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Investigating the anti-mineralocorticoid properties of synthetic progestins used in hormone therapy

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ABSTRACT

A more detailed understanding of the affinities and efficacies for transcriptional regulation by the synthetic progestins medroxyprogesterone acetate (MPA) and norethisterone acetate (NET-A) via the mineralocorticoid receptor (MR) is required, to better understand their relative risk profiles. Both MPA and NET-A bind to the MR, although with about 100-fold lower affinities than that of Prog. MPA and NET-A exhibit no agonist activity, but NET-A, unlike MPA, has similar antagonistic efficacy to Prog on the endogenous mineralocorticoid/glucocorticoid response element (MRE/GRE)-containing genes, α-glycolytic protein or orosomucoid-1 (Orm-1) and plasminogen activator inhibitor-1 (PAI-1). This study is the first to show that NET-A, but not MPA, can dissociate between transrepression and transactivation via the MR. Given the relatively low affinity and potency of MPA and NET-A for the MR, our results suggest that these progestins are unlikeley to exert significant effects via the MR at doses used in hormonal therapy. However, considering their relative free concentrations compared to endogenous hormones, the possibility that NET-A may exhibit significant MR antagonist activity, with some possible cardiovascular protective benefits, should not be excluded.

1. Introduction

Synthetic progestins have been used for decades for contraception and hormone replacement therapy (HRT). However, concerns have been raised regarding side-effects on breast cancer, cardiovascular complications and immune function and susceptibility to infections (reviewed in [1–5]); [6–9]. While a wide range of different progestins are currently available, particular concern has been raised regarding the use of MPA in both HRT and contraception [7,5]. NET-A and its derivatives are widely used in HRT, while MPA and NET-A are the two most commonly used injectable contraceptives particularly in the developing world. Many clinical studies have thus focused on these two progestins. MPA and NET used in HRT may increase the incidence of breast cancer [6,8], and MPA may have adverse effects on cardiovascular health [9]. Furthermore, MPA but not NET is implicated in increasing risks of HIV-1 infectivity and transmission [2]. As progestins are designed to mimic the actions of the endogenous hormone progesterone (Prog), their biological effects are assumed to be mediated by the progesterone receptor (PR). However, progestins exhibit a wide range of activities, most likely reflecting their differential affinities and activities via the mineralocorticoid- (MR), glucocorticoid- (GR) and androgen-receptors [1,3,4], thus raising the possibility that their side-effects, and some of the beneficial effects may be mediated by steroid receptors other than the PR. Relatively little research has been conducted to investigate detailed molecular mechanisms of progestins via different steroid receptors. Here we focus on the mechanism of action of MPA and NET-A via the MR, with a view to better understand and predict side-effects on cardiovascular and other physiological functions.

The MR is expressed in several tissues such as the kidney, heart, central nervous system, and the vasculature [1,4] and thus may play a role in responses to progestins targetting the MR. MR-mediated increase in transcription of target genes is termed transactivation, and occurs when ligand-activated MR binds to MREs in the promoters of specific genes [1,4]. Ligand-activated steroid receptors can also negatively regulate genes (transrepression), most likely via protein–protein interactions between the receptor and other transcription factors such as nuclear factor-kappa B (NFκB) and activator protein-1 (AP-1) [1,10].

Abbreviations: Prog, progesterone; MR, mineralocorticoid receptor; HRT, hormone replacement therapy; MPA, medroxyprogesterone acetate; NET, norethisterone; NET-A, norethisterone acetate; NET-EN, norethisterone enanthate; Ald, aldosterone; MRE/GRE, mineralocorticoid/glucocorticoid response element; GR, glucocorticoid receptor; PR, progesterone receptor; Orm-1, α-glycolytic protein or orosomucoid-1; PAI-1, plasminogen activator protein-1; AP-1, activator protein-1.

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Although contentious [11], there is some evidence that inappropriate activation of the MR by aldosterone (Ald), an endogenous mineralocorticoid, leads to deleterious effects on the cardiovascular system, while intervention with a MR antagonist is beneficial to patients with heart failure (reviewed in [12]). Ald has been shown to regulate expression of genes that may contribute to the development of cardiovascular damage such as Orm-1, an inflammation/acute phase-related gene, and PAI-1, an antithrombolytic factor that promotes tissue fibrosis [13], suggesting a beneficial mechanism for MR antagonists. Since progestins such as drospirenone and trimegestone were developed to have anti-mineralocorticoid properties to exhibit beneficial effects on blood pressure and cardiovascular function [1], ligands such as MPA and NET-A that reportedly lack anti-mineralocorticoid activity, may lead to cardiovascular complications. The mechanism of action and target genes, of MPA and NET-A, as compared to Prog, via the MR. remains poorly defined. Thus, the present study aimed to provide a comparative biochemical profile of the actions of these ligands via the human MR (hMR), in the same model system.

2. Materials and methods

2.1. Plasmids

pTAT-GRE-E1b-luc, driven by the E1b promoter containing two copies of the rat TAT-GRE, has been described previously [14]. The cytomegalovirus (CMV)-driven-β-galactosidase expression vector (pCMV-β-gal), the 7xAP-1-luc plasmid, the pRShMR [15] and the pRS-hGR α plasmids expressing the human MR and GR, respectively, were obtained from Guy Haegeman (University of Gent, Belgium), Stratagene (Houston, Texas, USA) and Prof. Evans (Howard Hughes Medical Institute, La Jolla, USA), respectively. FuGENE6 (Roche Molecular Biochemicals, South Africa) was used for all transfections, according to the manufacturer's instructions.

2.2. Inducing compounds

11β,21-Dihydroxy-3,20-dioxo-4-pregnen-18-al (Ald), 7α-acetylthio-3-oxo-17α-pregn-4-ene-21,17-carbolactone (spironolactone), 4-pregnene-3, 20-dione (Prog), 6α-methyl-17α-hydroxyprogesterone acetate (MPA), 17α-ethynyl-19-nortestosterone 17β-acetate (NET-A) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma–Aldrich, South Africa. All test compounds were dissolved in absolute ethanol and added to serum-free culturing medium in a final concentration of 0.1% ethanol. Vehicle control incubations contained 0.1% ethanol.

2.3. Whole cell binding assays

COS-1 monkey kidney cells were maintained as previously described [16]. Competitive whole cell binding assays were performed and analyzed essentially as described by Bamberger et al. (1995) [17], with the following modifications [16]. COS-1 cells were seeded into 24-well tissue culture plates at 1×10^5 cells per well. On day 2, cells were transfected with 0.375 μg pRShMR expression vector and 0.0375 μg of the pCMV-β-gal expression vector. On day 3, the cells were washed with phosphate-buffered saline (PBS) prior to incubation with 0.2 nM [^{3}H]-Ald (87.9 Ci/mmol) (PerkinElmer Life and Analytical Science, South Africa), in the absence or presence of increasing concentrations of unlabelled Ald, Prog, MPA or NET-A in serum-free DMEM for 16 h at 37 °C. Total binding ([^{3}H]-Ald only) was determined by scintillation counting and expressed as 100%. Specific bound [^{3}H]-Ald was calculated as the difference between total and non-specific binding ([^{3}H]-Ald plus 10 μM unlabelled Ald) and expressed as a relative% of total binding. K_i values for Prog, MPA and NET-A were determined from the heterologous displacement curves using the EC_{50}s, K_d for Ald, and concentration of [^{3}H]-Ald, as previously described [18].

2.4. Luciferase reporter assays

For transactivation: COS-1 cells were seeded into 96-well tissue culture plates at 1×10^4 cells per well. On day 2, cells were transiently transfected with 50 ng pTAT-GRE-E1b-luc, 5 ng pRShMR, and pCMV-β-gal expression vectors, respectively. On day 3, the cells were washed with PBS and incubated with 1 μM of either Ald, Prog, MPA or NET-A in serum-free DMEM for 24 h to investigate agonist activity. For antagonist activity, cells were incubated with 1 nM Ald in the absence and presence of increasing concentrations of Prog, MPA or NET-A.

For transrepression: COS-1 cells were seeded into 24-well tissue culture plates at a density of 5×10^4 cells per well. On day 2, cells were transfected with 0.045 μg pRShMR, 0.09 μg 7xAP-1-luc and 0.0225 μg pCMV-β-Gal expression vectors, respectively. On day 3, the cells were washed with PBS and incubated with serum-free medium containing 10 ng/ml PMA and 1 μM of each test compound in serum-free DMEM for 24 h. For both assays, cells were lysed and analyzed as previously described [19].

2.5. Isolation of total RNA and real-time quantitative RT-PCR analysis

H9C2 rat cardiomyocyte cell line were maintained as previously described for COS-1 cells [16]. Cells were seeded into 12-well plates at a density of 1×10^5 cells/well, transiently transfected with 70 ng pRShMR, and grown in serum-free DMEM for 48 h. Cells were incubated with 1 nM Ald in the absence and presence of 1 μM Prog, MPA, NET-A or Spironolactone for 24 h. Total RNA was isolated, cDNA prepared and realtime PCR performed as previously described [19]. The thermal cycling parameters were: initial denaturation at 95 °C for 5 min, 40 cycles at 95 °C for 15 s and 57 °C for 30 s, using primers previously described (Supplementary Table 1) [13,20].

2.6. Data manipulation and statistical analysis

The Graph Pad Prism® software was used for data analysis. Non-linear regression and one site competition were used in whole cell binding assays, whereas non-linear regression and sigmoidal dose response were used in transactivation (antagonist) experiments. One-way ANOVA analysis of variance and Newman-Keuls or Bonferroni (compares all pairs of columns) posttests were used for statistical analysis. Statistical significance of differences is indicated by *, ** or ***, to indicate $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively, whereas no statistical significance is indicated by $p > 0.05$. The letters a, b, c etc. are also used to denote statistically significant differences, where all those values which differ significantly from others, are assigned a different letter.

3. Results

3.1. MPA and NET-A have a similar binding affinity for the MR

Competitive whole cell binding assays were performed in the steroid-receptor-deficient COS-1 cell line expressing exogenous hMR. To obtain accurate K_d and K_i values an appropriate concentration of [^{3}H]-Ald, in the range two to ten times lower than the EC_{50}, was established as 0.2 nM, and the incubation time required for equilibrium to be reached for 0.2 nM [^{3}H]-Ald binding to the MR, was determined as sixteen hours (Supplementary Fig. 1). Homologous/heterologous curves with unlabelled steroids were then

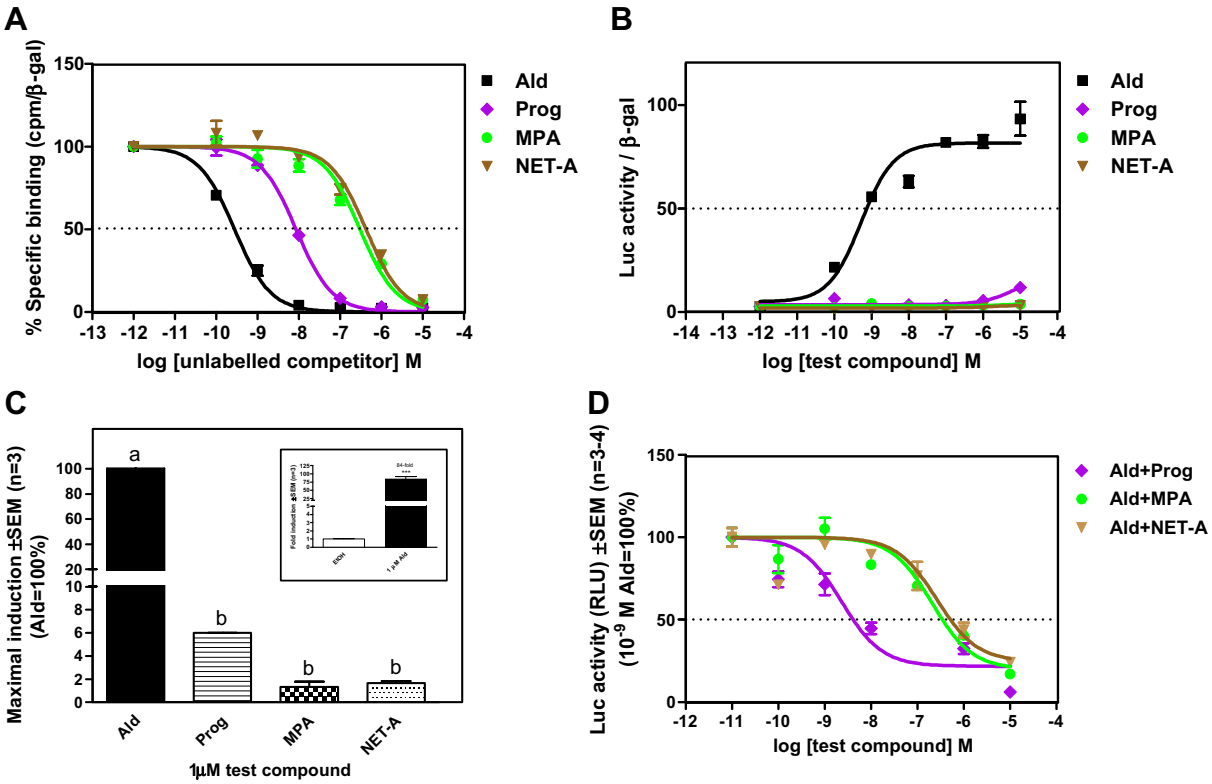

Fig. 1. (A) MPA and NET-A both compete with [^{3}H]-Ald for binding to the human mineralocorticoid receptor. The COS-1 cell line was transiently transfected with the pRShMR and pCMV-β-gal expression vectors, and incubated with 0.2 nM [^{3}H]-Ald in the presence of increasing concentrations of either unlabelled Ald (■), Prog (♦), MPA (●) or NET-A (▼) for 16 h. Results are plotted as % specific binding where total specific binding of [^{3}H]-Ald only is set as 100% and binding of unlabelled ligand is set as a % binding relative to Ald, after normalization for transfection efficiency with β-galactosidase levels. (B) and (C) Unlike Ald and Prog, MPA and NET-A do not display mineralocorticoid agonist activity on a reporter gene. The COS-1 cell line was transiently transfected with the pTAT-GRE-E1b-luc reporter plasmid, pRShMR and pCMV-β-gal expression vectors, and incubated with increasing concentrations of ligand for 24 h. (C) Induction at 1 μM (from B) is shown as a percentage relative to Ald = 100%. (D). Prog, MPA and NET-A can antagonize the agonist activity of Ald via the MR. COS-1 cells were transfected as above and incubated with 1 nM Ald (100%) or increasing concentrations of Prog (♦), MPA (●) or NET-A (▼) for 24 h. Results are representative experiments of at least three independent experiments with each condition performed in triplicate (±SEM).

obtained and results show that MPA and NET-A bind to the MR with a similar affinity, which is approximately 100-fold lower than that of Prog and 1000-fold lower than that of Ald (Fig. 1A). The curves for each competitor steroid indicate competitive binding to the same site as Ald (R^2 values for each MPA, NET-A and Prog were 0.9453, 0.9452 and 0.9837, respectively). The K_d for Ald was determined as 1.53×10^{-10} M, while K_i values for Prog, MPA and NET-A were determined as 1.69×10^{-9}, 1.97×10^{-7} and 2.29×10^{-7} M, respectively.

3.2. MPA and NET-A display weak MR antagonist-, but no significant agonist activity for transactivation on a reporter gene

We directly compared the mineralocorticoid properties of the progestins on a MRE/GRE-driven reporter construct via expressed hMR in COS-1 cells. Results show that MPA, NET-A and Prog display no significant agonist activity for the MR at concentrations from 0.1 nM to 1 μM (Fig. 1B and C). This is consistent with our finding that 1 μM Prog, MPA and NET-A fail to induce the N/C-interaction of the hMR (Supplementary Fig. 2). Antagonist activity via the MR was next investigated in the same model system. Both MPA and NET-A can similarly and fully antagonize the effects of 1 nM Ald, although they are 100–200-fold less potent than Prog (Fig 1D). Some antagonism was already observed for MPA and NET-A at about 10–100 nM, while full repression was observed at about 10 μM, with potencies (EC_{50} values) for antagonist activity of 3.1, 310 and 270 nM for Prog, MPA and NET-A, respectively.

3.3. MPA and NET-A display dissimilar mineralocorticoid properties for transrepression on a synthetic AP-1 promoter

We compared the transrepressive activity of MPA and NET-A in the COS-1 cell line with exogenously expressed hMR on a reporter plasmid containing AP-1 sites in the promoter. Fig. 2A shows 65% repression of the PMA-induced response with 1 μM Ald. Surprisingly, Prog and NET-A act as agonists for transrepression on the AP-1 promoter via the MR. No statistically significant difference was obtained in the transrepression efficacy for Ald versus NET-A via the MR, although the trend suggests that Prog and NET-A may have a lower efficacy than Ald. In contrast, MPA does not transrepress the PMA-induced response on the AP-1 promoter (Fig. 2B), revealing a significant and promoter-specific difference in the actions of MPA versus NET-A, via the MR. These data should reflect efficacy i.e. maximal response (Supplementary Table 2), as the fractional occupancy for all the ligands at 1 μM is near 100%.

3.4. Unlike MPA, Prog and NET-A antagonize the aldosterone-induced upregulation of the endogenous Orm-1 and PAI-1 genes

To determine whether the results observed on a synthetic promoter could be mimicked on endogenous promoters, mRNA levels of the Orm-1 and PAI-1 genes in the H9C2 cell line expressing exogenous hMR, were investigated. Both genes have previously been reported to be upregulated by 1 nM Ald in MR-expressing H9C2 cells, most likely binding of the liganded MR to a MRE [13].

Western blot analysis confirmed the presence of overexpressed MR and endogenous MR and GR, but not endogenous androgen

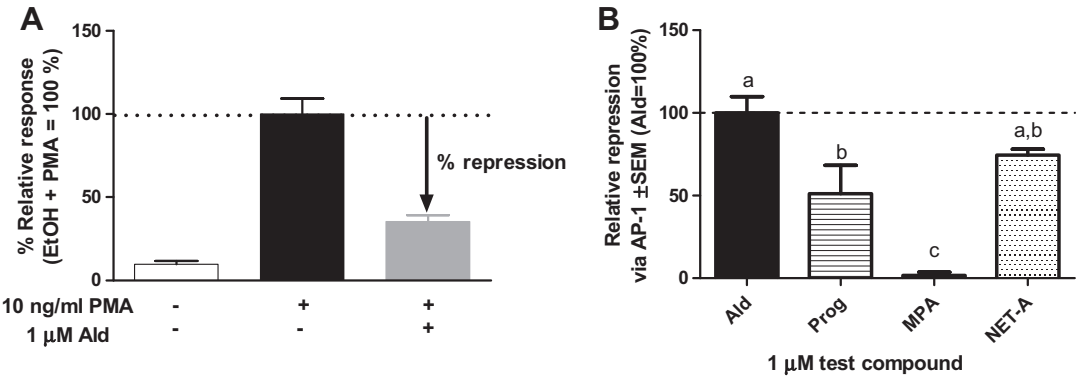

Fig. 2. (A) Transrepression activity of Ald on the synthetic AP-1 promoter via the hMR. The COS-1 cell line was transiently transfected with the pRShMR, pCMV-β-gal expression vectors and an AP-1-containing promoter-luciferase reporter construct, and incubated with vehicle (EtOH), EtOH in the presence of 10 ng/ml PMA, or 10 ng/ml PMA in the presence of 1 μM Ald for 24 h. EtOH + PMA was set as 100% and Ald response was calculated as a percentage of this. The % repression is indicated by the arrow. (B) Transrepression activity of Prog, MPA and NET-A on the synthetic AP-1 promoter via the hMR. The COS-1 cell line was transiently transfected as above and incubated with 10 ng/ml PMA and 1 μM ligands for 24 h. The % repression for the ligands was expressed as a % of the Ald response (100% repression). The result shown is the average of three independent experiments with each condition performed in triplicate (±SEM). β-Gal values were used to normalize for transfection efficiency.

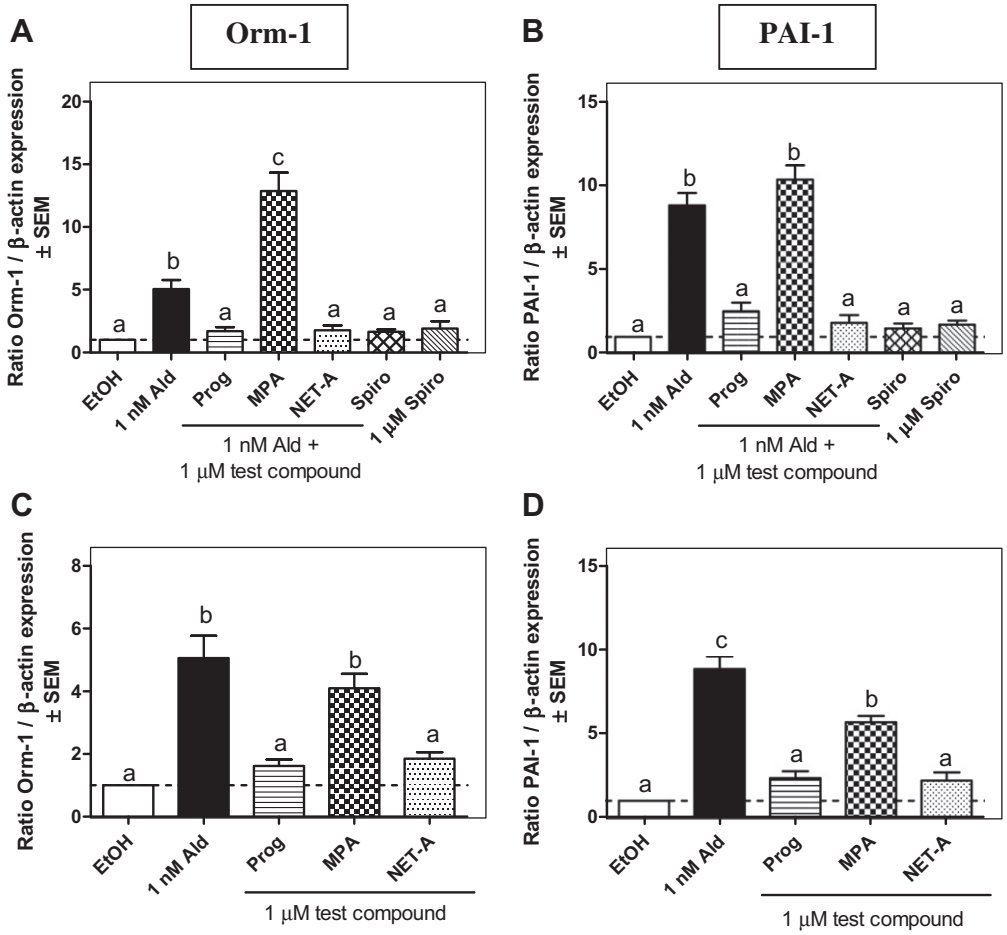

Fig. 3. Antagonist (A and B) and agonist (C and D) activity of Prog, MPA and NET-A, on the endogenous MRE-containing Orm-1 and PAI-1 genes in the H9C2 cell line. Cells were transiently transfected with the MR and incubated with (A and B) 1 nM Ald in the absence and presence of 1 μM Prog, MPA, NET-A or Spironolactone, or (C and D) 1 nM Ald or 1 μM Prog, MPA or NET-A. Orm-1, PAI-1 and β-actin mRNA were determined by QPCR. Ratios of the Orm-1/β-actin and PAI-1/β-actin genes were calculated using the Fit Points method described by Pfaffl (2001). Results shown are the averages of three independent experiments.

receptor- (AR) or PR (data not shown), while a functional assay supported the absence of endogenous AR and PR (Supplementary Fig. 3A–D). The use of 1 nM Ald with an affinity for the MR of 0.153 nM compared to 63 nM for the GR [21] ensured that the Ald response would be overwhelmingly due to the MR and not the GR. We observed a 5.1-fold increase in Orm-1 mRNA levels by 1 nM Ald at 24 h (Fig. 3A and C), while an 8.8-fold induction was observed for PAI-1 mRNA levels (Fig. 3B and D). Our result on the synthetic MRE/GRE showing that Prog is a potent MR antagonist was corroborated on the endogenous MRE/GRE-containing genes. Although MPA and NET-A showed a weak but similar antagonist potency for the MR on the synthetic promoter, they acted

differently on both endogenous promoters (Fig. 3A and B). NET-A antagonized the Ald-induced upregulation of both the Orm-1 and PAI-1 mRNA levels to similar extents as Prog, unlike MPA. Interestingly, MPA significantly enhanced the upregulation of Ald-induced Orm-1 mRNA levels (Fig. 3A).

4. Discussion

Given the published inconsistencies in affinities of progestins for steroid receptors [1,4], we determined precise equilibrium dissociation constants for the MR of Ald, Prog, MPA and NET-A in parallel in a receptor-deficient cell model using expressed hMR. The K_d we determined for Ald is lower than previously reported [15], while the K_i values we determined for MPA and NET-A are 40-fold lower than previously reported values [22,23]. These differences are possibly due to the absence of competing receptors in our system, as well as equilibrium having been established or other differences in methodologies [1].

We report for the first time the effects of MPA versus NET-A on MR-mediated gene regulation for both transactivation and transrepression on synthetic promoters in the same system. Our findings that Prog, MPA and NET-A are devoid of mineralocorticoid agonist activity via a synthetic MRE/GRE are consistent with most of the literature, although some have reported very weak partial mineralocorticoid agonist activity for Prog (reviewed in [1,4]), possibly reflecting increased receptor levels. Our results showing potent anti-mineralocorticoid activity of Prog are of the same order of magnitude with previously reported potencies [24,21]. Both MPA and NET-A exhibit MR antagonist activity, albeit less potent than Prog. While these findings are similar to one report [25], they are in contrast to others [26] (reviewed in [1]). Additionally, we show that Ald can repress AP-1 activity via the MR, in contrast to an earlier report [27]. These apparent differences in results most likely reflect methodological differences or suggest that steroid receptor activities are likely to be cell- and promoter-specific. Our finding that NET-A, but not MPA, can transrepress the PMA-induced response on the AP-1 promoter via the MR, without exhibiting agonist activity for MR transactivation via a synthetic MRE/GRE promoter, suggest that NET-A may be a promising agent for the treatment of certain inflammatory conditions.

We next determined whether MPA and NET-A could antagonize Ald-induced effects on the Orm-1 and PAI-1 genes, both associated with increased risk of cardiovascular disease [28,29]. The promoters of both these genes contain a functional MRE/GRE, and it has been reported that the effect of Ald on both Orm-1 and PAI-1 is direct, mediated by the binding of the liganded-MR to MREs in the promoter region [13]. Using a rat cardiomyocyte cell line expressing exogenous MR as a model for cardiovascular disease, we found that NET-A and Prog, but not MPA, completely inhibit the effect of Ald on the expression of endogenous genes, Orm-1 and PAI-1. In contrast, both MPA and NET-A acted as weak antagonists of the MR in a MRE/GRE-reporter assay in COS-1 cells. Interestingly, MPA increased the expression of the endogenous MRE/GRE-containing genes, while showing no agonist activity on the MRE/GRE reporter gene. In contrast, Prog and NET-A exhibited a similar lack of agonist activity on the reporter gene as well as the endogenous genes. It is likely that the agonist activity of MPA on the endogenous genes in the H9C2 cells is mediated by the endogenously expressed GR, rather than the overexpressed MR. Notably, NET-A does not display any glucocorticoid agonist properties [30], and thus the finding that NET-A has no agonist properties at a concentration of 1 μM is consistent with some GR activity of MPA.

Whether or not MPA or NET-A exert significant biological effects via the MR at concentrations relevant to hormonal therapy is a key question. Peak serum level reached with HRT (MPA = 0.2 nM and NET-A = 17.7 nM) [1,4] (Activelle package insert reg. No. 33/21.8.2/0532, Novo Nordisk Inc.) or contraception (MPA = 5–65 nM; NET-A = 59 nM) [1,2] are lower than the K_i for MPA and NET-A as well as their EC_{50}s for antagonist on the MRE/GRE reporter.

Thus our results suggest that these progestins are unlikely to exert significant anti-mineralocorticoid effects via the MR at doses used in hormonal therapy. However, it is possible that the progestins compete to some extent with Ald for binding to the MR *in vivo* considering that the serum levels for MPA and NET-A are typically about 100-fold that of Ald, with a similar percentage free ligand (reviewed in [1]). Furthermore, the affinity of NET-A is only about 3–10-fold higher than the serum concentrations of hormone therapy users, suggesting that NET-A, but not MPA, may exhibit some significant cell- and/or promoter-specific anti-mineralocorticoid effects via the MR *in vivo*, with possible cardiovascular protective benefits. Our finding that NET-A and Prog similarly antagonize the Ald-induced upregulation of the endogenous Orm-1 and PAI-1 genes, is consistent with this idea. Moreover, the NET-A results are consistent with clinical data showing no adverse effects of NET on CVD [31], while our MPA results may suggest an explanation for the observed adverse effects of MPA on CVD in the Women's Health Initiative study [8,9].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.086.

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