Evaluation of novel microtubules interfering agents myoseverin, tubulyzine and E2GG in primary cultures of rat hepatocytes

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Abstract. We investigated the effects of novel microtubules interfering agents (MIAs) in primary cultures of rat hepatocytes. Cells were treated for 24 h with a known compound colchicine and newly synthesized derivatives myoseverin, tubulyzine, and E2GG. We examined the effects of MIAs on microtubules network integrity and on the polymerization capability of isolated tubulin. All tested MIAs inhibited microtubules assembly with the following IC_{50} values: tubulyzine (4.4 ± 0.9 μmol/l), myoseverin (7.0 ± 0.8 μmol/l), E2GG (16 ± 2 μmol/l), colchicine (2.0 ± 0.4 μmol/l). The potency of MIAs to perturb microtubular network integrity (monitored by immune-histochemistry) increased in the order tubulyzine < myoseverin < E2GG < colchicine.

We described recently deleterious effects of MIAs on the expression of drug metabolizing enzymes, including CYP1A1. Here we observed inhibitory effects of novel MIAs on dioxin-inducible expression of CYP1A1 mRNA in rat hepatocytes. We conclude that novel MIAs exert analogical biological response as classical MIAs such as colchicine or nocodazole. This further supports the hypothesis that tubulin is the primordial target of MIAs within the cell and that perturbation of microtubules dynamics and/or integrity triggers the biological effects described here.

Key words: Microtubule — Tubulin polymerization — Cytochrome P450 — Cellular signaling — Myoseverin — Tubulyzine

Introduction

Microtubules perform multiple functions within a cell. Acting as a dynamic scaffold, microtubules determine the cell shape, direct cell movement, serve as tracks for intracellular traffic etc. Major task is formation of the spindle fibers for separating chromosomes during mitosis. As it is crucial point for cell proliferation, disruption of microtubules affects proliferating cells especially. Cancer cells proliferate rapidly, hence some cancer treatments take advantage of natural or artificial microtubule interfering agents (MIAs) ability to stall cancer cell proliferation. Sustained interest in development of new MIAs with different molecular mechanisms of action poses a question whether non-proliferating somatic cells might be affected as well considering the possible role of cytoskeleton in cell signaling.

Exact role of microtubules in cellular signaling is not fully understood yet. In our previous experiments, we have observed that MIAs restrict cellular signaling by glucocorticoid receptor (Dvořák et al. 2002, 2004, 2005a, 2006a; Pascussi et al. 2003) and aryl hydrocarbon receptor (AhR) (Dvořák et al. 2006b) by different mechanisms. These

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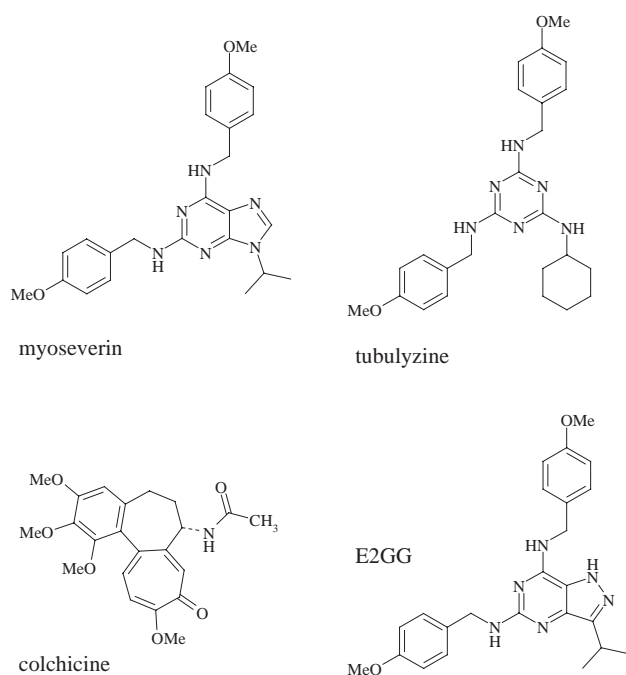E-mail: moulin@email.cz

Figure 1. Chemical structures of MIAs.

effects have severe consequences in the expression and activity of drug metabolizing enzymes and other cellular processes (Pascussi et al. 2003; Pascussi 2004; Modrianský and Dvořák 2005; Dvorak et al. 2005b). Regarding AhR function, we described down-regulation of AhR-controlled genes (HepG2 cells and rat hepatocytes) and restriction of AhR nuclear translocation (HepG2) by colchicine and nocodazole (Dvořák et al. 2006b). We observed similar behavior in primary cultures of human hepatocytes (submitted).

The fundamental question is, whether MIAs with different mechanism of action at molecular level will display different effects at biological/cellular level. For instance, microtubule stabilizer taxol had the same effects on the activation of c-jun-N-terminal kinase as microtubule destabilizers nocodazole and colchicine (Wang et al. 1998). In contrast, depolymerization of microtubules led to the induction of NF-κB-dependent gene expression, whereas these effects were blocked by the microtubule-stabilizing agent taxol (Rosette and Karin 1995).

Myoseverin

Novel microtubule-disassembling molecule, myoseverin, was identified from a library of 2,6,9-trisubstituted purines using a morphological differentiation screen (Rosania et al. 2000). Low toxicity of myoseverin suggested the substance may become a novel cytostatic agent. Myoseverin was

found to arrest the cell cycle at the G2/M transition (Chang et al. 2001). Comparison of myoseverin derivatives to taxol, vinblastine, nocodazole, and colchicine identify myoseverin's effect as being selectively reversible in addition to the lack of cytotoxic effects of these non-purine-based microtubule-disrupting molecules (Perez et al. 2002).

Tubulyzine

Microtubule destabilizing entities, the tubulyzines, were shown to target tubulin and possess an activity greater than that of myoseverin itself (Moon et al. 2002). As the binding site of tubulyzine, the 12Cysβ on tubulin was recently identified (Kim et al. 2006). Tubulyzines bind at the GTP binding site of β-tubulin (Kim et al. 2006). Interestingly, it was shown that the binding of tubulyzines to tubulin induces a conformational change in tubulin that prevents further interaction of the 239Cysβ with other reagents (Kim et al. 2006). Tubulyzine is a novel MIA and is not commercially available. The synthesis of tubulyzine is described in methods section.

E2GG

To analyze the effects of modifications of the myoseverin heterocyclic skeleton, we prepared its 8-azapurine and pyrazolo[4,3-*d*]pyrimidine analogues and compared their biological activities (Kryštof et al. 2006). Rearrangement of nitrogen atoms in the heterocycle changes the affinity of the compounds to purified tubulin, demonstrated in tubulin polymerization assays, and affects the proliferation of cancer cell lines. Surprisingly, compound E2GG, a pyrazolo[4,3-*d*]pyrimidine analogue of myoseverin, displayed inhibitory activity towards tubulin polymerization and also against the activity of cyclin-dependent kinases 1, 2 and 7 (Kryštof et al. 2006). Such a dual specificity-inhibitor offers a starting point for developing a novel class of antiproliferative agents.

Aim of our work was to compare the effects of novel MIAs to colchicine, a well-known MIA. We determined ability of all substances to interfere with tubulin polymerization *in vitro*, to affect microtubules integrity in primary rat hepatocytes, and to modulate AhR-dependent gene expression in primary rat hepatocytes. For structures of tested compounds, see Fig. 1.

Materials and Methods

Chemicals

Williams´ medium E, bovine serum, penicillin, streptomycin, L-glutamine, bisbenzimid (Hoechst No. 33258), colchi-

cine, myoseverin, and monoclonal anti-β-tubulin antibody were purchased from Sigma Chemicals (St. Louis, MO, USA). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was purchased from Ultra Scientific (RI, USA). Trizol$^{®}$ reagent was purchased from GibcoBRL Life Technologies (Cergy Pontoise, France). First-strand cDNA synthesis kit for RT-PCR (AMV), and LightCycler FastStart DNA Master^{PLUS} SYBR Green I were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Oligonucleotide primers used in RT-PCR reactions were purchased from Metabion International AG (Martinsried, Germany). 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.

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 mmol/l L-glutamine, 10 μmol/l streptomycin, 100 U/ml penicillin, 350 nmol/l insulin, and 1 μmol/l dexamethasone, was used for culture maintenance. The medium was enriched for plating with 5% foetal calf serum (v/v). Following 4 h stabilization, culture medium was replaced by a serum-free one and the cells were treated 24 h with DMSO (vehicle), TCDD (5 nmol/l) and/or with tested compounds in the presence of TCDD; i.e. colchicine (0.1, 1, 10 μmol/l), myoseverin (1, 10, 40 μmol/l), tubulyzine (1, 10, 40 μmol/l), and E2GG (1, 10, 40 μmol/l). Cultures were maintained at 37°C in 5% CO$_2$ (air : CO$_2$, 95 : 5) humidified incubator.

CYP1A1 mRNA determination

Following treatments total RNA was isolated using Trizol$^{®}$ reagent. Concentration of RNA was quantified by spectrometry at 260 nm and purity was assessed from the ratio of absorbances A$_{260nm}$/A$_{280nm}$. Reverse transcription (RT) was performed on 1 μg of total RNA using MMLV reverse transcriptase (Finnnzyme, Espoo, Finland) in a reaction volume of 12 μl containing 1× reaction buffer, 5 mmol/l MgCl$_2$, 0.5 mmol/l dNTP mixture, 7.5 μmol/l of oligo(dT)$_{18}$VN (Generi-Biotech, Hradec Králové, Czech Republic), 1 U/μl of RNase inhibitor TaKaRa (Otsu, Japan), 10 U/μl of MMLV reverse transcriptase, and 1 μg of RNA. Real-time PCR was performed using an iCycler (Bio-Rad, Hercules, CA) using SYBR$^{®}$ Green chemistry. Mastermix of a volume 25 μl contained 0.035 U/μl of HotStart *Taq*Polymerase (QIAGEN, Valencia, CA), 3 mmol/l MgCl$_2$, 200 nmol/l fluorescein, 1× buffer, 200 nmol/l dNTP, SybreGreen 1 : 2500 (Bio-Rad)

and 300 nmol/l of each primer. Following conditions were used: rat P450 1A1 primers: forward 5´-AGACCTTCCGA-CATTCA-3´; reverse: 5´-GTGTTTGTCCAGAGTGC-3´; 10 min/95°C, 30 cycles 95°C/15 s, 55°C/10 s, 72°C/12 s. rat GAPDH primers (Kemp et al. 2003): forward 5´-AC-CACAGTCCATGCCATCAC-3´; reverse 5´-TCCACCAC-CATGTTGCTGTA-3´; 10 min/95°C, 30 cycles 95°C/15 s, 58→54°C/5 s, 72°C/20 s. Purity of the product was verified by electrophoresis. Real-time PCR was carried out in an iCycler real-time PCR system (Bio-Rad Laboratories). All samples were run in quadruplicates and CT was automatically calculated. These transcripts were extensively optimized, run simultaneously with RNA- and RT-negative controls, and agarose gel electrophoresis was used to confirm the specificity of the priming. Primers were designed using Vector NTI software (Invitrogen). Relative expression of P450 1A1 mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

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 as described above. 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.

Tubulin polymerization

Preparation of lamb brain tubulin. Tubulin was purified from soluble lamb brain homogenate by ammonium sulfate fractionation and ion exchange chromatography according to the published method (Lee et al. 1973; Andreu et al. 1984). The protein was stored in liquid nitrogen. Its concentration was determined spectrophotometrically with a Perkin-Elmer Lambda 800 spectrophotometer assuming an extinction coefficient at 275 nm of 1.07 l·g^{-1}·cm^{-1} in 0.5% SDS in neutral aqueous buffer or 1.09 l·g^{-1}·cm^{-1} in 6 mol/l guanidine hydrochloride.
Tubulin polymerization. Microtubule assembly was assayed in 20 mmol/l sodium phosphate, 1 mmol/l EGTA, 3.4 mol/l glycerol, and 0.1 mmol/l GTP, pH 6.95. The polymerization reaction (20 μmol/l tubulin) was started by increasing the temperature from 4 to 37°C and the mass of formed polymer was monitored in thermostated cuvettes by measuring turbidimetry at 350 nm in a Beckman DU 7400 spectrophotometer as described (Barbier et al. 2001). IC$_{50}$ (inhibition

of tubulin polymerization to 50%) values were interpolated from graphs showing the percentage of turbidity inhibition as a function of the concentration of the tested compounds. Concetration range for colchicin was 0,5–8 μmol/l, for myoseverin 2–16 μmol/l, for tubulyzine 0,5–8 μmol/l and for E2GG 5–30 μmol/l. To determine the inhibitory effect, we calculated the difference between the polymerization plateau value and the depolymerization plateau value and expressed it in percent of inhibition relative to the control. IC$_{50}$ values were calculated from the equation of linear regression of secondary plots of percentage of inhibition against drug concentration according to the method previously described (Barbier et al. 2001; Kryštof et al. 2006).

Synthesis of tubulyzine and E2GG

Synthesis of E2GG was described elsewhere (Kryštof et al. 2006). Synthesis of tubulyzine (2-cyclohexylamino-4,6-bis(4-methoxybenzylamino)-[1,3,5]triazine) was firstly described in Moon et al. (2002). The set of 1,3,5-triazines was synthesized using combinatory chemistry. Syntheses were based on the binding of substrate on the carrier. Here we synthesized tubulyzine by classical means of subsequent substitutions of chlorine atoms by nitrogen nucleophiles (amines) in cyanurchloride.

Synthesis was performed as follows:

2-(4-methoxybenzylamino)-4,6-dichloro-[1,3,5]triazine (compound 1): To the stirred solution of cyanuric chloride (3.0 g; 16.4 mmol) in ether (90 ml) was added dropwise solution of 4-methoxybenzylamine (4.5 g; 32.8 mmol) in ether (40 ml) at 0°C. The mixture was stirred at 0–5°C for 3 h and left to stand for 18 h. The precipitated solid was collected on a filter and washed with small amount of ether. The ether filtrate was concetrated *in vacuo* and product crystallized from heptane. Yield 3.5 g (75%). MS (ESI, *m/z*): 286.1[M+H]$^+$. ^{1}H NMR (DMSO-d_6)δ 3.72 (s, 3H, CH$_3$); 4.66 (m, 2H, CH$_2$); 6.86 (t, 2H, arom); 7.27 (t, 2H, arom). IR (cm^{-1}): 3270, 3173, 3001, 1640, 1551, 1404,1237, 1169. Anal. Calcd. for C$_{11}$H$_{10}$Cl$_2$N$_4$O (285.1); C, 46.34; H, 3.54; N, 19.65. Found C, 46.28; H, 3.61; N, 19.44.

2-cyclohexylamino-4-(4-methoxybenzylamino)-6-chloro-[1,3,5]triazine (compound 2): To the stirred suspension of compound 1 (3.4 g; 11.9 mmol) and cyclohexylamine (1.18 g; 11.9 mmol) in water (40 ml) and dioxane (40 ml) was added dropwise solution of sodium bicarbonate (1.0 g; 11.9 mmol) in water (10 ml). The reaction mixture was heated to 45–47°C for 14 h. On cooling, the precipitated solid was filtered off, washed with water, dried and crystallized from ethanol. Yield 2.9 g (70%). MS (ESI, *m/z*): 349.1[M+H]$^+$. ^{1}H NMR (DMSO-d_6)δ 1.21 (m, 6H, CH$_2$); 1.71 (m, 4H, CH$_2$); 3.63

(bs, 1H, CH); 3.70 (s, 3H, CH$_3$); 4.30 (m, 2H,CH$_2$); 6.85 (t, 2H, arom); 7.20 (t, 2H, arom). IR (cm^{-1}): 3268, 3170, 2927, 2849, 1569, 1522, 1426, 1170. Anal. Calcd. for C$_{17}$H$_{22}$ClN$_5$O (347.8); C, 58.70; H, 6.37; N, 20.13. Found C, 58.79; H, 6.29; N, 20.44.

Tubulyzin (compound 3): The mixture of compound 2 (400 mg; 1.15 mmol), 4-methoxybenzylamine (220 mg; 1.6 mmol) and triethylamine (232 mg; 2.3 mmol) in n-butanol (4 ml) was heated with stirring at 110°C for 5 h. The reaction mixture was evaporated *in vacuo* to dryness, triturated with diluted hydrochloric acid (1 : 20) and extracted with chloroform (2 × 10 ml). Purification by elution with chloroform through a short column (4 cm i.d.) filled with silica gel 60 (230–400 mesh, 50 g) gave compound 3. Sample for analysis was prepared by crystallization from ethanol-water mixture. Yield 0.331 g (64%). MS (ESI, *m/z*): 449.5[M+H]$^+$. ^{1}H NMR (DMSO-d_6)δ 1.17 (m, 6H, CH$_2$); 1.66 (m, 4H, CH$_2$); 3.60 (bs, 1H, CH); 3.70 (s, 6H, CH$_3$); 4.33 (m, 4H,CH$_2$); 6.85 (t, 4H, arom); 7.17 (t, 4H, arom). IR (cm^{-1}): 3410, 3266, 2928, 2851, 1564, 1513, 1347, 1246, 1173, 1034. Anal. Calcd. for C$_{25}$H$_{32}$N$_6$O$_2$ (448.6); C, 66.94; H, 7.19; N, 18.74. Found C, 66.87; H, 7.11; N, 18.86.

The IR spectra were recorded in KBr wafers on an ATI Unicam Genesis FTIR instrument. The NMR spectra were registered on a Bruker Avance 300 MHz DRX spectrometer. Elemental analyses were performed with an elemental analyser EA 1108 (Fissons Instruments). Mass spectrometric experiments were performed using an LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA).

Statistics

Results were expressed as mean ± S.D. (standard deviation). Paired Student´s *t*-test was applied to all analyses.

Results and Discussion

General demonstration of a substance affinity to its intended target is a primary task. Many an approach is at hand and microtubules assembly *in vitro* serves to show whether a substance is capable of interfering with the dynamic event of tubulin association.

All substances tested in this work inhibit microtubule assembly with variable potency (Fig. 2). The inhibitory constants IC$_{50}$ were as follows: tubulyzine (4.4 ± 0.9 μmol/l), myoseverin (7.0 ± 0.8 μmol/l), E2GG (16 ± 2 μmol/l), colchicine (2.0 ± 0.4 μmol/l). The constants describe the *in vitro* MIA capability, but give only limited information on the potency of the corresponding MIA within a cell as well as relation to any unintended protein targets. One of the issues involved is the actual cellular concentration of the MIA, which is likely cell type-dependent because of
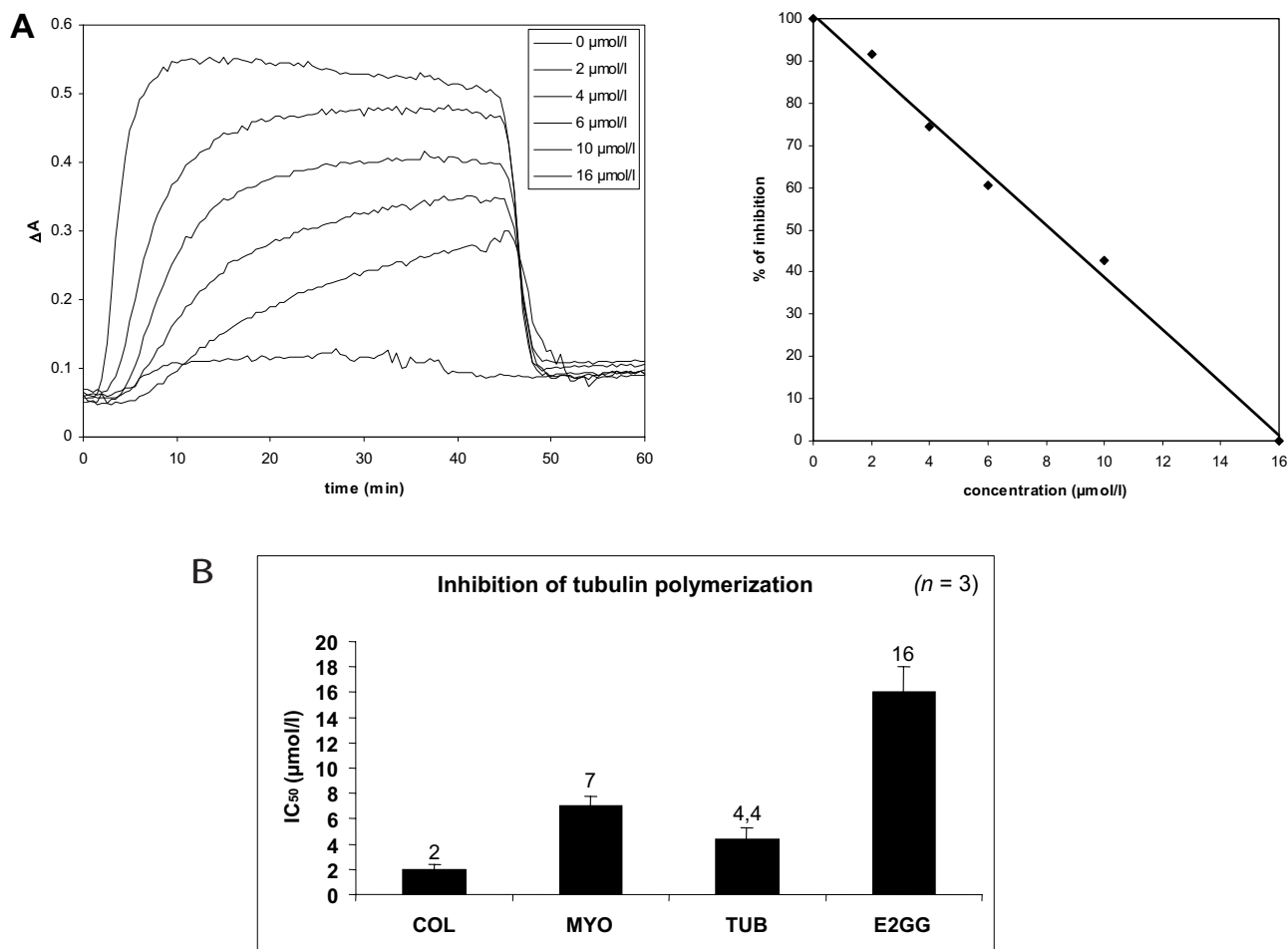
Figure 2. Effects of MIAs on tubulin polymerization. Microtubule assembly was assayed as described in Materials and Methods section. **A.** Effect of myoseverin on tubulin polymerization. Concentration dependence curves for inhibition of polymerization by indicated concentrations of myoseverin. After 45 min, the samples were cooled to 8°C to demonstrate reversibility of the polymerization. The inset (right plot) shows the percentage of turbidity inhibition as a function of myoseverin concentration. **B.** Bar graphs show means ± S.D. of IC_{50} values obtained from three independent experiments. COL, colchicine; MYO, myoseverin; TUB, tubulizine; E2GG, pyrazolo[4,3-*d*]pyrimidine analogue of myoseverin. Results are expressed as the percentage of turbidity inhibition as a function of the concentration of the tested compounds.

transport proteins and xenobiotic metabolism, the other is the abundance of any possible binding sites.

Hepatocytes are a complex cellular model utilized for its involvement in xenobiotic metabolism and generally non-trivial frequency of a number of proteins present in other more specialized cell types with some exceptions. It is also a model of non-proliferating cells, an important consideration because MIAs are designed specifically to interfere with proliferation. We treated rat hepatocytes for 24 h with colchicine (1 μmol/l), myoseverin (1, 10, 40 μmol/l), tubulyzine (1, 10, 40 μmol/l), E2GG (1, 10, 40 μmol/l), and DMSO (vehicle). While all substances disrupt the integrity of microtubules (Fig. 3) their potency is dif-

ferent from that demonstrated in *in vitro* polymerization assay. E2GG is very interesting in this view as it interacts with other proteins besides tubulin (Kryštof et al. 2006). Neither assay, however, can assure us E2GG effect on microtubules is a combination of protein-E2GG interactions. Hepatocytes remain in G0 or G1 phase of the cell cycle even when challenged with MIAs (Dvořák et al. 2006b). Therefore effects on CDKs are less likely to indirectly affect microtubules integrity and dynamics.

Cell signaling is a bounty of proteins and small molecules involved in relaying extracellular stimuli to genetic response. Along the path lie hurdles to overcome which serve as regulatory check points. MIAs, while demonstrated to interact with
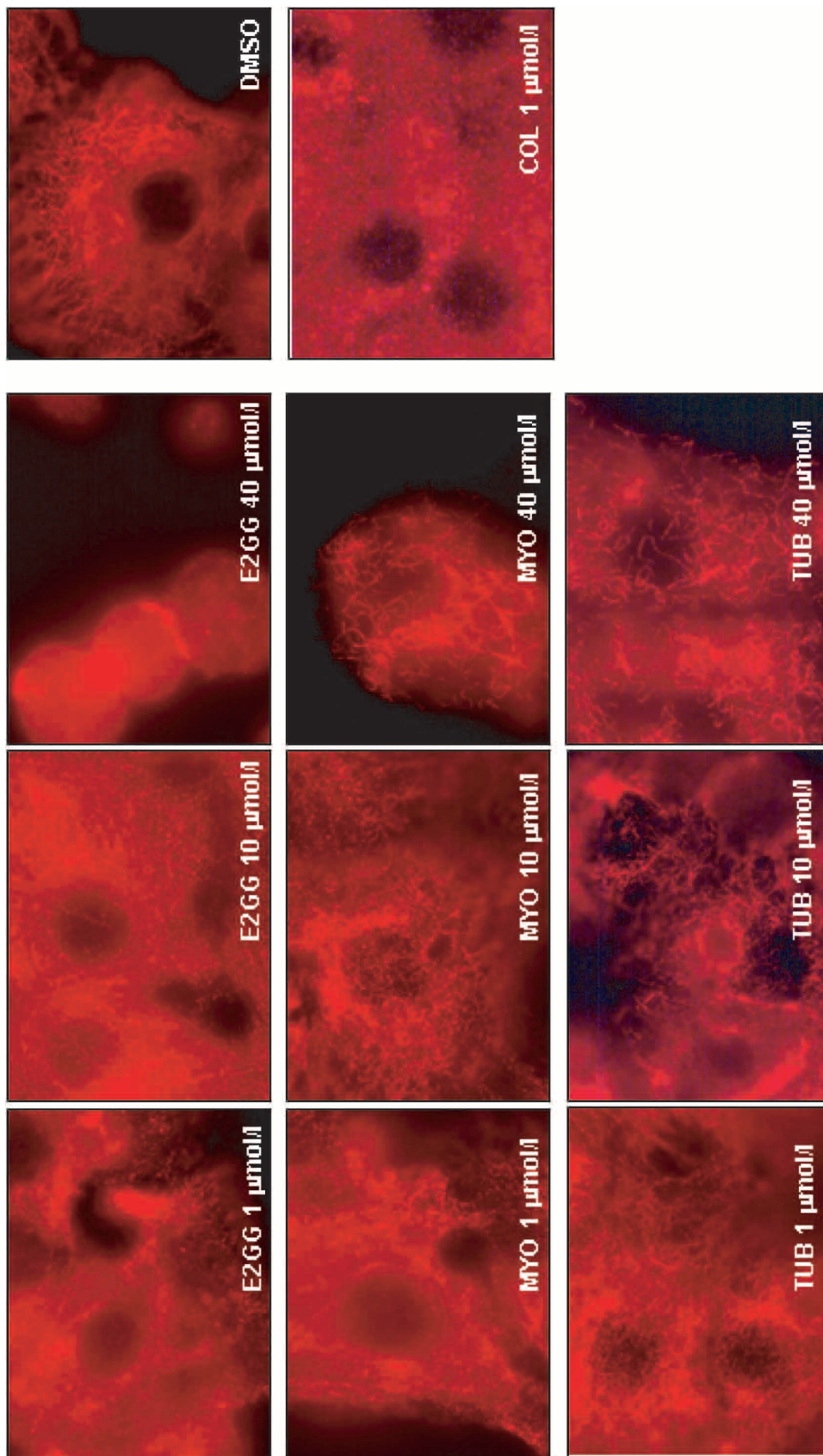
Figure 3. Effects of MIAs on microtubules integrity in primary rat hepatocytes. Cells were treated for 24 h with colchicine (COL; 1 μmol/l), myoseverin (MYO; 1, 10, 40 μmol/l), tubulyzine (TUB; 1, 10, 40 μmol/l), and pyrazolo[4,3-*d*]pyrimidine analogue of myoseverin (E2GG; 1, 10, 40 μmol/l) and DMSO (vehicle). 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. Examination was performed in two independent experiments. Showed are representative images.
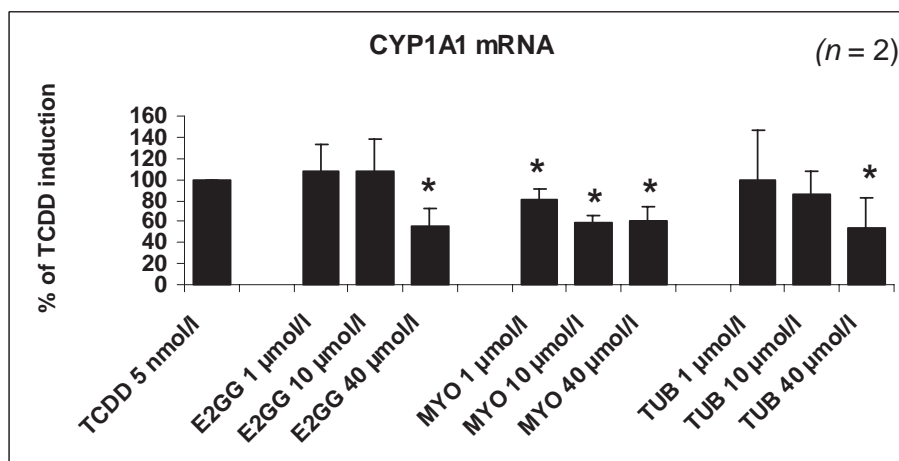
Figure 4. Effects of MIAs on dioxin-dependent CYP1A1 mRNA induction in primary rat hepatocytes. Cells were treated for 24 h with DMSO (vehicle), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nmol/l) and tested compounds in the presence of TCDD; i.e with myoseverin (MYO; 1, 10, 40 μmol/l), tubulyzine (TUB; 1, 10, 40 μmol/l), and pyrazolo[4,3-d]pyrimidine analogue of myoseverin (E2GG; 1, 10, 40 μmol/l). The levels of CYP1A1 and GAPDH mRNAs were determined by RT-PCR as described in Materials and Methods section. The data obtained were normalized on the GAPDH level. Bar plots represent means ± S.D. from two independent experiments. * the value is significantly different from TCDD value at $p < 0.05$.

tubulin to an extent, are likely to affect interaction of tubulin with their associated proteins, e.g. chaperones (Galigniana et al. 1998), positioned at certain check points. Detecting transcriptional activity of a receptor, must be a "commuting" receptor undergoing cytosolic to nuclear translocation, may support the involvement of microtubules or its associate proteins in the event. The important information is that affecting receptor activity may have cytotoxic consequences. Therefore we also examined the effects of newly synthesized MIAs on dioxin-dependent CYP1A1 mRNA induction in primary rat hepatocytes and compared it to the known activity of colchicine (Dvořák et al. 2006b). Cells were treated for 24 h with DMSO, TCDD (5 nmol/l) and tested compounds in the presence of TCDD. All tested MIAs down-regulated CYP1A1 mRNA (Fig. 4). The potency of these compounds to inhibit inducible expression of CYP1A1 was comparable and increased in the following order: E2GG < tubulyzine < myoseverin.

Out of the substances tested, E2GG appears to have promising biological activity because its microtubules disruption is more potent in cellular environment than in a defined one component system. Moreover, its effect on receptor signaling is less severe than of the other substances tested making it less likely a culprit of drug-drug interactions.

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