RARβ and RARγ mRNAs in HeLa cell line, as the model of proliferating cells. Cultures were treated with colchicine (COL; 0.1 μM and 1 μM), nocodazole (NOC; 1 μM and 10 μM), taxol (TAX; 1 μM and 10 μM), all-*trans*-retinoic acid (ATRA; 1 μM) and/or with DMSO as vehicle for control. ATRA increased the levels of all RARs mRNA by factors 1.15, 7.4 and 1.14 for RARα, RARβ and RARγ, respectively (Fig. 3b). Induction of RARβ by ATRA was reported also elsewhere (Geisen et al., 1997). The effects of MIAs on the expression of RARs mRNAs were less pronounced than in rat hepatocytes. The level of RARβ mRNA was not altered by MIAs. The potency of MIAs to down-regulate RARα and RARγ increased in order NOC < COL < TAX; i.e. the same as in hepatocytes (Fig. 3b). Note: we did not use NOC and TAX in 40 μM concentration due to high toxicity in HeLa cells.

3.3. *Effects of microtubule interfering agents and ATRA on the level of RARα protein*

While the analyses of RARs mRNAs reveal about the effects of tested substances on gene expression of the receptors, the analyses of the protein content comprise additional information on the stability/degradation of the protein products—receptors. Rat hepatocytes were treated 24 h with colchicine (COL; 0.1 μM and 1 μM), nocodazole (NOC; 1 μM, 10 μM and 40 μM), taxol (TAX; 1 μM, 10 μM and 40 μM), all-*trans*-retinoic acid

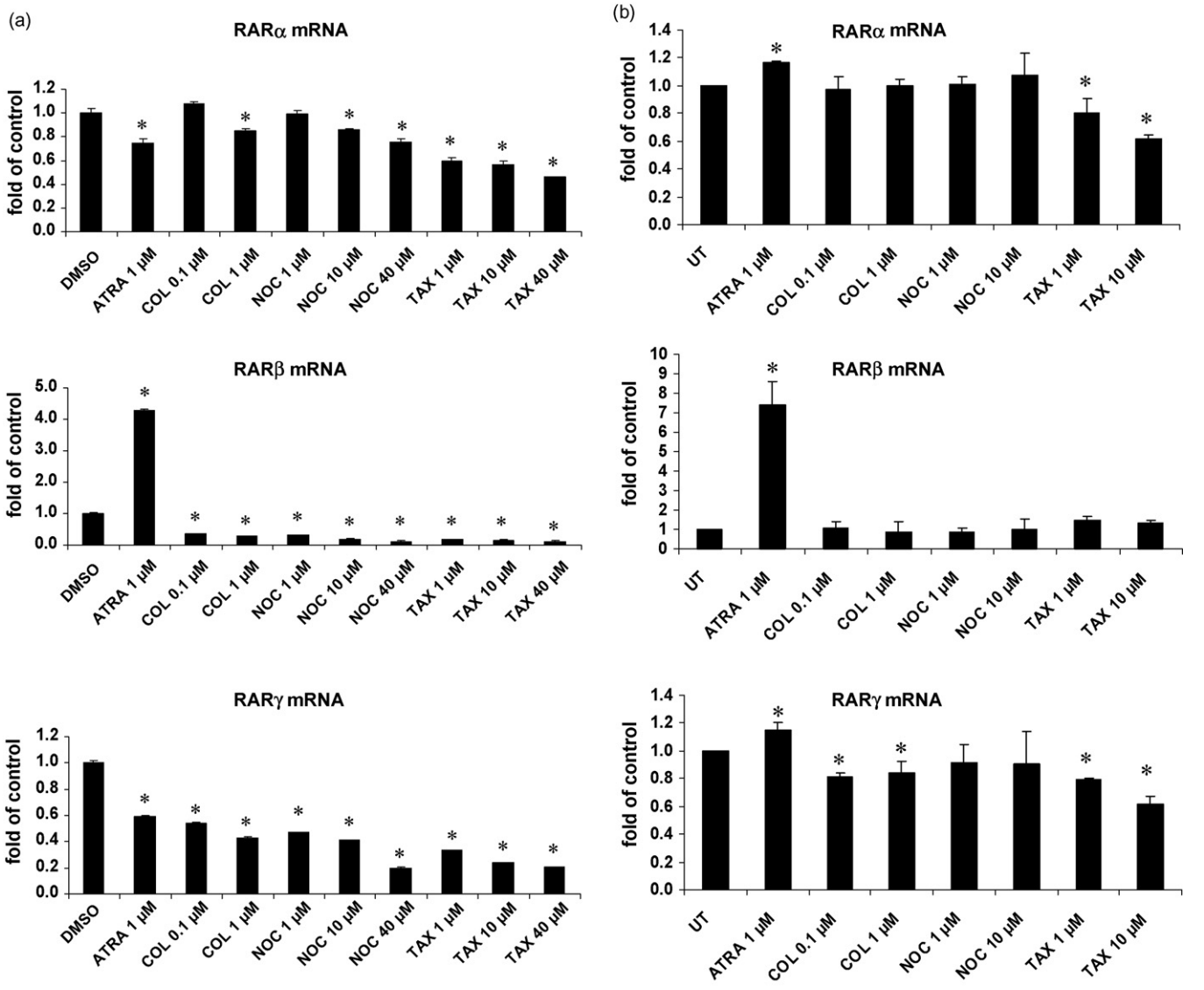

Fig. 3. Effects of microtubule interfering agents and ATRA on the expression of retinoic acid receptors mRNAs. (Panel a) Primary rat hepatocytes were treated 24 h with all-*trans*-retinoic acid (ATRA; 1 μM), colchicine (COL; 0.1 μM and 1 μM), nocodazole (NOC; 1 μM, 10 μM and 40 μM), taxol (TAX; 1 μM, 10 μM and 40 μM) and/or with DMSO as vehicle for control. (Panel b) Cultures were treated with colchicine (COL; 0.1 μM and 1 μM), nocodazole (NOC; 1 μM and 10 μM), taxol (TAX; 1 μM and 10 μM), all-*trans*-retinoic acid (ATRA; 1 μM) and/or with DMSO as vehicle for control. Total RNA was isolated and the levels of RARα, RARβ, RARγ and GAPDH mRNAs were determined by RT-PCR. The data were normalized per GAPDH content in the respective samples. Bar graphs represent means ± S.D. of three independent experiments. *Significantly different from the control value (DMSO) at $p < 0.05$.

(ATRA; 1 μM) and/or with DMSO as vehicle for control. Total protein extracts were isolated and subjected to Western blot analyses. For protein detection, we have limited our attention to RARα receptor. ATRA and MIAs decreased the levels of RARα protein, similarly as they did at the level of mRNA (Fig. 4A).

In parallel, we have examined the effects of MIAs and ATRA on the level of RARα protein in HeLa cells. Cultures were treated 24 h with colchicine (COL; 0.1 μM and 1 μM), nocodazole (NOC; 1 μM and 10 μM), taxol (TAX; 1 μM and 10 μM), all-*trans*-retinoic acid (ATRA; 1 μM) and/or with DMSO as vehicle for control. All tested MIAs and ATRA decreased the level of RARα protein (Fig. 4B). The effects of MIAs were more pronounced in HeLa cells as compared to those in rat hepatocytes.

3.4. Effects of colchicine and ATRA on RAR-dependent catalytic activity of trans-glutaminase

The expression of *trans*-glutaminase (*t*-GLUT) is under transcriptional control of RARα. Hence, the alteration in *t*-GLUT catalytic activity reflects the changes in the number and transcriptional activity of RARα. It gives additional information to the analyses at the level of protein and mRNA, and regarding *in vivo* or clinical situation, it is the most relevant parameter in the cascade mRNA-protein-activity. Rat hepatocytes were treated 24 h with colchicine (COL; 1 μM), all-*trans*-retinoic acid (ATRA; 1 μM) and/or with DMSO as vehicle for control. ATRA, a ligand of RARα, caused an increase in *t*-GLUT activity by factor 1.23. In contrast, colchicine diminished *t*-GLUT activity down to 49% of the activity of control cells (Fig. 5).

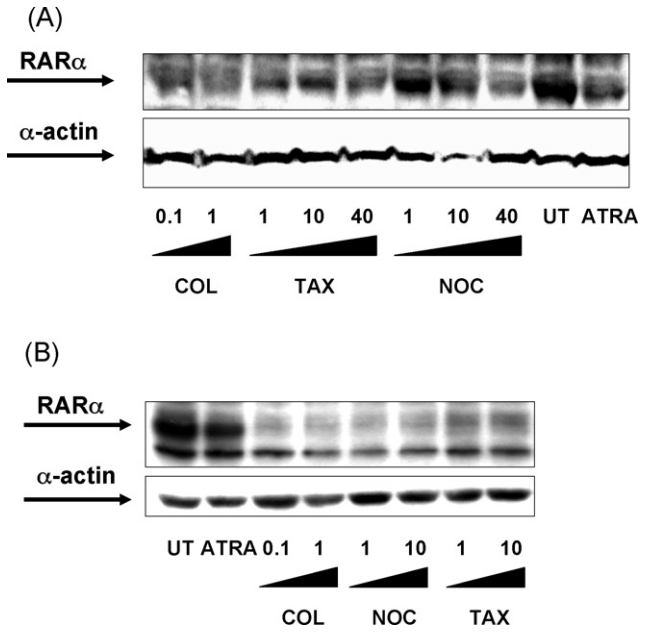

Fig. 4. Effects of microtubule interfering agents and ATRA on the level of RARα protein. Shown are representative Western blots of RARα. (Panel A) Rat hepatocytes were treated 24 h with colchicine (COL; 0.1 μM and 1 μM), nocodazole (NOC; 1 μM, 10 μM and 40 μM), taxol (TAX; 1 μM, 10 μM and 40 μM), all-*trans*-retinoic acid (ATRA; 1 μM) and/or with DMSO as vehicle for control. (Panel B) HeLa cells were treated 24 h with colchicine (COL; 0.1 μM and 1 μM), nocodazole (NOC; 1 μM and 10 μM), taxol (TAX; 1 μM and 10 μM), all-*trans*-retinoic acid (ATRA; 1 μM) and/or with DMSO as vehicle for control. Total protein extracts were isolated and after Western blot analysis the membrane was probed with anti RARα and anti actin antibodies. A similar profile was obtained from three independent experiments.

3.5. *Effects of proteasome inhibitor MG-132 on colchicine- and ATRA-mediated decrease of RARα protein and transcriptional activity*

Finally, we have investigated the involvement of proteasome in MIAs- and ATRA-elicited alterations in RARα protein and transcriptional activity. Rat hepatocytes were treated 24 h with colchicine (COL; 1 μM), all-*trans*-retinoic acid (ATRA; 1 μM) and/or with DMSO in the presence or absence of proteasome inhibitor (MG-132; 1 μM). The levels of RARα protein and catalytic activity of *t*-GLUT were determined. The decrease of RARα protein by ATRA was reversed by MG-132, implying ligand-dependent RARα degradation. In contrast, the effects of MIAs were proteasome-independent and decrease in RARα protein content was rather due to RARα gene down-regulation (Fig. 6A). MG-132 had no significant effect on basal *t*-GLUT activity. Co-treatment with MG-132 partly reversed inhibitory effect of colchicine, and it further augmented the increase of *t*-GLUT activity by ATRA (Fig. 6B).

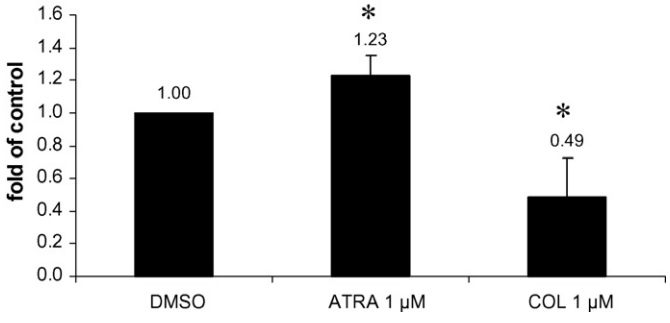

Fig. 5. Effects of colchicine and ATRA on catalytic activity of *trans*-glutaminase. Rat hepatocytes were treated 24 h with colchicine (COL; 1 μM), all-*trans*-retinoic acid (ATRA; 1 μM) and/or with DMSO as vehicle for control. Catalytic activity of *t*-GLUT was measured as described in Section 2. Bar graphs represent means ± S.D. of four independent experiments. *Significantly different from the control value (DMSO) at $p < 0.05$.

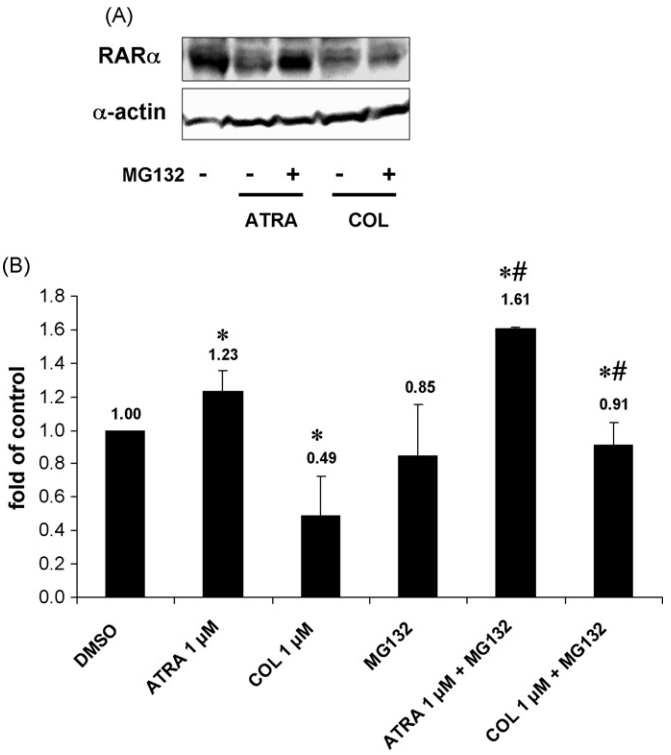

Fig. 6. Effects of proteasome inhibitor MG-132 on RARα protein content and transcriptional activity. Rat hepatocytes were treated 24 h with colchicine (COL; 1 μM), all-*trans*-retinoic acid (ATRA; 1 μM) and/or with DMSO in the presence or absence of proteasome inhibitor (MG-132; 1 μM). (Panel A) Total protein extracts were isolated and after Western blot analysis the membrane was probed with anti RARα and anti actin antibodies. A similar profile was obtained from two independent experiments. (Panel B) Catalytic activity of *t*-GLUT was measured as described in Section 2. Bar graphs represent means ± S.D. of three independent experiments. *Significantly different from the control value (DMSO) at $p < 0.05$. #Significantly different from the treatments in the absence of MG-132 at $p < 0.05$.

4. Discussion

Retinoids have been studied as chemopreventive agents in clinical trials due to their established role in regulating cell growth, differentiation and apoptosis in preclinical models (Zanardi et al., 2006). In this work we describe differential effects of MIAs and ATRA on the regulation of RAR genes in primary rat hepatocytes. In particular, we show that: (i) ATRA down-regulates RARα and RARγ mRNAs and up-regulates RARβ mRNA. MIAs down-regulate RARα, RARβ and RARγ mRNAs; (ii) ATRA decreases RARα protein content

by proteasome-dependent degradation. MIAs decrease RARα protein in proteasome-independent manner; (iii) transcriptional activity of RARα (monitored as catalytic activity of *t*-GLUT) was increased by ATRA and inhibited by colchicine.

The data obtained have two aspects:

- *Mechanistic aspect*. The role of microtubules in cellular signaling by steroids and their cognate nuclear receptors is still unresolved issue. We have previously reported the inhibition of transcriptional activity of GR (Pascussi et al., 2003; Dvorak et al., 2004b) and AhR (Dvorak et al., 2006; Dvorak et al., 2005a) by MIAs in human and rat hepatocytes, respectively. Both receptors under investigation, i.e. GR and AhR undergo cytosol to nucleus shuttling. The mechanism of MIAs inhibitory activity against these two receptors comprised at least partly restriction of nuclear import of GR and AhR. Assuming the involvement of microtubules in cellular signaling by these receptors, we should consider at least three mechanisms: (i) microtubules serve as supporting rails in cytosol to nucleus shuttling of the receptors or the factors in charge; (ii) microtubules associated proteins play certain role in the process; (iii) GTPase activity of microtubules is involved in the process. Hence, focusing on exclusively nuclear receptor (such as RARα), which does not require nuclear translocation, we can exclude option "(i)". Primordial question is: are the effects of MIAs direct or mediated via another receptor/transcriptional factor? While the expression of RXRs in human hepatocytes (Pascussi et al., 2000) and rat hepatocytes (Steineger et al., 1997; Yamaguchi et al., 1999) is under transcriptional control of GR, there is lack of information on the transcriptional factors/receptors controlling the expression of RARs. It was reported that dexamethasone (DEX) enhanced RARβ mRNA inducibility by ATRA in rat hepatocytes. This increase was inhibited by cycloheximide whereas the increase produced by ATRA treatment alone was not affected. These results indicate that DEX also enhances ATRA-dependent transcriptional activation in cultured hepatocytes by inducing RXRα. Since MIAs restrict cellular signaling by GR, the inhibitory effects of MIAs on the expression of RARs could be explained by the involvement of GR in transcriptional control of RARs. Different inhibitory potency of MIAs against RARs (RARα < RARγ < RARβ) would then be probably due to different contribution of GR in the process of the expression of individual RARs (different number of GRE in RARs promoters). Indeed, we have observed analogical effects on GR-controlled gene expression in human hepatocytes. The inhibitory potency of colchicine and nocodazole on gene expression increased in order PXR < CAR < TAT; note: TAT being exclusively GR-regulated gene (Pascussi et al., 2003).
- *Clinical aspect*. MIAs are clinically used as anticancer drugs (vincristine, vinblastine, taxol) or in the treatment of gout or familial Mediterranean fever (colchicine). We described recently that MIAs restrict cellular signaling by GR in primary human hepatocytes. It resulted in down-regulation of PXR and CAR receptors and P450 enzymes with possible consequences in drug metabolism (Pascussi et al., 2003). For instance, the co-administration of COL and coumarine-based anti-coagulants constitutes a precarious situation because this type of drugs is metabolized by CYP enzymes, in particular CYP3A4 and CYP2C8. Recent reports attributed the drug–drug interaction to down-regulation of CYP2C9 by microtubule disruption and subsequent retardation of anticoagulant metabolism (Gras-Champel et al., 2005; Rojas et al., 2005). Similarly, attention should be paid when using drugs acting via RARs together with MIAs. For instance, synergistic cytotoxicity exhibited by combination treatment with selective retinoid ligands and taxol was reported (Vivat-Hannah et al., 2001).

Finally, the data presented in this paper are the first report on the effects of MIAs on the expression and activity of RARs. Future research should focus on the role of GR in regulation of RARs. Since GR transcriptionally regulates also RXRs and thyroid receptor, the effects of MIAs on these receptors should be examined as well.

Acknowledgements

This research was supported by grant MSM 6198959216 from the Ministry of Education, Youth and Sports of the Czech Republic, by grant GACR 303/04/P074 from the Grant Agency of the Czech Republic, and grant VEGA no. 2/5017/5.

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