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Inhibition of multidrug resistance-associated protein 2 (MRP2) activity by the contraceptive nomegestrol acetate in HepG2 and Caco-2 cells

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ABSTRACT

Multidrug resistance-associated protein 2 (MRP2) plays a key role in hepatic and intestinal disposition of *endo*- and xenobiotics. Several therapeutic agents modulate MRP2 activity resulting in pharmacological interactions. Nomegestrol acetate (NMGA) is a progestogen increasingly used in contraceptive formulations. The aim of this work was to evaluate the effect of NMGA on MRP2 activity in HepG2 and Caco-2 cells as models of human hepatocytes and enterocytes, respectively. NMGA (5, 50 and 500 nM; 48 h) decreased MRP2-mediated transport of 2,4-dinitrophenyl-S-glutathione in HepG2 cells, with no effect on MRP2 protein expression. Acute exposure (1 h) to the same concentrations of NMGA failed to affect MRP2 activity, ruling out an inhibitory action directly induced by the drug. In contrast, acute incubation with a lysate of HepG2 cells pre-treated with NMGA, containing potential metabolites, reproduced MRP2 inhibition. Preincubation of lysates with sulfatase but not with β-glucuronidase abolished the inhibitory action, strongly suggesting participation of NMGA sulfated derivatives. Western blot studies in plasma vs. intracellular membrane fractions ruled out internalization of MRP2 to be responsible for the impairment of transport activity. MRP2-mediated transport of 5(6)-carboxy-2′,7′-dichlorofluorescein was not affected in Caco-2 cells incubated for 48 h with either 5, 50 or 500 nM NMGA. Conversely, acute exposure (1 h) of Caco-2 cells to NMGA-treated HepG2 lysates decreased MRP2 activity, being this effect also prevented by pre-treatment of the lysates with sulfatase. Taken together, these findings demonstrate an inhibitory effect of NMGA sulfated metabolites on hepatic and intestinal MRP2 function. Extrapolated to the in vivo situation, they suggest the possibility of pharmacological interactions with coadministered drugs.

1. Introduction

Drug transporters of the ABC (ATP binding cassette) superfamily play a key role in the disposition of *endo*- and xenobiotics. Among them, the multidrug resistance-associated protein 2 (MRP2/*ABCC2*) transports a wide range of prescription drugs and their metabolites, hormones, signaling molecules and glutathione species. MRP2 substrates comprise anionic compounds conjugated with glutathione, glucuronic acid or sulfate as well as non-conjugated compounds (Klaassen and Aleksunes 2010). In the liver, MRP2 localizes to the canalicular membrane of the hepatocytes and mediates the efflux of substrates into the bile, including those involved in the formation of bile salt-independent bile flow (Klaassen and Aleksunes 2010). In the intestine, MRP2 localizes to the apical membrane of the enterocyte and limits the uptake of drugs, environmental toxicants and micronutrients incorporated with the diet (Mottino et al. 2000). Also, a role of intestinal MRP2 in the excretion of substances administered systemically has been demonstrated (Gotoh et al. 2000; Villanueva et al. 2012). Due to the presence in tissues of crucial pharmaco-toxicological relevance, alterations in MRP2 function constitute a challenge to the maintenance of

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the internal homeostasis.

Inhibition of MRP2 activity by drugs or their metabolites represents a common phenomenon underlying drug-drug interactions and drug side effects. Acute inhibition may result from a direct interaction of a drug or its metabolites with the transporter without changes in its protein expression. For instance, MRP2 inhibition by non-steroidal anti-inflammatory drugs (NSAIDs) is well acknowledged (El-Sheikh et al. 2007). In this regard, NSAIDs have been reported to impair biliary excretion and thus enterohepatic recirculation and pharmacokinetics of the glucuronide conjugate of mycophenolic acid, an immunosuppressant drug and well-known MRP2 substrate (Fukuda et al. 2011). Alternatively, loss of transporter localization at the apical membrane (e.g. by endocytic retrieval) induced by drugs may lead to reduced MRP2 activity in spite of preserved total expression (Crocenzi et al. 2012; Mottino et al. 2002; Tocchetti et al. 2016). In general, reduced clearance and increased bioavailability of MRP2 substrates can be expected upon transporter inhibition produced by a particular drug. In this case, reduction in the treatment safety and increased toxicity, especially for coadministered drugs with a narrow therapeutic index, could be expected.

Nomegestrol acetate (NMGA) is a synthetic progestogen originally used alone for the treatment of gynecological disorders or combined with estradiol as hormonal replacement therapy in postmenopausal women. In the last years, a combination of NMGA and estradiol (E2) has been approved for biphasic combined oral contraception (COC) (Mueck and Sitruk-Ware 2011; Shields-Botella et al. 2003). Unlike other contraceptive drugs, NMGA exhibits reduced binding to nuclear receptors other than the progesterone receptor, thus resulting in less side effects (Lello 2010). In addition, the 24/4 administration scheme of NMGA/placebo permits a better compliance than COCs based on a multiphasic regimen (Ruan et al. 2012). Therefore, NMGA-E2 formulations appear as an alternative that may gain even more acceptance and thus may be more often prescribed than other contraceptives in the near future. In spite of the increase in NMGA prescription, many pharmacokinetic properties as well as its interaction potential are not fully elucidated.

Serum NMGA concentration rises rapidly after oral intake of the combined formulation (2.5 mg NMGA/1.5 mg E2) with C_{max} values of about 40 nM being reached within 2–3 h. During steady state, NMGA plasma concentrations of about 33 nM were reported in healthy women. Noteworthy, higher concentrations are expected in women with decreased hepatic function (Lello 2010; Ruan et al. 2012). NMGA is mainly metabolized by the liver, where it undergoes phase I and phase II reactions including formation of sulfated and glucuronidated metabolites with minimal progestational activity (Lello 2010). Less than 2% of the oral administered dose is excreted without modification (Huang et al. 2015; Lello 2010; Ruan et al. 2012). Currently, there is scarce information available about the interaction of NMGA or its metabolites with drug metabolizing and transporter proteins.

Modulation of MRP2 by physiological and pharmacological progestogens was already assessed in MRP2-overexpressing MDCK cells, where a clear inhibition of MRP2 activity by norgestimate and progesterone was observed. Since the onset of the effect takes place no later than 10 min after progestogen addition, the observations likely result from acute transporter inhibition without changes in the transporter expression (Lindenmaier et al. 2005). To date, it is unknown whether MRP2 inhibition by NMGA may take place. This bears striking relevance since the increasing prescription of NMGA (e.g. under long-term protocols) may result in drug-drug interactions with MRP2 substrates, particularly in first-pass metabolism tissues like intestine and liver.

The aim of this work was to evaluate the effect of NMGA concentrations in the range achieved in plasma of women taking combined oral contraceptives or under hormonal replacement therapy on the activity of MRP2 in HepG2 and Caco-2 cells, used as models of human hepatocytes and enterocytes, respectively. Our data suggests an acute inhibition of MRP2 by NMGA sulfated metabolites without changes in the transporter expression or localization in HepG2 cells. Furthermore, MRP2 inhibition by these metabolites was also observed in Caco-2 cells.

2. Materials and Methods

2.1. Chemicals

Cell culture media, fetal bovine serum, L-glutamine, penicillin and streptomycin, non-essential amino acids, 1-chloro-2,4-dinitrobenzene (CDNB), 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDFDA), Hank's buffered saline solution (HBSS), doxorubicin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), β-glucuronidase from bovine liver, MK571, nomegestrol acetate (NMGA) and sulfatase from *Helix pomatia* type H-1 were from Sigma-Aldrich (St. Louis, USA). Aprotinin, leupeptin, pepstatin and pefabloc were from Carl Roth (Karlsruhe, Germany). DMSO was from Merck (Darmstadt, Germany). The glutathione-conjugated derivative of CDNB, 2,4-dinitrophenyl-S-glutathione (DNP-SG), was synthesized with the use of 1-fluoro-2,4-dinitrobenzene and glutathione as described by Sokolovsky et al. (1964). All other chemicals were of analytical grade purity or higher.

2.2. Cell Culture

HepG2 and Caco-2 cells were purchased from the American Tissue Culture Collection (Rockville, USA). HepG2 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Caco-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Both cell lines were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 (Rigalli et al. 2015).

For studies in HepG2 cells, 5×10^5 cells/well were seeded in 6-well plates and incubated for 24 h or 72 h, depending on the particular experiment. For MRP2 activity assays after long-term exposure to NMGA, MRP2 expression studies and preparation of NMGA metabolites, cells were cultured for 24 h prior to NMGA addition. For MRP2 activity assays after short-term treatment with NMGA or NMGA metabolites and MRP2 localization studies, cells were cultured for 72 h before addition of NMGA. Then, the corresponding treatment medium was added. Treatment details are provided in the corresponding subsection.

For MRP2 activity studies in Caco-2 cells, 3×10^5 cells/well were seeded in 12-well Transwell® permeable supports (cat. No. 3401; Corning Inc., Corning, USA) and cultured for 21 days as described by Hubatsch et al. (2007). Culture medium in both apical and basolateral compartments was replaced every second day. To evaluate monolayer integrity, transepithelial electrical resistance (TER) was monitored every second day using an Epithelial Voltohmmeter (EVOM; World Precision Instruments, Sarasota, USA). All cell monolayers used in the experiments had TER values at 37 °C above 300 Ω cm^2, indicating a continuous monolayer (Hubatsch et al. 2007).

2.3. Preparation of NMGA Metabolite-containing Lysates

HepG2 cells were seeded and cultured as described in 2.2. Then, cells were treated with NMGA (1 µM) or vehicle (DMSO, 0.1% V/V) for 48 h to allow NMGA biotransformation. After incubation, cell lysates were obtained through sonication (3 times, 5 s, 30% amplitude) in 300 µL/well isotonic saline solution. Lysates are referred to as “NMGA-treated cell lysates” and “control cell lysates” throughout the manuscript.

To evaluate whether sulfated and/or glucuronidated metabolites of NMGA exert an acute inhibitory effect on MRP2, cell lysates subjected to enzymatic hydrolysis with sulfatase and/or β-glucuronidase were

used in MRP2 activity studies. Control- and NMGA-treated cell lysates were obtained through sonication (3 times, 5 s, 30% amplitude) in 300 µL/well sodium acetate 0.1 M pH = 5.00 and incubated overnight at 37 °C with sulfatase (12.5 U/mL) and/or β-glucuronidase (5000 U/mL) (Fraiser and Jiang, 2009; Walle et al. 1999). Cell lysates incubated in the same conditions in absence of enzymes were used as a control.

All lysates were diluted 1:10 in HBSS efflux medium and used in MRP2 activity studies in both HepG2 and Caco-2 cells as described below.

2.4. MRP2 Activity Measurements

2.4.1. Studies in HepG2 Cells

For long-term NMGA treatments, cells were seeded and incubated as described in 2.2. Then, cells were incubated for 48 h in treatment medium consisting of growth medium with different NMGA concentrations (0.5–500 nM) aimed to cover the reported range in plasma of individuals under NMGA treatment (Ruan et al. 2012). For short-term incubation with NMGA or NMGA metabolites, cells were seeded and cultured as described in 2.2 and subjected to MRP2 activity studies. In this case, NMGA (5–500 nM) or lysates of NMGA-treated cells (i.e. containing NMGA metabolites) were added only during the DNP-SG efflux period (1 h) (see below). NMGA was dissolved in DMSO and added from a 1000× stock solution. The final concentration of DMSO in culture media was always kept below 0.2% V/V.

MRP2 activity in HepG2 cells was assessed by quantifying the efflux of its model substrate DNP-SG into the incubation medium, which directly correlates with MRP2 transporter activity (Zhang et al. 2001). For this purpose, cells treated as described above were rinsed and incubated with DNP-SG precursor, CDNB (0.5 mM, 30 min, 10 °C), which passively diffuses across the plasma membrane and under these conditions it is spontaneously conjugated (i.e. independently of glutathione-S-transferase activity) with glutathione yielding DNP-SG (Elferink et al. 1989). Then, cells were rinsed with ice cold PBS and further incubated at 37 °C in HBSS to allow DNP-SG excretion by MRP2. For short-term treatments, NMGA or NMGA metabolites were added only in this step. After 1 h, aliquots of efflux medium were taken, deproteinized by addition of perchloric acid (10% V/V) and centrifuged (14,000*g*, 2 min, 4 °C). Supernatants were used for DNP-SG quantification by HPLC using a Waters600 device (Waters, Milford, USA) equipped with a C18 column (Luna 5 µ, Phenomenex, Torrance, USA). Isocratic elution was performed using a mobile phase of acetonitrile and water-ortophosphoric acid 0.1% V/V (1:3). Flow rate was set at 1 mL/min and detection was performed spectrophotometrically at 365 nm. DNP-SG quantification was performed based on the peak area (Rigalli et al. 2015). MRP2-independent DNP-SG efflux was quantified using the selective MRP inhibitor MK571 (20 µM) (Lindenmaier et al. 2005) and subtracted from all experimental groups.

To evaluate whether differential DNP-SG accumulation between control and long-term NMGA-treated cells takes place during incubation with CDNB, intracellular DNP-SG content was determined at the end of incubation. For this purpose, cells treated with NMGA (5–500 nM; 48 h) or vehicle were incubated with CDNB as described above. Immediately after rinsing, cell lysates were obtained through sonication (3 times, 5 s, 30% amplitude) in 300 µL/well isotonic saline solution. Then samples were deproteinized and DNP-SG was quantified as described above.

To confirm the inhibitory effect of NMGA metabolites, MRP2 activity was also evaluated by assessing the efflux of the MRP2 substrate doxorubicin (Vlaming et al. 2006) as previously described with minor modifications (Rigalli et al. 2016). Briefly, cells were seeded as described in 2.2 and incubated in fresh medium containing doxorubicin (10 µM; 1 h). Subsequently, cells were rinsed and incubated at 37 °C in HBSS in the presence of control or NMGA-treated cell lysates (prepared as detailed in 2.3.) for 1 h to allow doxorubicin efflux. Finally, doxorubicin was quantified in efflux media by spectrofluorometry with 485 and 538 nm as excitation and emission wavelengths, respectively. MK571 (20 µM) was used to quantify MRP2-independent doxorubicin efflux.

2.4.2. Studies in Caco-2 cells

Cells were seeded on semipermeable filters and cultured for 21 days as described in 2.2. After this time of culture, Caco-2 cells undergo spontaneous differentiation to form a confluent monolayer of enterocyte-like polarized cells, which structurally and functionally resembles the small intestine epithelium (Hidalgo et al. 1989; Hilgers et al. 1990; Hubatsch et al. 2007). Regarding expression of the most relevant intestinal transporters (including MRP2), a similar pattern between differentiated Caco-2 cells and human small intestine was reported (Englund et al. 2006; Hilgendorf et al. 2007; Taipalensuu et al. 2001). Thus, Caco-2 monolayers grown on permeable supports constitute an optimal in vitro model system for drug transport studies (Hubatsch et al. 2007). For long-term NMGA treatments, treatment medium containing NMGA (5–500 nM) was added on both apical and basolateral sides of the cell monolayer for 48 h, then cells were rinsed and MRP2 activity studies were performed.

MRP2 activity was evaluated by assessing the ratio between the apical-to-basolateral (AB) and the basolateral-to-apical (BA) directional transport of the prototypical MRP2 substrate 5(6)-carboxy-2′,7′-dichlorofluorescein (CDF) as described by Schexnayder and Stratford (2015). The CDF non-fluorescent promoiety, CDFDA, is taken up passively by Caco-2 cells and immediately hydrolyzed by intracellular esterases to the fluorescent CDF (Zamek-Gliszczynski et al. 2003). Briefly, prior to the activity measurement, cell monolayers were washed with HBSS for 15 min at 37 °C. Then, filter inserts were transferred to new plates and CDFDA (10 µM, in HBSS) was added to the donor compartment (apical for AB direction experiments, basolateral for BA direction experiments). The receiving compartment (basolateral for AB direction experiments, apical for BA direction experiments) was filled with prewarmed HBSS. For MRP2-independent CDF efflux determination, MK571 (20 µM) was added to both compartments. For short-term studies, NMGA metabolite-containing lysates were prepared as described in 2.3. and added to both compartments. Plates were then placed in a 37 °C incubator and samples from the receiving compartment were withdrawn with volume replacement every 30 min up to 120 min. CDF was quantified using a Fluoroskan Ascent microplate fluorometer (Thermo Fisher Scientific, Vantaa, Finland) set at 485 nm/520 nm for excitation and emission wavelengths, respectively. Apparent permeability coefficients (P_{app}, $cm.s^{-1}$) of CDF across cell monolayers in both AB and BA directions were calculated using the following equation:

$$P_{app} = \frac{1}{C_0 A} \times \frac{dQ}{dt}$$

where dQ/dt is the appearance rate of CDF on the receiving compartment, C_0 is the initial CDFDA concentration in the donor compartment (10 µM) and A is the Transwell® support surface area (1.12 cm^2). CDF efflux ratio was obtained by dividing P_{app} in the BA direction by P_{app} in the AB direction. MRP2-independent CDF efflux was quantified using the selective MRP inhibitor MK571 (20 µM) (Lindenmaier et al. 2005) and subtracted from all experimental groups.

2.5. Preparations of Plasma (PM) and Intracellular (IM) Membrane Fractions of HepG2 Cells

For MRP2 localization studies, cells were seeded and cultured as described in 2.2. Then, culture medium was replaced by NMGA-treated or control cell lysates diluted 1:10 in HBSS and cells were incubated for 30 min.

At the end of the incubations, PM and IM were obtained by differential centrifugation (Kubitz et al. 2005; Tocchetti et al. 2018). Briefly, HepG2 cells were washed twice, scraped and collected in 250 mM sucrose, 5 mM EGTA, 1 mM $MgCl_2$, 20 mM Tris (pH 7.40) buffer

supplemented with aprotinin (1 μg/mL), leupeptin (5 μg/mL), pepstatin (1 μg/mL) and pefabloc (1 mg/mL) as protease inhibitors. Cells were then lysed by passing through a 25G needle. The PM fraction was obtained by centrifugation at 1000*g* for 5 min at 4 °C. The resulting supernatant was centrifuged at 100,000*g* for 60 min at 4 °C to obtain the IM fraction. Once prepared, PM and IM fractions were subjected to western blot studies for MRP2 and β-actin detection as described in 2.6.

2.6. Western Blot Studies

To evaluate the effect of NMGA on MRP2 protein expression, HepG2 cells were seeded and incubated as described in 2.2. Then, cells were exposed to treatment medium consisting of growth medium and 5, 50 and 500 nM NMGA for 48 h. At the end of treatments, cells were rinsed with PBS and incubated for 30 min in RIPA buffer (Thermo Fisher Scientific, Waltham, USA) supplemented with aprotinin (1 μg/mL), leupeptin (5 μg/mL), pepstatin (1 μg/mL) and pefabloc (1 mg/mL) as protease inhibitors. Cell lysis was accomplished by passing through a 25G needle. Cell lysates, PM and IM fractions obtained as described in 2.5. were subjected to protein quantification using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) and used in western blot studies as previously described (Rigalli et al. 2013). Briefly, proteins were separated by SDS-PAGE, transferred onto a PVDF membrane and incubated in blocking buffer (3% BSA in TBS with 0.3% Tween 20) for 1 h. Then, membranes were sequentially incubated with primary and secondary antibody solutions and developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, USA). Primary antibodies were: anti-MRP2: M_2III-6 from Enzo Life Sciences (Farmingdale, USA); anti-GAPDH: G-9 from Santa Cruz Biotechnology (Heidelberg, Germany) and anti-β-actin: AC-74 from Sigma-Aldrich (St. Louis, USA). Immunoreactive bands were quantified using ImageJ software (NIH, Bethesda, USA).

2.7. Cell Viability Studies

HepG2 and Caco-2 cell viability was assessed for all the treatments in which MRP2 activity was affected by measuring the conversion of MTT to its formazan as previously described (Rigalli et al. 2011). The rate of conversion in all treated groups was not statistically different from the respective control groups (Fig. A.2 in Appendix A).

2.8. Statistical Analysis

All results are expressed as mean ± standard deviation. All experiments were performed at least in triplicates. Statistical comparisons were performed through the Student's *t*-test or one-way ANOVA followed by ANOVA Tukey's post-hoc test, for 2 or > 2 experimental groups, respectively. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of NMGA on MRP2 Transport Activity and Expression in HepG2 Cells

We first evaluated the effect of therapy-associated plasma concentrations of NMGA on MRP2 activity in HepG2 cells. After 48 h of treatment, NMGA (5, 50 and 500 nM) led to a strong, dose-independent decrease in DNP-SG efflux (−82%, −86% and − 86%, respectively) via MRP2. These differences cannot be attributed to alterations in DNP-SG synthesis rate or accumulation since the intracellular load of DNP-

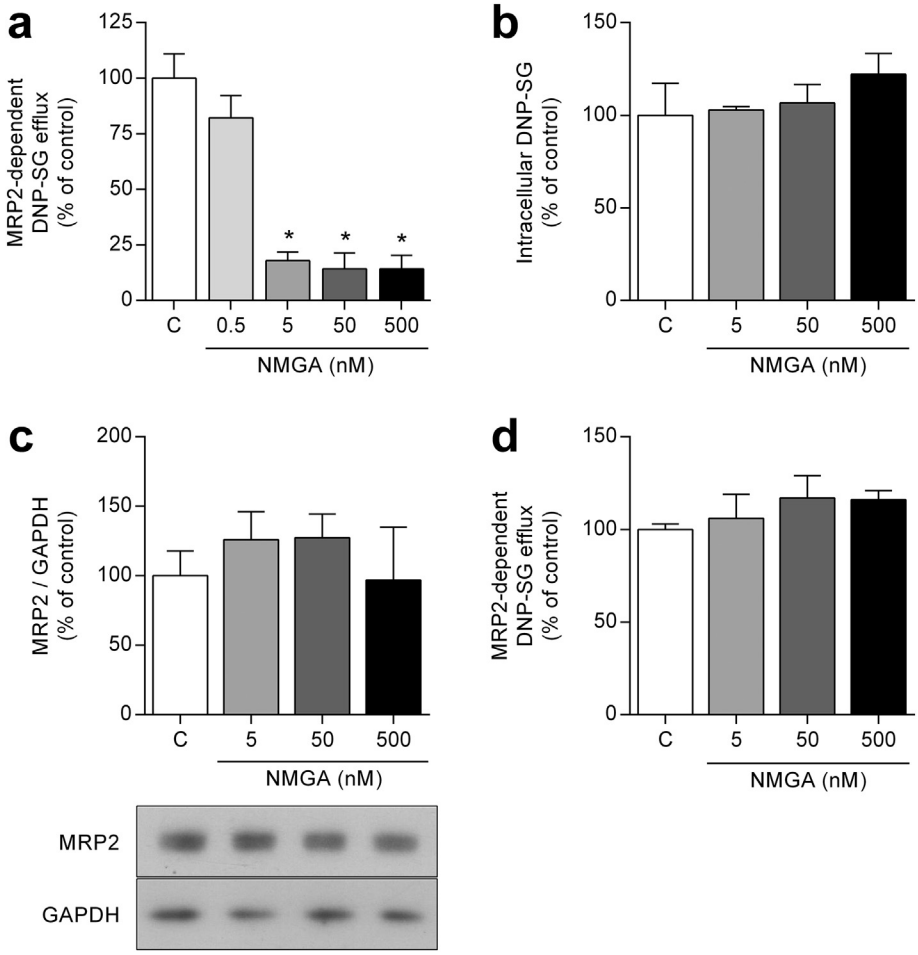

Fig. 1. Effect of NMGA on MRP2 transport activity and expression in HepG2 cells. a. To evaluate the effect of a long-term exposure to NMGA on MRP2 activity, DNP-SG efflux was quantified in control (C) and NMGA-treated cells (0.5, 5, 50 and 500 nM; 48 h). Data are presented as % of C and expressed as mean ± standard deviation (n = 3). *: $p < 0.05$ vs C. b. Intracellular content of DNP-SG was quantified in control and NMGA-treated cells (5, 50 and 500 nM; 48 h) to compare the amount of DNP-SG available to be extruded after CDNB loading. Data are presented as % of C and expressed as mean ± standard deviation (n = 3). c. To evaluate the effect of NMGA on MRP2 protein expression, cells were treated with 5–500 nM NMGA or vehicle for 48 h and then subjected to western blot analysis. Representative blots for MRP2 and GAPDH (loading control) are shown together with the respective densitometric analysis. Data are presented as % of control group (C) and expressed as mean ± standard deviation (n = 3). d. To determine whether NMGA itself inhibits MRP2 activity, DNP-SG extrusion was quantified in the presence of NMGA (5, 50 and 500 nM) in efflux medium. Data are presented as % of C and expressed as mean ± standard deviation (n = 3). Experiments in the presence of the selective MRP inhibitor MK571 (20 μM) were performed in all MRP2 activity studies shown in this figure to quantify and deduct unspecific DNP-SG leakage.

SG right before the efflux phase (i.e. amount of DNP-SG available to be extruded) was identical between groups (Fig. 1b). No changes in DNP-SG efflux were observed in cells treated with NMGA 0.5 nM (Fig. 1a).

Western blot studies were performed to establish whether the effect of NMGA on MRP2 activity was associated with a decrease in MRP2 expression. As shown in Fig. 1c, HepG2 cells exhibited no significant changes in MRP2 protein expression after exposure to NMGA (5, 50 and 500 nM; 48 h). We hypothesized that NMGA might affect intrinsic MRP2 activity by a direct interaction with the transporter. To test this possibility, we evaluated whether the decrease in MRP2 activity also takes place in response to a short exposure to NMGA (i.e. during the efflux phase). As seen in Fig. 1d, NMGA (at all concentrations tested)

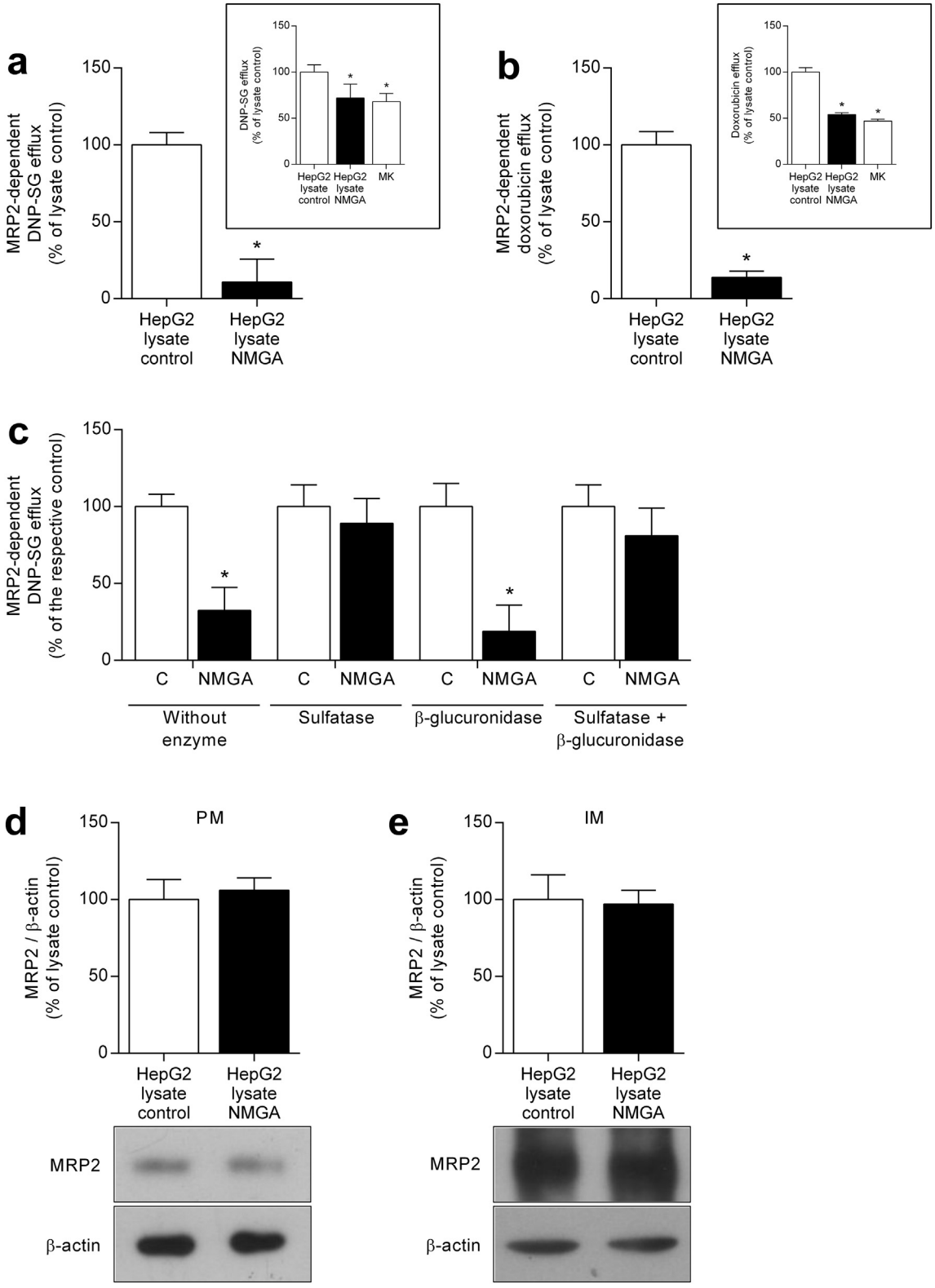

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Fig. 2. Effect of NMGA metabolites on MRP2 activity and localization in HepG2 cells. a. To assess the effect of NMGA-treated cell lysate on MRP2 activity, DNP-SG extrusion was quantified in the presence of control or NMGA-treated HepG2 cell lysates in efflux medium. A comparison between the effect of the lysates and MK571 (MK) on DNP-SG efflux is also presented (inset). Data are presented as % of lysate control and expressed as mean ± standard deviation (n = 3). *: $p < 0.05$ vs lysate control. b. The effect of NMGA metabolites on MRP2 activity was also evaluated by quantifying the efflux of doxorubicin. The comparative effect of the lysates and MK571 (MK) on doxorubicin efflux is presented (inset). Data are presented as % of lysate control and expressed as mean ± standard deviation (n = 3). *: $p < 0.05$ vs lysate control. c. To evaluate whether sulfated and/or glucuronidated NMGA metabolites are responsible for NMGA-treated cell lysate inhibitory effects, MRP2 activity assays with lysates subjected to enzymatic hydrolysis with sulfatase and/or β-glucuronidase in efflux medium were performed. Data are presented as % of the respective control and expressed as mean ± standard deviation (n = 3). *: $p < 0.05$ vs the respective control. Experiments in the presence of the selective MRP inhibitor MK571 (20 μM) were performed in all MRP2 activity studies shown in this figure to quantify and deduct unspecific DNP-SG leakage. d and e. Alteration of MRP2 distribution between plasma (PM) and intracellular (IM) membranes in response to NMGA was evaluated by western blot. Representative blots for detection of MRP2 and β-actin (loading control) in PM and IM fractions are shown together with the respective densitometric analysis. Data are presented as % of lysate control and expressed as mean ± standard deviation (n = 3).

did not result in alterations in MRP2 activity when added during DNP-SG efflux period, thus ruling out a direct inhibitory effect of NMGA.

3.2. Effect of NMGA Metabolites on MRP2 Activity and Localization in HepG2 Cells

As NMGA itself failed to impair MRP2 activity, we speculated that the products of NMGA biotransformation generated intracellularly might be responsible for the inhibitory effect. Thus, we repeated the MRP2 activity assay but using the lysate of cells treated with NMGA instead of NMGA itself (see section 2.3.). We found a significant decrease in MRP2-mediated DNP-SG efflux (−89% vs control cell lysate) (Fig. 2a). The magnitude of this inhibition was of similar extent as the magnitude of the inhibition after NMGA long-term incubation (Fig. 1a). A similar outcome was observed after performing MRP2 activity studies using doxorubicin as substrate (−86% vs control cell lysate) (Fig. 2b). These results suggest that NMGA metabolites may be responsible for NMGA-inhibitory effect on MRP2.

Since the principal products of hepatic NMGA biotransformation were shown to be glucuronidated and sulfated metabolites (Lello 2010), they constitute the main candidates to explain the current MRP2 inhibitory effect. To further evaluate this possibility, NMGA-treated cell lysates were subjected to enzymatic hydrolysis with sulfatase and/or β-glucuronidase prior to performing MRP2 activity assay. Incubation of lysates with sulfatase and with the combination of sulfatase + β-glucuronidase prevented MRP2 inhibition by NMGA-treated cell lysates, as determined using DNP-SG as model substrate. Conversely, lysates incubated with β-glucuronidase alone preserved their inhibitory potential (Fig. 2c). Taken together, these findings suggest that sulfated (but not glucuronidated) NMGA metabolites are responsible for MRP2 inhibition.

Changes in MRP2 efflux activity without changes in total protein expression may take place due to alteration of either transporter subcellular localization or intrinsic activity. Rapid translocation of MRP2 from plasma membrane (PM) to subapical, intracellular domains (IM) are a common feature of hepatic and intestinal MRP2, with consequences in transport function (Crocenzi et al. 2012; Tocchetti et al. 2018). To test the potential of NMGA metabolites to reduce MRP2 content at the PM (ultimately leading to impairment in MRP2 activity), HepG2 cells were acutely exposed to NMGA-treated cell lysates and then subjected to membrane fractionation and western blot analysis. MRP2 distribution between PM and IM was not affected by treatment with NMGA-treated cell lysate (Fig. 2d and e), ruling out MRP2 internalization by NMGA metabolites as a mechanism underlying MRP2 inhibition.

3.3. Effect of NMGA and NMGA Metabolites on MRP2 Transport Activity in Caco-2 Cells

We further evaluated the ability of NMGA to modulate MRP2 activity in differentiated Caco-2 cells, a model of human intestinal epithelium. As seen in Fig. 3a, CDF efflux ratio was not significantly affected by NMGA treatment (5, 50 and 500 nM; 48 h). Apparent permeability coefficients (P_{app}) in both AB and BA directions (as individual components of CDF efflux ratio) were neither affected (Table A.1).

To evaluate whether a deficient biotransformation could explain the absence of MRP2 inhibition by NMGA in Caco-2 cells, we performed transport activity studies incubating the cell monolayer with NMGA-treated HepG2 cell lysates (i.e. containing inhibitory metabolites). As a result, we found a significant decrease in CDF efflux ratio (−75% vs control HepG2 cell lysates) (Fig. 3b) due to a decreased CDF permeability in BA direction (Fig. A.1d and Table A.1), reaching a similar magnitude of inhibition as in HepG2 cells (Fig. 2a). Moreover, we repeated the experiment using NMGA-treated HepG2 cell lysates previously subjected to enzymatic hydrolysis with sulfatase. Like in HepG2, sulfatase treatment prevented the decrease in intestinal MRP2 activity by the cell lysates (Fig. 3c). Table A.1 and Fig. A.1 in Appendix A provide complete data regarding P_{app} and CDF efflux vs time for all experimental groups. These results provide further evidence supporting the essential role of NMGA biotransformation to exert the inhibitory effect on MRP2. In contrast to HepG2 cells, Caco-2 cells seem unable to synthesize enough inhibitory NMGA metabolites.

4. Discussion

MRP2 is a membrane transporter mediating the efflux of a wide range of *endo*- and xenobiotics. Its expression in the canalicular membrane of the hepatocytes and in the brush border membrane of the enterocytes confers MRP2 a major role promoting the biliary excretion and limiting the intestinal absorption of different substances, respectively, thus modulating their bioavailability and/or toxicity (Klaassen and Aleksunes 2010). MRP2 expression is subject to modulation by endogenous and exogenous substances. In this regard, inhibition of MRP2 activity by progesterone and norgestimate has been reported (Lindenmaier et al. 2005). In line with this previous report, we here demonstrated a significant inhibition of hepatic MRP2 by pharmacologically relevant concentrations of NMGA, a progestogen increasingly used in COC therapy (Fig. 1a). Since no changes in the initial load of DNP-SG (Fig. 1b) or in the transporter expression were observed (Fig. 1c), a direct modulation of the efflux activity is likely involved, agreeing well with previous reports for other progestogens (Lindenmaier et al. 2005). Indeed, modulation of MRP2 activity by drugs has been extensively described (Tocchetti et al. 2016). NSAIDs (El-Sheikh et al. 2007) and the anti-gout probenecid (Horikawa et al. 2002), among others, have been reported to inhibit MRP2 activity. In our study, inhibitory effects were observed even at NMGA 5 nM, about one order of magnitude below the maximal NMGA concentration reported in plasma of women taking NMGA (Huang et al. 2015; Lello 2010; Ruan et al. 2012). Therefore, although inhibition must always be analyzed in terms of relative affinities between the interacting compounds, our findings indicate a strong potential of NMGA to inhibit the transport of MRP2 substrates during contraceptive treatment.

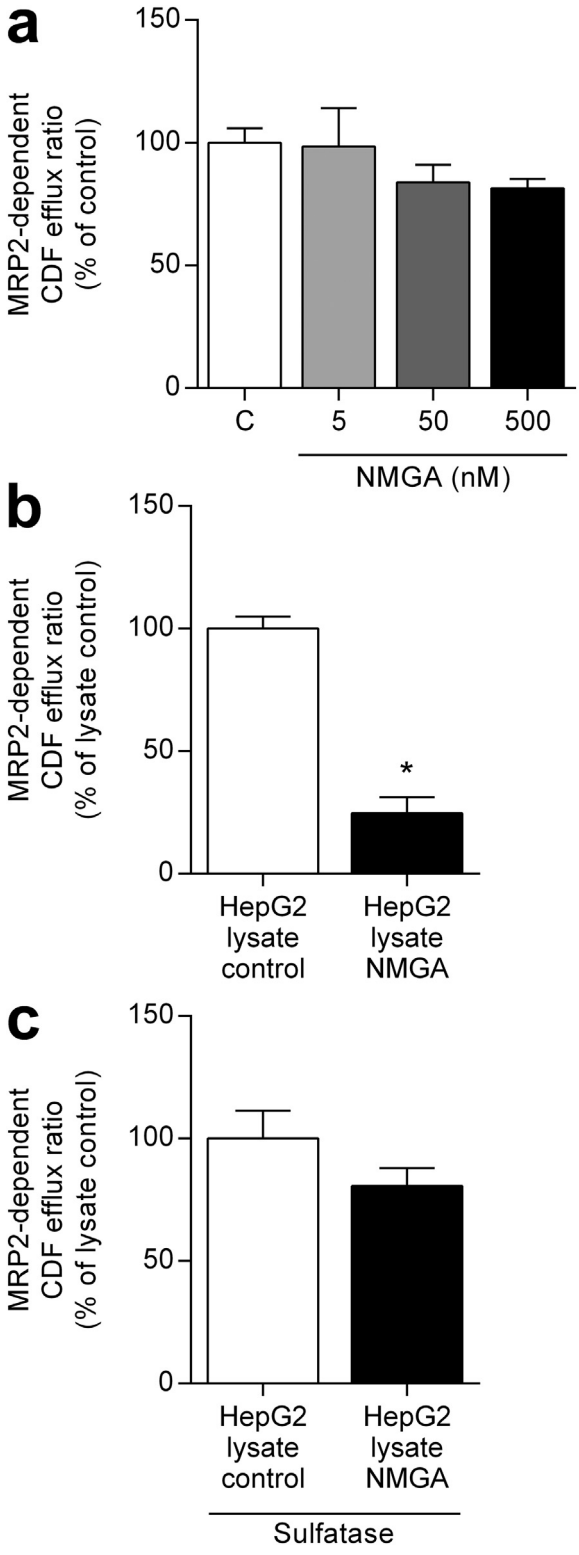
Our data demonstrated MRP2 inhibition by NMGA in HepG2 cells only after 48 h of preincubation with the progestogen. Since no changes in the expression are required, NMGA biotransformation may be

suggested as a likely mechanism explaining this delay between NMGA addition and transporter inhibition. Furthermore, MRP2 inhibition by a lysate of HepG2 cells pre-treated for 48 h with NMGA (Fig. 2a and b) also supports the presence of intracellularly generated metabolites as perpetrators of the transporter inhibition. Transporter translocation from the plasma membrane to intracellular membranes (i.e. endocytic retrieval) has been also associated to impairment of MRP2 activity (Crocenzi et al. 2012). Sometimes, biotransformation is a pre-requisite for a drug or endogenous compound to exert such effect. That is the case of estradiol-17beta-D-glucuronide (Mottino et al. 2002; Tocchetti et al. 2018). Our observations in membrane fractionation experiments, however, did not evidence differences in MRP2 localization after exposure to a lysate of NMGA pre-treated cells (i.e. containing inhibitory metabolites) (Fig. 2d and e).

Although NMGA has been described to undergo phase II biotransformation and yield sulfated and glucuronidated metabolites (Ruan et al. 2012), to date there is no available information on the exact identity of these metabolites. Prevention of MRP2 inhibition by treatment of the lysates with sulfatase (Fig. 2c) strongly indicates sulfated metabolites to be the main inhibitory species. MRP2 substrates comprise a wide range of drug sulfated and glucuronidated conjugates, probably including NMGA derivatives (Ahmed et al. 2008; Klaassen and Aleksunes 2010; Zamek-Gliszczynski et al. 2005). Since conjugated MRP2 substrates have been already reported to inhibit the transport of other substrates (Jäger et al. 2003), a direct interaction between NMGA sulfated conjugate(s) and MRP2 is possibly involved in the inhibition of DNP-SG efflux observed in HepG2 cells. Since the nature and the abundance of NMGA sulfated metabolites in our model are currently unknown, we cannot conclude on the exact mechanism of MRP2 inhibition by NMGA. The absence of a concentration-dependence (Fig. 1a) suggests a non-competitive mechanism. However, because the inhibitory species are sulfated metabolites and saturation of metabolite formation and/or cellular retention is possible, one could expect a similar amount of metabolites to accumulate intracellularly in spite of the increasing concentrations of NMGA. In that case, no different inhibitory potency would be detected and a competitive mechanism may still be taking place.

The pathophysiological role of hepatic MRP2 inhibition is well-acknowledged. For instance, a decrease in MRP2 expression and function was related to an impairment in the efflux of MRP2 substrates during cholestasis of pregnancy (Cao et al. 2002) and in models of obstructive cholestasis, where an increase in morphine and morphine-3-glucuronide plasma levels (MRP2 substrates) was reported (Hasegawa et al. 2009). Interestingly, a clinical study described sulfated progesterone metabolites as a prognostic factor for intrahepatic cholestasis of the pregnancy (Abu-Hayyeh et al. 2016). Although the association with MRP2 has not been investigated, considering the predominant transport of sulfated conjugates by MRP2, a contribution of MRP2 inhibition to the disease cannot be ruled out. Our data, pointing to an inhibition of hepatic MRP2 by NMGA sulfated conjugates, also suggest a potential of the drug to impair biliary secretion and this way the excretion of *endo*- and xenobiotics.

Intestinal MRP2 plays a major role limiting the absorption and the oral bioavailability of its substrates, thus mediating drug-drug interactions (Dahan and Amidon 2010; Lagas et al. 2012). Our results indicating no direct inhibition of MRP2 by NMGA in Caco-2 cell Transwell® culture (Fig. 3a; Fig. A.1; Table A.1), unlike previous observations in HepG2 (Fig. 1a), suggest a lack of biotransformation capacity in intestinal cells (i.e. absence of the inhibitory metabolite(s) present in HepG2 cells). Similar discrepancies between hepatic and intestinal MRP2 inhibition were already described for the phytoestrogen genistein. In fact, while acute inhibition of hepatic MRP2 by genistein is well-acknowledged (Jäger et al. 1997), no effect on the transporter activity was observed in Caco-2 cells incubated with the phytoestrogen (Schexnayder and Stratford 2015). Interestingly, in our study intestinal MRP2 inhibition was observed after incubation of Caco-2 cells with lysates from NMGA-treated HepG2 cells (i.e. containing inhibitory metabolite(s)) and this effect was again reverted by treatment of lysates with sulfatase (Fig. 3c). Considering that NMGA undergoes extensive enterohepatic circulation (Huang et al. 2015), our observations suggest the possibility of intestinal MRP2 inhibition by NMGA sulfated metabolite(s) reaching the intestine after biliary excretion. Noteworthy, reduced expression of particular members of the sulfotransferase families



(caption on next page)

Fig. 3. Effect of NMGA and NMGA metabolites on MRP2 transport activity in Caco-2 cells. a. To assess the effect of a long-term treatment with NMGA on MRP2 activity, CDF efflux was quantified in control (C) and NMGA-treated cells (5, 50 and 500 nM; 48 h). Data are presented as % of C and expressed as mean ± standard deviation (n = 3). b and c. To further characterize the effect of NMGA metabolites previously found to acutely inhibit MRP2 in HepG2 cells, CDF efflux was quantified in the presence of NMGA-treated HepG2 cell lysates in efflux medium (b). To evaluate the participation of sulfated metabolites of NMGA, experiments were repeated after sulfatase treatment of lysates (c). Data are presented as % of lysate control and expressed as mean ± standard deviation (n = 3). *: $p < 0.05$ vs lysate control. Experiments in the presence of the selective MRP inhibitor MK571 (20 μM) were performed in all MRP2 activity studies shown in this figure to quantify and deduct unspecific CDF leakage.

SULT1, SULT2 and SULT4 was described in Caco-2 cells compared to the intestine (Meinl et al. 2008). A higher sulfotransferase activity towards NMGA in the human intestine compared to Caco-2 cells may account for a more significant formation of NMGA metabolites also in the enterocyte and thus a more potent inhibition of intestinal MRP2.

In conclusion, we have observed a significant inhibition of hepatic and intestinal MRP2 by NMGA. Considering long-term extension of contraceptive treatments, coadministration with other drugs substrate of MRP2 is highly possible. In the case of compounds with a narrow therapeutic window, coadministration may lead to an increased risk of toxicity and reduced treatment safety.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejps.2018.07.017.

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