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## Accepted Manuscript

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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**

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**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  $\mu$ M) down-regulated RAR $\alpha$  and RAR $\gamma$  mRNAs (decrease 23% and 41%, respectively) whereas it up-regulated RAR $\beta$  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 RAR $\alpha$  < RAR $\gamma$  < RAR $\beta$ . The levels of RAR $\alpha$  protein were decreased by both MIAs and ATRA. The effects of ATRA were reversed by proteasome inhibitor MG132, implying ligand-dependent RAR $\alpha$  degradation. In contrast, the effects of MIAs were proteasome-independent and decrease in RAR $\alpha$  protein content was due to RAR $\alpha$  gene down-regulation. We monitored transcriptional activity of RAR $\alpha$ . For this purpose, we measured catalytic activity of *trans*-glutaminase - target gene of RAR $\alpha$ . *Trans*-glutaminase activity was increased by ATRA (1.23 fold increase) and decreased by colchicine (decrease 51%). Co-treatment with proteasome inhibitor MG132 partly reversed inhibitory effect of colchicine, and it further augmented the increase of *trans*-glutaminase activity by ATRA. We have also observed decrease of RAR $\alpha$  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.

## 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 [1]. 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 [1,2]. 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 [3,4]. Similarly, inhibitory effects of MIAs on aryl hydrocarbon receptor (AhR)-dependent cytochrome P450 CYP1A1 expression were reported [5-8]. 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 [9-11]. 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 [12-18] and AhR [5-7] 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 [19].

Retinoic acid receptors (RARs) are typical nuclear receptors that are involved in essential endogenous processes and they are often targets in human pharmacotherapy [20]. 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 [21]. 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 [22]. 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

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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 [23-27], 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 non-proliferating cells. We evaluated: i) Expression of RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$  mRNAs; ii) Expression and stability of RAR $\alpha$  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.

## Materials and methods

### *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- $\beta$ -tubulin antibody and Kodak X-Omat AR photographic film were purchased from Sigma Chemicals (St. Louis, MO, USA). Trizol<sup>®</sup> Reagents, was purchased from GibcoBRL Life Technologies (Cergy Pontoise, France). Complete<sup>™</sup> 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.

### *Rat hepatocytes cultures*

Primary rat hepatocytes were isolated by two-step collagenase perfusion according to a published protocol [28]. Following isolation, the cells were plated on collagen-coated culture dishes using cell density  $2 \times 10^5$  cells/cm<sup>2</sup>. Williams' medium E supplemented with 2 mM L-glutamine, 10  $\mu$ M streptomycin, 100 U/mL penicillin, 350 nM insulin, and 1  $\mu$ M 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

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and the cells were ready for the treatments. Cultures were maintained at 37°C in 5% CO<sub>2</sub> (air:CO<sub>2</sub>, 95:5) humidified incubator.

#### *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<sub>2</sub> (air:CO<sub>2</sub>, 95:5) humidified incubator. For the treatments, the cells were plated on Petri dishes (100 mm I.D.) in a density of 2 x 10<sup>6</sup> 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.

#### *mRNA analyses*

Rat hepatocytes were plated on collagen-coated Petri dishes (100 mm I.D.) in a density of 1 x 10<sup>7</sup> 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  $A_{260\text{nm}}/A_{280\text{nm}}$ . 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 10x PCR buffer, 0.75 µl of 50 mM MgCl<sub>2</sub>, 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 sec), annealing (57°C, 30 sec), extension (72°C, 90 sec), and a final extension at 72°C for 10 min. The oligonucleotide sequences of primers used in PCR are rat RAR $\alpha$ : sense 5'-CAGATGCACAACGCTGGC-3', antisense 5'-CCGACTGTCCGCTTAGAG-3'; rat RAR $\beta$ : sense 5'-ATGCTGGCTTCGGTCCTC-3', antisense 5'-CTGCAGCAGTGGTGACTG-3'; rat RAR $\gamma$ :

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sense 5'-GTGGAGACCGAATGGACC-3', antisense 5'-GACAGGGATGAACACAGG-3' and GAPDH: sense 5'-TGAACGGGAAGCTCACTGG-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 [29]. 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 (RAR $\alpha$  and  $\gamma$ , GAPDH) and 8 µl (RAR $\beta$ ) RT mixture, 2.5 µl of 10x PCR buffer, 0.75 µl of 50 mM MgCl<sub>2</sub>, 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 - RAR $\alpha$  and  $\gamma$ , GAPDH) and (62°C, 1 min - RAR $\beta$ ), 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 RAR $\alpha$  sense 5'-ACCCCCTCTACCCCGCATCTACAAG-3', antisense 5'-CATGCCCACTTCAAAGCACTTCTGC-3'; human RAR $\beta$  sense 5'-ATTCCAGTGCTGACCATCGAGTCC-3', antisense 5'-CCTGTTTCTGTGTCATCCATTTCC-3'; human RAR $\gamma$  sense 5'-TACCACTATGGGGTTCAGC-3', antisense 5'-CCGGTCATTTTCGCACAGCT-3' [30].

### *Protein analyses*

Rat hepatocytes were seeded on 6-well dishes in a density of 2 x 10<sup>6</sup> 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<sub>2</sub>; 0.5 mM DTT; 0.1% v/v NP-40; anti-protease cocktail, 0.2% w/v sodium dodecylsulfate). The mixture was incubated

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for 20 min on ice and then centrifuged (12000 g/ 10 min/ 4°C). Supernatant was collected and the protein content in extracts was determined by the bicinchoninic acid method.

The extracts were analyzed on SDS-PAGE gels (7.5%) according to the general procedure [31]. Protein transfer onto nitrocellulose membrane was carried out as described [32]. 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; RAR $\alpha$ (C-20) rabbit polyclonal; dilution 1/1000) and  $\alpha$ -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.

#### *Histochemical detection of microtubules disruption*

Rat hepatocytes were plated on collagen-coated glass slides at density  $5 \times 10^5$  cells/cm<sup>2</sup>. Cells were treated for 24 h with colchicine (COL; 0.1  $\mu$ M, 1  $\mu$ M), nocodazole (NOC; 1  $\mu$ M, 10  $\mu$ M, 40  $\mu$ M), taxol (TAX; 1  $\mu$ M, 10  $\mu$ M, 40  $\mu$ M); 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- $\beta$ -tubulin antibody for 60 min. Secondary antibody used was Alexa Fluor 596-conjugated anti-mouse IgG. Microscopy (400 x magnification) was performed on Olympus microscopes and digital images of all cultures were taken and stored as JPG files.

#### *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.

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#### *Tissue trans-glutaminase activity assay*

Rat hepatocytes were plated on collagen-coated Petri dishes (100 mm I.D.) in a density of  $1 \times 10^7$  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  $\mu$ M), colchicine (COL; 1  $\mu$ M) 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 [<sup>3</sup>H] putrescine into N, N'-dimethylcaseine [33]. One of the functions of tissue transglutaminase is to form a protein polymer by epsilon(gamma-glutamyl)lysine and, specifically gamma-glutamylpolyamine cross-links. A slight modification of the method commonly used for tissue transglutaminase activity in liver tissue [34] enabled us to adapt that assay in which the protein concentration either in PBMC or cell culture samples was reduced six times [35].

## **Results**

#### *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 (Figure 1). The effects of MIAs on microtubules network were monitored by immune-histochemical detection of  $\beta$ -tubulin (Figure 2). Based on these results, we used following concentrations of MIAs in ongoing experiments: colchicine (COL; 0.1  $\mu$ M, 1  $\mu$ M), nocodazole (NOC; 1  $\mu$ M, 10  $\mu$ M, 40  $\mu$ M), taxol (TAX; 1  $\mu$ M, 10  $\mu$ M, 40  $\mu$ M). The effects of MIAs in HeLa cells we described elsewhere [10].

#### *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  $\mu$ M) and MIAs, i.e. colchicine (COL; 0.1  $\mu$ M and 1  $\mu$ M), nocodazole (NOC; 1  $\mu$ M, 10  $\mu$ M and 40  $\mu$ M), taxol (TAX; 1  $\mu$ M, 10  $\mu$ M and 40  $\mu$ M) on the expression of retinoic acid receptors RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$  mRNAs. Primary cultures of rat hepatocytes were treated 24 h with tested compounds, total RNA was isolated and the levels of RAR $\alpha$ , RAR $\beta$ , RAR $\gamma$  and GAPDH mRNAs were determined by RT-PCR. ATRA selectively modulated the

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expression of RAR genes (Figure 3a); it down-regulated RAR $\alpha$  and RAR $\gamma$  mRNAs (decrease 23% and 41%, respectively) whereas it up-regulated RAR $\beta$  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 RAR $\alpha$  < RAR $\gamma$  < RAR $\beta$  (Figure 1).

In parallel, we have examined the effects of MIAs and ATRA on the expression of RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$  mRNAs in HeLa cell line, as the model of proliferating cells. Cultures were treated with colchicine (COL; 0.1  $\mu$ M and 1  $\mu$ M), nocodazole (NOC; 1  $\mu$ M and 10  $\mu$ M), taxol (TAX; 1  $\mu$ M and 10  $\mu$ M), all-trans retinoic acid (ATRA; 1  $\mu$ M) 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 RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$ , respectively (Figure 3b). Induction of RAR $\beta$  by ATRA was reported also elsewhere [36]. The effects of MIAs on the expression of RARs mRNAs were less pronounced than in rat hepatocytes. The level of RAR $\beta$  mRNA was not altered by MIAs. The potency of MIAs to down-regulate RAR $\alpha$  and RAR $\gamma$  increased in order NOC < COL < TAX; i.e. the same as in hepatocytes (Figure 3b). Note: we did not use NOC and TAX in 40  $\mu$ M concentration due to high toxicity in HeLa cells.

#### *Effects of microtubule interfering agents and ATRA on the level of RAR $\alpha$ 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  $\mu$ M and 1  $\mu$ M), nocodazole (NOC; 1  $\mu$ M, 10  $\mu$ M and 40  $\mu$ M), taxol (TAX; 1  $\mu$ M, 10  $\mu$ M and 40  $\mu$ M), all-trans retinoic acid (ATRA; 1  $\mu$ M) 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 RAR $\alpha$  receptor. ATRA and MIAs decreased the levels of RAR $\alpha$  protein, similarly as they did at the level of mRNA (Figure 4a).

In parallel, we have examined the effects of MIAs and ATRA on the level of RAR $\alpha$  protein in HeLa cells. Cultures were treated 24 h with colchicine (COL; 0.1  $\mu$ M and 1  $\mu$ M), nocodazole (NOC; 1  $\mu$ M and 10  $\mu$ M), taxol (TAX; 1  $\mu$ M and 10  $\mu$ M), all-trans retinoic acid (ATRA; 1  $\mu$ M) and/or with DMSO as vehicle for control. All tested MIAs and ATRA decreased the

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level of RAR $\alpha$  protein (Figure 4b). The effects of MIAs were more pronounced in HeLa cells as compared to those in rat hepatocytes.

#### *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 RAR $\alpha$ . Hence, the alteration in t-GLUT catalytic activity reflects the changes in the number and transcriptional activity of RAR $\alpha$ . 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  $\mu$ M), *all-trans* retinoic acid (ATRA; 1  $\mu$ M) and/or with DMSO as vehicle for control. ATRA, a ligand of RAR $\alpha$ , 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 (Figure 5).

#### *Effects of proteasome inhibitor MG-132 on colchicine- and ATRA-mediated decrease of RAR $\alpha$ protein and transcriptional activity*

Finally, we have investigated the involvement of proteasome in MIAs- and ATRA-elicited alterations in RAR $\alpha$  protein and transcriptional activity. Rat hepatocytes were treated 24 h with colchicine (COL; 1  $\mu$ M), *all-trans* retinoic acid (ATRA; 1  $\mu$ M) and/or with DMSO in the presence or absence of proteasome inhibitor (MG-132; 1  $\mu$ M). The levels of RAR $\alpha$  protein and catalytic activity of t-GLUT were determined. The decrease of RAR $\alpha$  protein by ATRA was reversed by MG132, implying ligand-dependent RAR $\alpha$  degradation. In contrast, the effects of MIAs were proteasome-independent and decrease in RAR $\alpha$  protein content was rather due to RAR $\alpha$  gene down-regulation (Figure 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 (Figure 6b).

## **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 [21]. 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 RAR $\alpha$  and RAR $\gamma$  mRNAs and up-regulates RAR $\beta$  mRNA. MIAs

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down-regulate RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$  mRNAs; (ii) ATRA decreases RAR $\alpha$  protein content by proteasome-dependent degradation. MIAs decrease RAR $\alpha$  protein in proteasome-independent manner; (iii) Transcriptional activity of RAR $\alpha$  (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 [1,19] and AhR [8,9] 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 RAR $\alpha$ ), 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 [37] and rat hepatocytes [38,39] 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 RAR $\beta$  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 RXR $\alpha$ . 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 (RAR $\alpha$  < RAR $\gamma$  < RAR $\beta$ ) 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 [1].

**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

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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 [1]. 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 anti-coagulant metabolism [3,4]. 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 [22].

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.

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**List of non-standard 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

**Figure legends**

**Figure 1. Effects of MIAs on viability of primary rat hepatocytes.** Cells were treated for 24 h with colchicine (COL; 0.1  $\mu$ M, 1  $\mu$ M), nocodazole (NOC; 1  $\mu$ M, 10  $\mu$ M, 40  $\mu$ M), taxol (TAX; 1  $\mu$ M, 10  $\mu$ M, 40  $\mu$ M); 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  $\pm$  SD from three independent experiments. \* = the value is significantly different from that of DMSO-treated cells at  $p < 0.05$ .

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

**Figure 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  $\mu$ M), colchicine (COL; 0.1  $\mu$ M and 1  $\mu$ M), nocodazole (NOC; 1  $\mu$ M, 10  $\mu$ M and 40  $\mu$ M), taxol (TAX; 1  $\mu$ M, 10  $\mu$ M and 40  $\mu$ M) and/or with DMSO as vehicle for control. **Panel B:** Cultures were treated with colchicine (COL; 0.1  $\mu$ M and 1  $\mu$ M), nocodazole (NOC; 1  $\mu$ M and 10  $\mu$ M), taxol (TAX; 1  $\mu$ M and 10  $\mu$ M), all-trans retinoic acid (ATRA; 1  $\mu$ M) and/or with DMSO as vehicle for control. Total RNA was isolated and the levels of RAR $\alpha$ , RAR $\beta$ , RAR $\gamma$  and GAPDH mRNAs were determined by RT-PCR. The data were normalized per GAPDH content in the respective samples. Bar graphs represent means  $\pm$  SD of three independent experiments. \* = the value significantly different from the control value (DMSO) at  $p < 0.05$ .

**Figure 4. Effects of microtubule interfering agents and ATRA on the level of RAR $\alpha$  protein.** Shown are representative Western blots of RAR $\alpha$ . **Panel A:** Rat hepatocytes were treated 24 h with colchicine (COL; 0.1  $\mu$ M and 1  $\mu$ M), nocodazole (NOC; 1  $\mu$ M, 10  $\mu$ M and

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40  $\mu\text{M}$ ), taxol (TAX; 1  $\mu\text{M}$ , 10  $\mu\text{M}$  and 40  $\mu\text{M}$ ), all-trans retinoic acid (ATRA; 1  $\mu\text{M}$ ) and/or with DMSO as vehicle for control. **Panel B:** HeLa cells were treated 24 h with colchicine (COL; 0.1  $\mu\text{M}$  and 1  $\mu\text{M}$ ), nocodazole (NOC; 1  $\mu\text{M}$  and 10  $\mu\text{M}$ ), taxol (TAX; 1  $\mu\text{M}$  and 10  $\mu\text{M}$ ), all-trans retinoic acid (ATRA; 1  $\mu\text{M}$ ) and/or with DMSO as vehicle for control. Total protein extracts were isolated and after western blot analysis the membrane was probed with anti RAR $\alpha$  and anti actin antibodies. A similar profile was obtained from three independent experiments.

**Figure 5. Effects of colchicine and ATRA on catalytic activity of trans-glutaminase.** Rat hepatocytes were treated 24 h with colchicine (COL; 1  $\mu\text{M}$ ), all-trans retinoic acid (ATRA; 1  $\mu\text{M}$ ) and/or with DMSO as vehicle for control. Catalytic activity of t-GLUT was measured as described in Materials and Methods section. Bar graphs represent means  $\pm$  SD of four independent experiments. \* = the value significantly different from the control value (DMSO) at  $p < 0.05$ .

**Figure 6. Effects of proteasome inhibitor MG-132 on RAR $\alpha$  protein content and transcriptional activity.** Rat hepatocytes were treated 24 h with colchicine (COL; 1  $\mu\text{M}$ ), all-trans retinoic acid (ATRA; 1  $\mu\text{M}$ ) and/or with DMSO in the presence or absence of proteasome inhibitor (MG-132; 1  $\mu\text{M}$ ). **Panel A:** Total protein extracts were isolated and after western blot analysis the membrane was probed with anti RAR $\alpha$  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 Materials and Methods section. Bar graphs represent means  $\pm$  SD of three independent experiments. \* = the value is significantly different from the control value (DMSO) at  $p < 0.05$ . # = the value is significantly different from the treatments in the absence of MG132 at  $p < 0.05$ .

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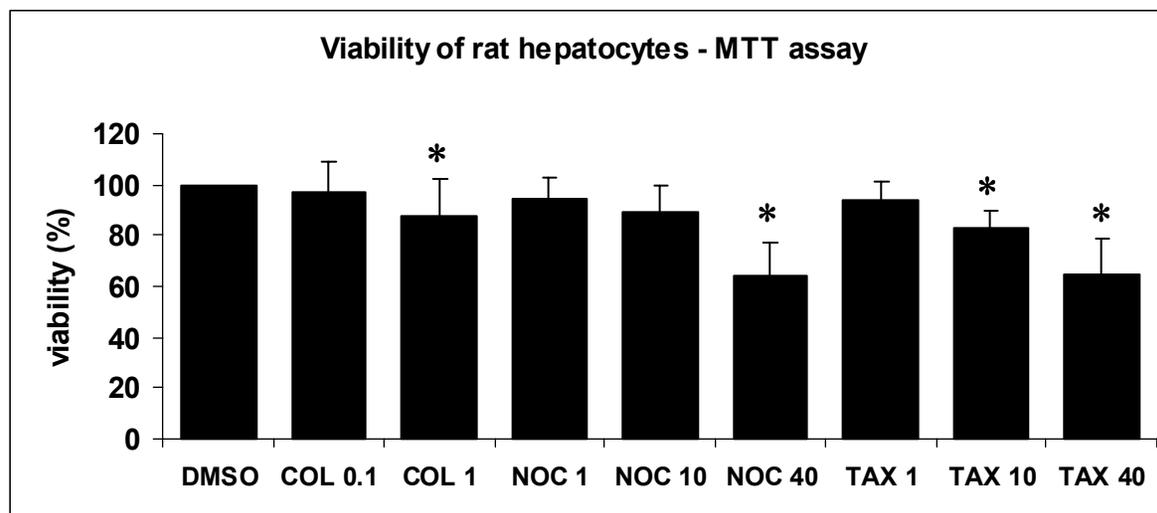


Figure 1

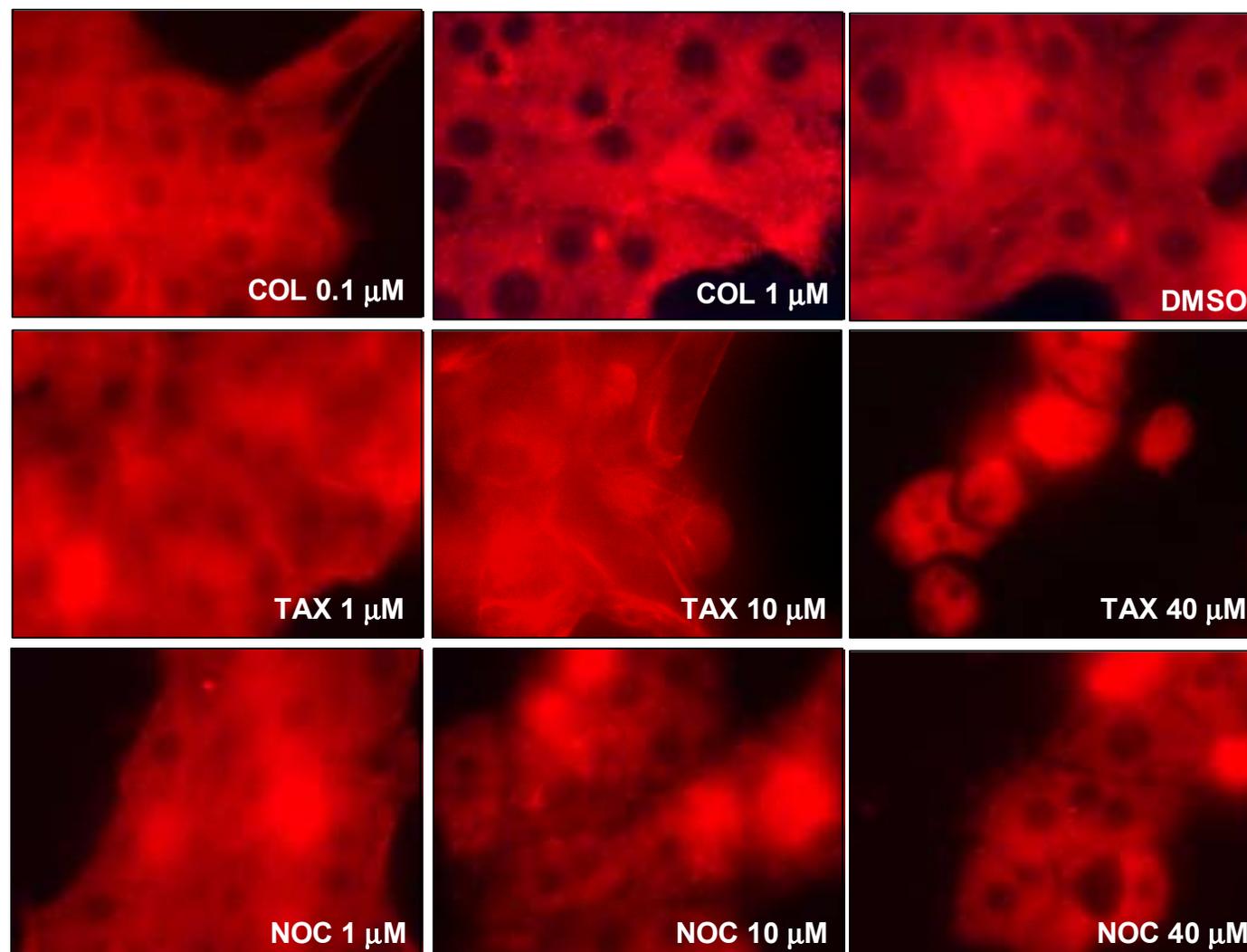


Figure 2

## Primary Rat Hepatocytes

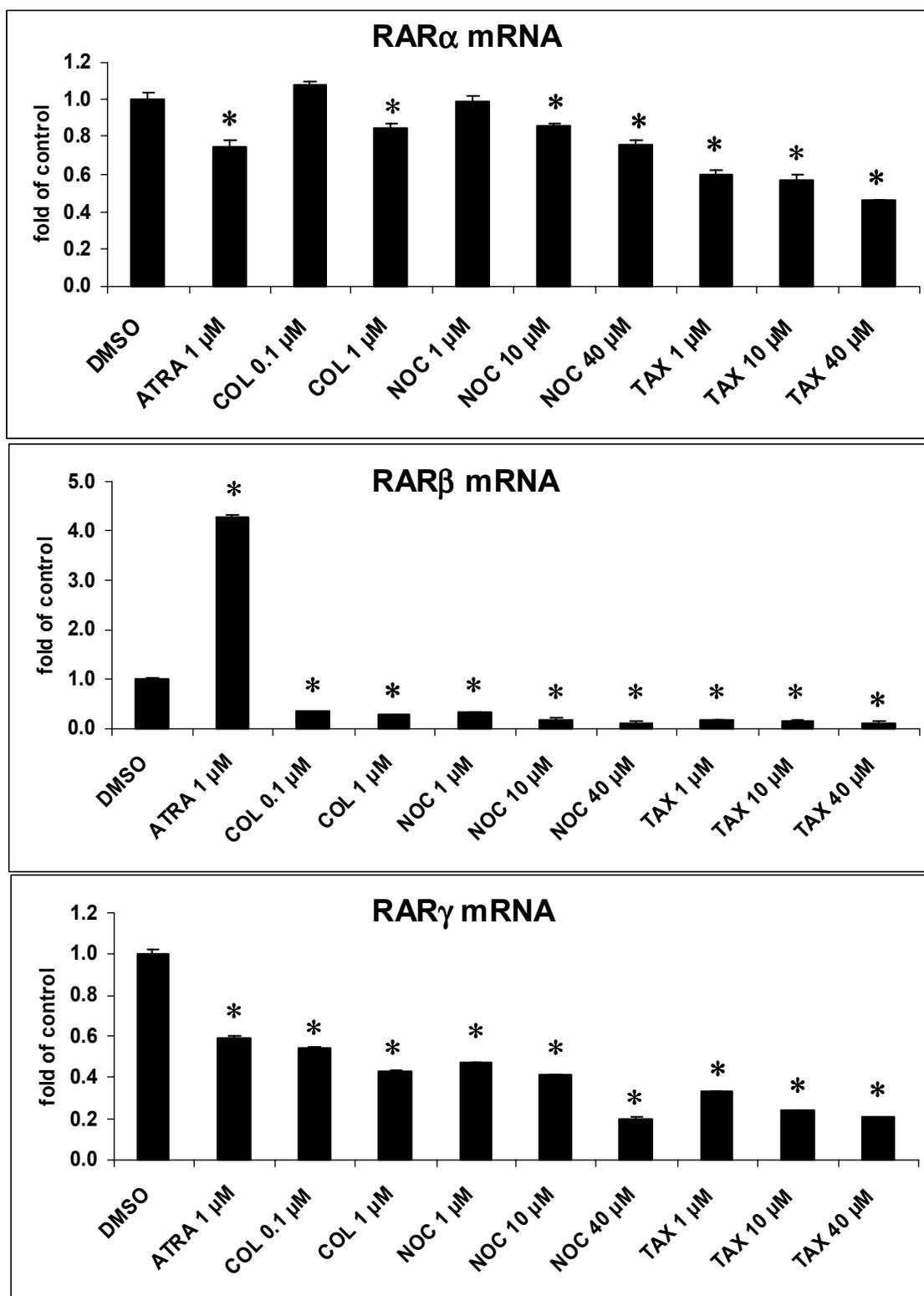


Figure 3a

## HeLa cells

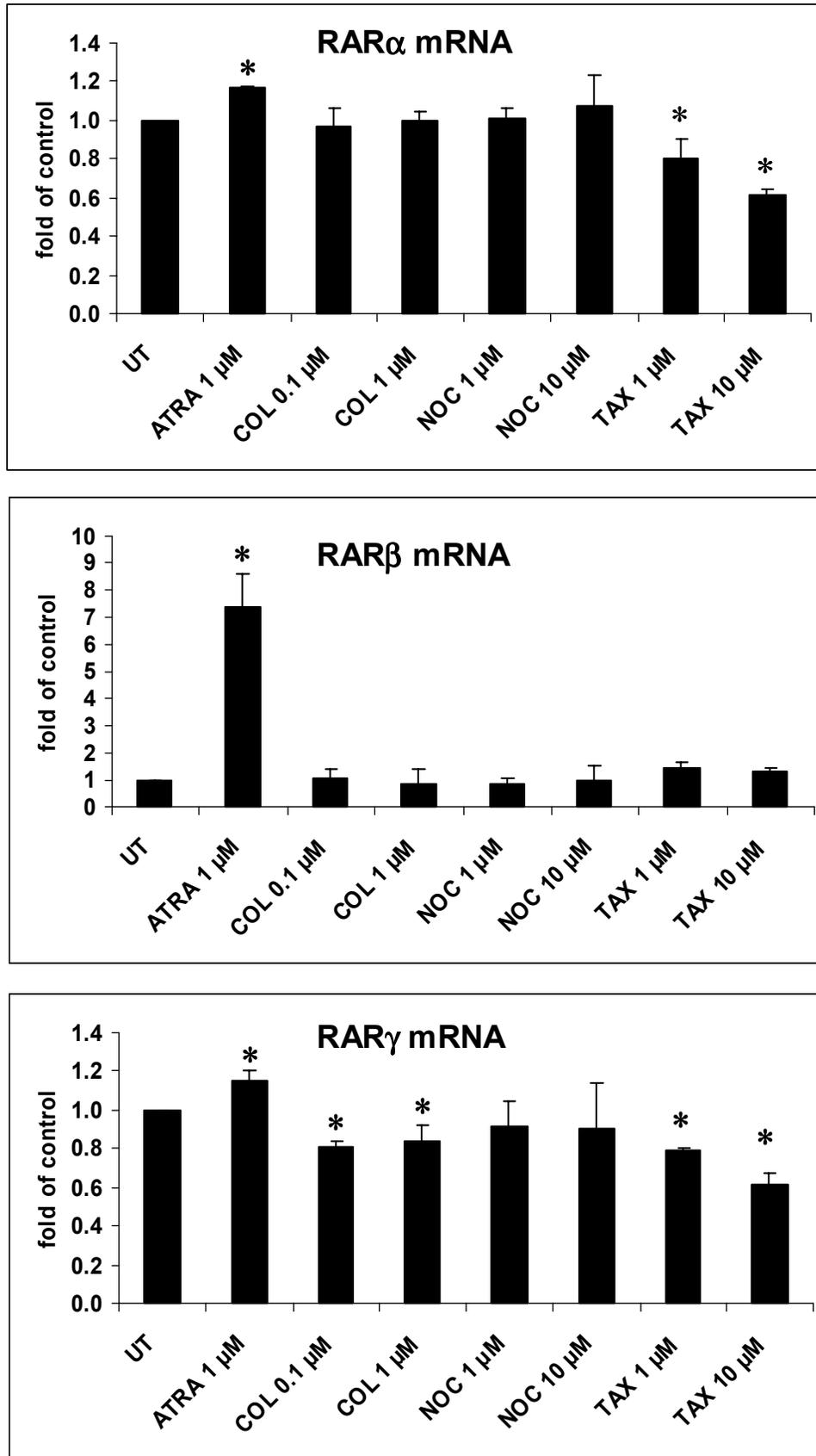
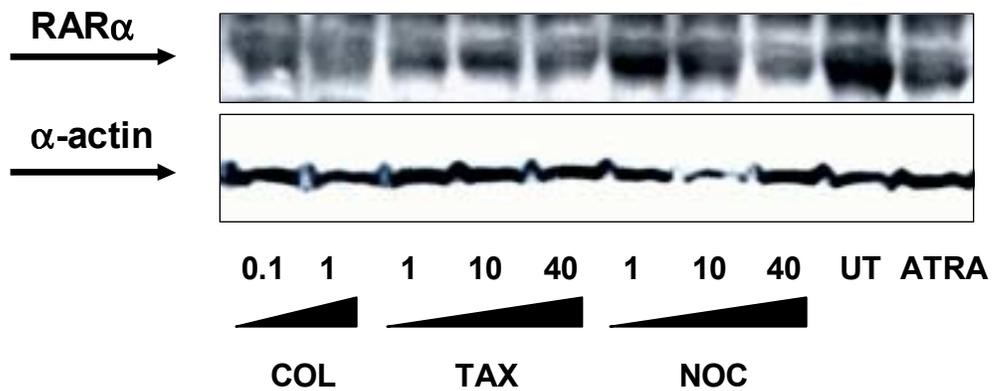
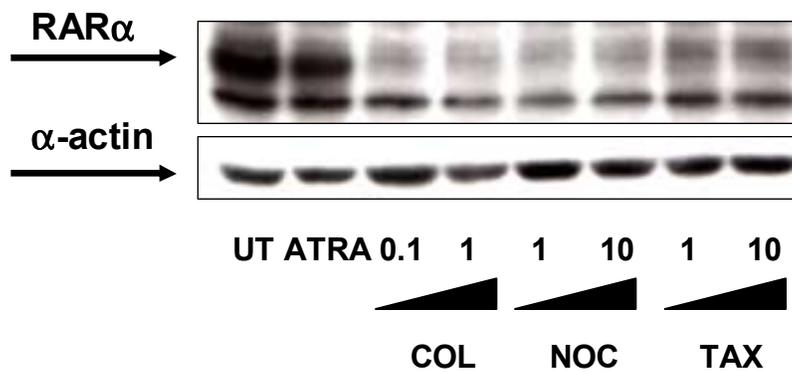
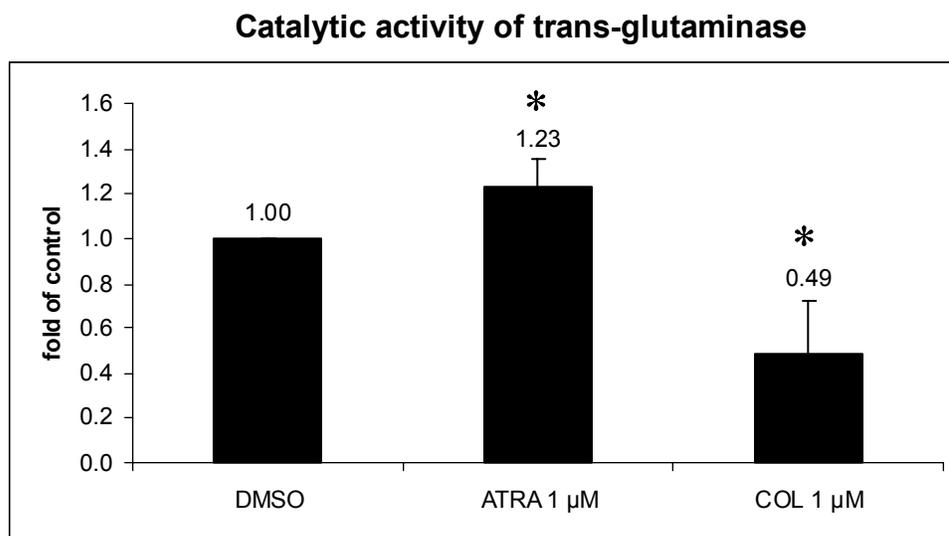
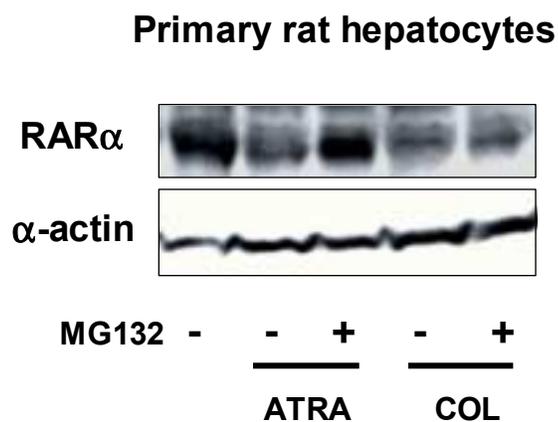


Figure 3b

**A****Primary Rat Hepatocytes****B****HeLa Cells****Figure 4**

**Figure 5**

A



B

## Catalytic activity of trans-glutaminase

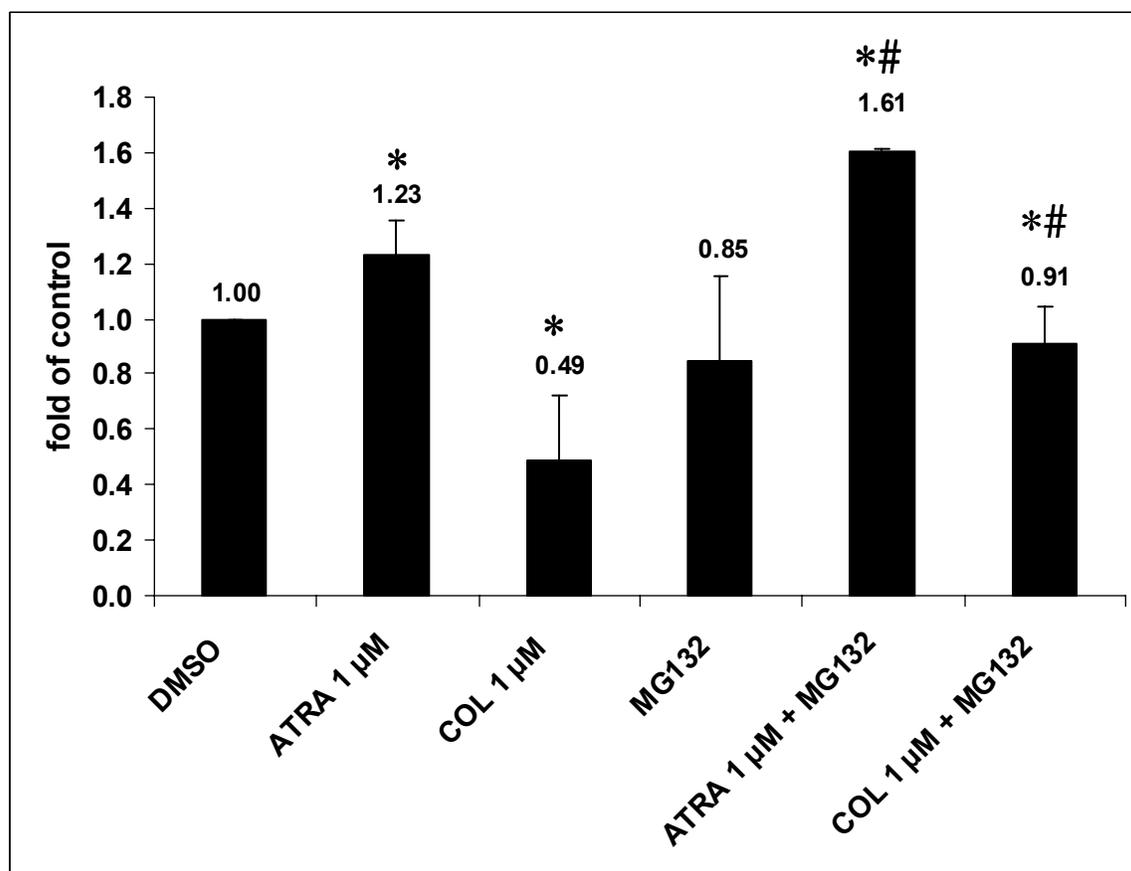


Figure 6