TRAP220 is modulated by the antineoplastic agent 6-Mercaptopurine, and mediates the activation of the NR4A subgroup of nuclear receptors

K D Senali Abayratna Wansa and George E O Muscat
Institute for Molecular Bioscience, University of Queensland St Lucia, 4072 Queensland, Australia

(Requests for offprints should be addressed to G.Muscat@imb.uq.edu.au)

Abstract
The NR4A1–3 (Nur77, NURR1 and NOR-1) subfamily of nuclear hormone receptors (NRs) has been implicated in Parkinson’s disease, schizophrenia, manic depression, atherogenesis, Alzheimer’s disease, rheumatoid arthritis, cancer and apoptosis. This has driven investigations into the mechanism of action, and the identification of small molecule regulators, that may provide the platform for pharmaceutical and therapeutic exploitation. Recently, we found that the purine antimetabolite 6-Mercaptopurine (6-MP), which is widely used as an anti-neoplastic and anti-inflammatory drug, modulated the NR4A1–3 subfamily. Interestingly, the agonist-mediated activation did not involve modulation of primary coactivators’ (e.g. p300 and SRC-2/GRIP-1) activity and/or recruitment. However, the role of the subsequently recruited coactivators, for example CARM-1 and TRAP220, in 6-MP-mediated activation of the NR4A1–3 subfamily remains obscure. In this study we demonstrate that 6-MP modulates the activity of the coactivator TRAP220 in a dose-dependent manner. Moreover, we demonstrate that TRAP220 potentiates NOR-1-mediated transactivation, and interacts with the NR4A1–3 subgroup in an AF-1-dependent manner in a cellular context. The region of TRAP220 that mediated 6-MP activation and NR4A interaction was delimited to amino acids 1–800, and operates independently of the critical PKC and PKA phosphorylation sites. Interestingly, TRAP220 expression does not increase the relative induction by 6-MP, however the absolute level of NOR-1-mediated trans-activation is increased. This study demonstrates that 6-MP modulates the activity of the NR4A subgroup, and the coactivator TRAP220.

Introduction

All members of the NR superfamily display a highly-conserved structural organisation, with an amino terminal region AB that encodes activation function 1 (AF-1); followed by the C region which encodes the DNA binding domain (DBD); a linker region D and the C-terminal E region. The DE region encodes the ligand binding domain (LBD) and a transcriptional domain, denoted as activation function 2 (AF-2) (Gronemeyer & Laudet 1995). The NR4A1–3 subfamily of orphan nuclear receptors is well conserved in the DBD (~91–95%) and the C-terminal LBD (~60%), but divergent in the N-terminal AB region (e.g. AF-1).

A decade ago, gene products were identified that appeared to belong to the nuclear receptor superfamily on the basis of their nucleic acid sequence identity. The endogenous signalling molecules which bound to these proteins were unknown and thus the term ‘orphan receptor’ was adopted. Thus the orphans forecast an enormous yet unexploited opportunity for the discovery of important new therapeutic agents.

6-Mercaptopurine (6-MP) belongs to the thiopurines, a group of substances structurally related to endogenous
purine bases like adenine, guanine, and hypoxanthine. Azathioprine (parent compound of 6-MP) and other thiopurine drugs have been administered in the treatment of leukaemia and a number of autoimmune conditions, such as lupus erythematosus, pemphigus, and myasthenia gravis. More recently their use has been extended to the management of patients with chronic inflammatory bowel diseases (IBDs) including Crohn’s disease and ulcerative colitis, and also multiple sclerosis. However, the precise mechanism of action of the thiopurine drugs remains unclear, despite more than 40 years of use. (Cara et al. 2004). It has been shown that this purine anti-metabolite has anti-proliferative and cytotoxic effects resulting primarily from the inhibition of purine de novo biosynthesis at multiple steps and incorporation into nucleic acids as thionguanine nucleotides (Cara et al. 2004).

Our previous work has expanded the function of purine anti-metabolites by demonstrating that 6-MP targets the NR4A1–3 subfamily of NRs and regulates its activity (Ordentlich et al. 2003, Wansa et al. 2003). This suggested that the signalling pathways that inhibit proliferation via inhibition of de novo purine and/or nucleic acid biosynthesis are involved in the regulation NR4A1–3 activity. The absence of a conventional ligand binding pocket in the NR4A1–3 LBD may account for the inability of 6-MP to directly bind to the NR4A1–3 subfamily (Baker et al. 2003, Wang et al. 2003). 6-MP is a specific activator of the NR4A1–3 subfamily of NRs, as FXR, RXR, LXRα, RORα and ERα are not activated by 6-MP (Ordentlich et al. 2003). Furthermore general transactivators (GALVP16), and the myogenic transactivators (MyoD, Myogenin and MFE2C), and the coactivators steroid receptor coactivator-2 (SRC-2) and p300 are not activated by 6-MP (Wansa et al. 2003).

Ligand-dependent transcriptional activation by NRs is a process that involves a stepwise recruitment of various coactivators to the promoters of hormone-regulated genes assembled into chromatin. Ligand-dependent recruitment of the p160 factors (SRCs) in concert with other factors such as CREB binding protein (CBP), p300, p300/CBP-associated factor (PCAF), and coactivator-associated arginine methyltransferases 1(CARM1) bring histone acetyltransferase and histone methylation activity to NR complexes. The resulting modification of chromatin structure facilitates the recruitment of the thyroid receptor-associated proteins/vitamin D receptor-interaction proteins (TRAP/DRIp) complex, helping to recruit RNA polymerase II to the promoters of the hormone regulated-genes. (Glass & Rosenfeld 2000).

In this study, we determined whether the auxiliary coactivators CARM1 and TRAP220/DRIp205 could be modulated by 6-MP. The results here identify 6-MP as a regulator of TRAP220 activity. Moreover, we demonstrate that TRAP220 interacts with the NOR-1 and Nur77 in an AF-1-dependent manner. The region of TRAP220 that mediates 6-MP activation was delimited to amino acids 1–800. This study demonstrates that 6-MP not only mediates activation of the NR4A1–3 subfamily, but also modulates TRAP220 activity.

Material and methods

Transient transfections

Proliferating C2C12 cells were grown in DMEM supplemented with 10% Serum supreme foetal calf serum (Biowhittaker, Edward Kellar Pty Ltd, Hallam, Victoria, Australia) in 6% CO2 . Cells grown in 12-well dishes to 50% confluence were transiently transfected with 1µg (per well) POMC-TK-LUC (NurRE-5oxo-tk-LUC) reporter plasmid (Maira et al. 1999) together with 0·16µg (per well) pSG5-NOR-1-FL or pSG5 cotransfected with pcDNA-TRAP220 (0·01, 0·1 and 0·3 µg per well) alone using a DOTAP/Metafectene (Biotex Laboratories, GmbH, Munich, Germany) liposome mixture in HEBs (42 mM HEPES, 275 mM NaCl, 10 mM KCl, 0·4 mM Na2HPO4 and 11 mM Dextrose, pH 7·1). The DNA/DOTAP/Metafectene mixture was added to the cells in 1 ml phenol red free DMEM, containing 10% charcoal-stripped foetal calf serum and incubated for 14 h. Medium was replaced 16–24 h later and/or 6-MP (Sigma) was added and cells grown for a further 24–48 h. Cells were harvested after 48 h and assayed for luciferase activity.

GAL4 hybrid assay

C2C12 cells were passaged into 12-well plates and transfected at 50–80% confluence with the reporter, G5E1b-LUC (0·33–1µg per well), and 0·16µg (per well) of the GAL chimeric constructs SV40-GAL4 DBD, CMV-GAL4 DBD, GAL-NOR-1-FL, CMV-GAL-CARM1, GAL-TRAP220 and the chimeric constructs GAL-TRAP220-aa1–1000, GAL-TRAP220aa1–800, GAL-TRAP220-aa1–300, GAL-TRAP220-aa330–660, GAL-TRAP220-aa660–970, GAL-TRAP220-aa970–1300, GAL-TRAP220-aa330–1567, GAL-TRAP220-aa1290–1567, GAL-TRAP220-aa800–1567, GAL-TRAP220S756A/S757A, GAL-TRAP220S796A/S797A. Furthermore, C2C12 cells were transfected with 0·33–1µg (per well) reporter G5E1b-LUC and 0·16µg (per well) of the GAL chimeric constructs CMV-GAL4 DBD, CMV-GAL-NOR-1-FL and GAL-TRAP220aa1–1000 and were then cotransfected with either pcDNA-SRC-2, pcDNA-TRAP220 or SG5-NOR-FL (0·66µg per well) using DOTAP/Metafectene liposome mixture in HEBs per well. The DNA/DOTAP/Metafectene mixture was added to the cells in 1 ml of DMEM, containing 10%
FCS, and incubated for 14 h. Medium was replaced 16–24 h later and/or 6-MP (Sigma) was added and cells grown a further 24–48 h. Cells were harvested after 48 h and assayed for luciferase activity. Phenol red-free DMEM containing 10% CSF was used for transfections with 6-MP in the procedure, as described previously (Wansa et al. 2002, 2003).

**Mammalian two-hybrid assay**

Plasmids (1 µg per well G5E1b-LUC reporter and 0·33 µg per well GAL4 DBD or GAL-TRAP220 chimeric constructs) were co-transfected/expressed in human choriocarcinoma Jeg3 cells grown in 12-well plates with either VP160, VP16-NOR-1 or VP16-Nur77 chimeric constructs (0·33 µg per well) in DMEM containing 5% charcoal-stripped FCS by the DOTAP/metalenectet-mediated procedure as described previously, then assayed with respect to their ability to transactivate the reporter (G5E1b-LUC) (Chen et al. 2000).

**Luciferase assays**

Luciferase activity was assayed using a Luclite kit (PerkinElmer Pty Ltd, Rosville, Victoria, Australia) according to the manufacturer’s instructions. Briefly, cells were washed once in PBS and resuspended in 80µl phenol red-free DMEM and 80µl Luclite substrate buffer. Cell lysates were transferred to a 96-well plate and relative luciferase units were measured for 5 sec in a Wallac Trilux 1450 microbeta luminometer (PerkinElmer, Wansa et al. 2002, 2003).

**Plasmids, plasmid construction and primer sequences**

The expression plasmids SV40 GALO (Kato et al. 1990), CMVGAL0, (Casanova et al. 1994, Willy et al. 1995), pCMV-GAL-CARM1, pCMV-SRC-2 (Chen et al. 2000, 2002), pcDNA-TRAP220 (Rachez & Freedman 2000), GAL-NOR-FL (Wansa et al. 2003) and the reporter plasmid G5E1b-LUC (Lillie & Green 1989) have been described elsewhere. All primers used in this study were obtained from GeneWorks Pty Ltd, Thebarton, South Australia, Australia. CMV-GAL-NOR-1-FL was constructed by excising NOR1 cDNA from pSG5-NOR-FL and recloning into the EcoRI site of the CMVGAL0 vector. 5’ GCC GTC GAC AT ATG CCC TGC GTG CAA GCC CAG and 3’ GCC GTC GAC TCA GAA AGG CAG GGT GTC AAG GAA primers containing Sall sites were used to PCR NOR-1 cDNA; and 5’ GCC GTC GAC AT ATG CCC TGT ATT CAA GCT CAA and 3’ GCC GTC GAC TCA GAA AGA CAA TG TGT GTC CAT primers containing SaII sites were used to PCR Nur77 cDNA from pSG5-NOR-FL and pSG5-Nur77-FL expression plasmids, respectively. The PCR products were cloned into the SaII site of SV40-VP160. 5’ GCC GTC GAC AT ATG CCC TGC GTG CAA GCC CAG and 3’ GCC GTC GAC TCA TGT GCC TTT CCC AGA TGA TGA were used to PCR and clone NOR-1-AB and 5’ GCC GTC GAC AT ATG CCC TGT ATT CAA GCT CAA and 3’ GCC GTC GAC TCA GAA AGG CAG GGT GTC AAG GAA primers were used to PCR and clone NOR-1-DE. 5’ GCC GTC GAC AT ATG CCC TGT ATT CAA GCT CAA and 3’ GCC GTC GAC TCA GAA AGA CAA TG TGT GTC CAT were used to PCR and clone Nur77-AB and 5’ GCC GTC GAC AT ATG CCC TGT ATT CAA GCT CAA and 3’ GCC GTC GAC TCA GAA AGA CAA TG TGT GTC CAT were used to PCR and clone Nur77-DE. PCR products were amplified from pSG5-NOR-1-FL and pSG5-Nur77-FL expression plasmids and the cloned Sall site of SV40-VP160. Bold type indicates restriction enzyme sites.

Primers used for PCR amplification of TRAP220 sub-domains cloned into SV40 GAL0 were: TRAP220aa1—800 GMUQ 805 5’ GCC GTC GAC ATA TGA GTT CTC TCC TGG AAC G and GMUQ 807 3’ GCC GTC GAC CTA ATG CCC AGA GCT TGA AG; TRAP220aa1—330- GMUQ 808 5’ CCA TCG ATA CCA TGA GTT CTC TCC TGG AAC G and GMUQ 809 3’ GCC GAG CTC CTA CTA CTG AGT GAT CAG TTT ATC ATA C; TRAP220aa1—800—1567-GMUQ 810 5’ CCA TCG ATA CCA TGA GTT CTC AGA GTA CCC and GMUQ 811 3’ GCC GAG CTC CTA ATT CCC AAT CAG GGC C; TRAP220aa1290—1567-GMUQ 812 5’ CCA TCG ATA CCA TGA GTT CTC AGA GTA TAG ATA AAC TG and GMUQ 813 3’ GCC GAG CTC CTA ATT CCC AAT CAG GGC C; TRAP220aa330–660-GMUQ 814 3’ GCC GAA TTC ACC ATG TTT GAG CTA TCA AAG G and GMUQ 815 3’ GCC GAA TTC ACC ATG TTT GAG CTA TCA AAG G and GMUQ 816 3’ GCC GAA TTC CTA GGT GCC ATT GCC TTT C; TRAP220aa970–1300-GMUQ 817 5’ GCC GAA TTC ACC ATG AGT AAT AGT ACT CTC and GMUQ 818 3’ GCC GAA TTC CTA GAC AAG CCC ATG ATT C and GMUQ 819 3’ GCC GAA TTC CTA ATT CCC AAT CAG GGC C; TRAP220aa1—1000 has been described elsewhere (Harris et al. 2002). Bold type indicates the nucleotides mutated on TRAP220.

**Site directed mutagenesis**

Using Stratagene Quick change site directed mutagenesis kit as per manufacturers’ instructions (Stratagene, Cedar Creek, Texas, USA), GAL-TRAP220-aa1—800 chimeras
that simultaneously carried double amino acid mutations were constructed. The primers were TRAP220S656A/S657A-5’ CCT TTA GAA AGG AAC GCC GCT TCC GGC TCA CCC CG and 3’ CG GGG TGA GCC GGA AGC GGC GTT CTG CCT TTC TAA AGG G; TRAP220S756A/S757A- 5’ GG ATG GTC CGA CTA GCC GCT TCA GAC AGC ATT GGC CC and 3’ GG GCC AAT GCT GCT TGA AGC GGC TAG TCG GAC CAT CC; TRAP220S796A/S797A – 5’ CCT CTT CGA GAT GCT GCA AGC TCT GGG CAT TCT CAG and 3’ CTG AGA ATG CCC AGA GCT TG C ATC TCG AAG AGG GG.

Results

6-MP modulates the activity of TRAP220/DRIP205

Previously we demonstrated that general transcriptional activators, myogenic trans-activators, and the primary coactivators, SRC-2 and p300, were not 6-MP targets (Wansa et al. 2003).

In the current study we investigated whether any subsequently recruited cofactors in the NR4A activation could be modulated by 6-MP. We utilised the GAL4 hybrid system, whereby putative activation domains are fused to the DBD of the yeast transcription factor GAL4. If these regions encode a modular activation domain, they complement the GAL4 DBD to produce a functional transactivator and induce the transcription of the GAL-responsive reporter construct G5E1b-LUC. G5E1b-LUC contains five copies of the GAL4 binding site upstream of a minimal E1b promoter. The coactivators, CARM1 and TRAP220, were fused to the GAL4 DBD and were examined for the ability of these chimeras to regulate the expression of the G5E1b-LUC reporter in C2C12 myogenic cells in the presence and absence of 6-MP (Fig. 1A). We observed that 6-MP did not activate the cofactor CARM1, however, the coactivator TRAP220 which contained amino acids 1–1000 was significantly activated by 6-MP (Fig. 1B). This suggested that 6-MP modulated the activity of TRAP220.

To investigate the dose-dependent nature of 6-MP-mediated modulation of TRAP220 we examined the ability of TRAP220 relative to NOR-1 to be activated by 0·1–100µM 6-MP. GAL-NOR-FL and GAL-TRAP220 were responsive to 6-MP at concentrations of 10µM and the activity of these chimeras was further potentiated at concentrations of 33µM, 50µM and 100µM (Fig. 1C). Thus, NOR-1 and TRAP220 were both modulated by 6-MP. The EC50 values were in the range of 33–50µM, however, at those concentrations 6-MP produced a more efficacious activation of NOR-1 relative to TRAP220.

Figure 1 6-MP modulates the activity of TRAP220/DRIP205. (A) A diagrammatic representation of the Gal4 hybrid assay is shown. (B and C) GAL-TRAP220-aa1–1000, GAL-CARM1 and GAL-NOR-1-FL (0·16µg) were co-transfected into C2C12 proliferating myoblasts together with the reporter plasmid G5E1b-LUC (0·83µg) in presence and absence of 50µM of 6-MP. (C) Dose response activity to 6-MP is shown for GAL4-DBD, GAL-NOR1-FL and GAL-TRAP220-aa1–1000 at concentrations of 0·1, 0·3, 1, 3·3, 10, 33, 50 and 100µM. Fold induction is expressed as the difference in fold activation obtained after cotransfection of a GAL-chimeric construct in the presence of 6-MP relative to the same GAL-chimeric construct transfected in the absence of 6-MP. The mean luciferase fold activation/induction and S.D. values (bars) were derived from a minimum of 2–3 independent triplicate experiments.

The region between amino acids 1–800 of TRAP220 mediates the activation by 6-MP

In order to further characterise the region of TRAP220 that is activated by 6-MP, we constructed a comprehensive set of TRAP220 deletions by PCR and cloned these segments into the GAL4 DBD (Fig. 2A) and examined the ability of these chimeras to regulate the expression/transcription of the G5E1b-LUC reporter in the presence and absence of 6-MP. In the GAL4 hybrid
system we observed, as before (Figs 1B and 1C) the region between amino acids 1–1000 of TRAP220 retained the potential to be activated significantly by 6-MP. Further delimiting of TRAP220 demonstrated that the region activated by 6-MP was between amino acids 1–800, which was activated 18-fold over the TRAP220-mediated transcription of the reporter gene in the absence of 6-MP (Fig. 2B). The importance of amino acids 1–800 of TRAP220 in mediating 6-MP activation was reinforced by the observation that domains downstream of amino acids 800 were not activated by 6-MP. For example, deletions containing amino acid regions 660–970, 970–1300, 300–1567 and 1290–1567 of TRAP220 did not mediate 6-MP activation.
activation. Furthermore, additional delimitation of the 
first 800 amino acids into smaller segments spanning 
amino acids, 1–300 and 300–660 TRAP220 were not 
activated by 6-MP (Figs 2A and B).

**The characterized protein kinase A and C sites in 
TRAP220 are not involved in 6-MP mediated 
modulation of TRAP220 activity**

It has been proposed that phosphorylation regulates 
p300/CBP and SRC-1 coactivator function (Janknecht 
& Nordheim 1996, Rowan et al. 2000, See et al. 2001, 
Moreover, in vitro and in vivo approaches identified 
that TRAP220 is phosphorylated, the major phosphor-
ylation sites were subsequently identified to be protein 
kinase A (PKA), C (PKC) and mitogen-activated protein 
kinase (MAPK) sites. Furthermore, the effect of MAPK 
activation on TRAP220-mediated PPARγ transcription 
suggested that this kinase signal pathway positively 
influences TRAP220 function (Misra et al. 2002).
The region of TRAP220 (amino acids 1–800) that 
mediates 6-MP activation contains the defined PKC 
and PKA phosphorylation sites (Fig. 3A). We mutated 
these PKA and PKC sites within TRAP220 and investigated 
the effect of the mutations on 6-MP-mediated activation 
of TRAP220. We changed the two critical serines in each 
of the three defined kinase motifs to alanines. We ob-
served that the mutants GAL-TRAP220-S656A/S657A, 
GAL-TRAP220-S756A/S757A and GAL-TRAP220-S796A/S797A did not compromise the ability of 6-MP 
to modulate the activity of TRAP220 (Fig. 3B).
TRAP220 expression stimulates the activity of NOR-1

Our previous studies had demonstrated the direct in vitro binding of SRC-2 and TRAP220 to Nur77 and NOR-1. Furthermore, we demonstrated that SRC-2 modulated the activity of Nur77 and NOR-1 (Wansa et al. 2003). Activation of gene expression by the classical nuclear hormone receptors is dependent on the recruitment of the SRCs that recruit a number of cofactors in a sequential manner that possess intrinsic histone acetyltransferase (HAT) activity, and precipitate the assembly of a higher order structure that includes the TRAP/DRIP protein complex that regulates localised nucleosome structure (Glass & Rosenfeld 2000). The key member of the TRAP/DRIP complex is the TRAP220/DRIP205 subunit that has also been shown to interact with many NRs, such as vitamin D receptor (VDR), thyroid receptor (TR), androgen receptor (AR), estrogen receptor (ER), glucocorticoid receptor (GR) and peroxisome proliferator activated-receptor (PPAR) (Zhu et al. 1997, Hittelman et al. 1999, Sharma & Fondell 2002, Burakov et al. 2000, Ren et al. 2000, Wang et al. 2002, Acevedo & Kraus 2003).

Hence, we wished to investigate whether TRAP220 modulated the activity of NOR-1 in the GAL4 hybrid system. In these assays the activity of NOR-1 is independent of its binding to its cognate binding motifs. If TRAP220 regulates the transcriptional activity of NOR-1, then the potential of the GAL4-NOR-1-FL fusions to trans-activate gene expression should be increased in this assay (Fig. 4A). C2C12 cells were cotransfected with GAL-NOR-1-FL and the G5E1b-LUC reporter in the presence and absence of an SRC-2 or TRAP220 expression vector (Fig. 4B). Transfection of GAL-NOR-1-FL efficiently induced transcription relative to the GAL4 DBD, this level of activity was stimulated by the addition of SRC-2 by ~3-fold (Fig. 4B) as we had previously shown (Wansa et al. 2003). Furthermore, we observed that NOR-1 activity was also stimulated in the presence of TRAP220 by ~3 fold, this suggested that NOR-1-mediated transcriptional activation is coactivated by TRAP220.

Subsequently, we examined whether TRAP220 potentiates NOR-1-mediated activation of a native NR4A response element. We utilized a heterologous reporter gene that contains native NOR-1 response elements i.e. the NurRE-5 POMC-tk-LUC reporter contains five copies of the naturally occurring NR4A response element (TGATATTACCTCCAAATGCCA) from the pro-opiomelanocortin (POMC) gene, (Fig. 4C). This native response element is responsive to physiological stimuli, binds NR4A dimers and efficaciously responds to NR4A mediated transactivation (Maira et al. 1999). C2C12 muscle cells were cotransfected with the reporter plasmid NurRE-5 POMC-tk-LUC and the NOR-1 expression vector. We observed efficient NOR-1-dependent transactivation of the NurRE-5 POMC reporter by ~60-fold (Fig. 4D) as previously reported. Moreover, increasing concentrations of TRAP220 expression plasmid potentiated NOR-1-mediated activation of the native response element by ~4–9-fold, consistent with the GAL4 hybrid assay (Fig. 4A). Higher concentrations of TRAP220 resulted in squelching of NOR-1-mediated transcription (data not shown).

In addition, 6-MP induced NOR-1-mediated transactivation of the NurRE-POMC reporter by >5-fold, as previously reported (Wansa et al. 2003). Interestingly, TRAP220 expression did not further potentiate the induction of NOR-mediated transactivation by 6-MP, however, the absolute level of NOR-1 mediated reporter gene activation was certainly increased in the presence of TRAP220 and 6-MP (Fig. 4D). These experiments indicate NOR-1-mediated transcriptional activation is potentiated by TRAP220.

NOR-1 and Nur77 interact with TRAP220 in an AF-1-dependent manner in a cellular context: amino acids 1–800 of TRAP220 are necessary for nuclear receptor recruitment

Our previous studies demonstrated that NOR-1 could recruit TRAP220 in vitro. To investigate whether NOR-1 and Nur77 could interact with TRAP220 in a cellular context in vivo we utilised the mammalian two-hybrid assay. In these experiments the yeast GAL4 DBD domain is fused to TRAP220 and expressed in transfected JEG-3 cells with NOR-1 or Nur77 linked to the transactivation domain (AD) of the herpes simplex virus, VP16. Transactivation of a GAL4-dependent reporter gene (G5E1 bLUC) is only achieved when the coexpressed proteins interact physically (Fig. 5A).

JEG-3 cells (and not the C2C12 muscle cells) were utilized for the mammalian two-hybrid assays, because the activity of NOR-1, Nur77 and TRAP220 is high in C2C12, COS-1 and 293 cells (data not shown) (Castro et al. 1999, Wansa et al. 2002, 2003). Furthermore, Castro et al. (1999) reported that the NR4A activity in this cell line is very weak, consequently background activity is low, thus enabling in vivo interactions to be observed (and increasing the sensitivity of the assay). This feature of JEG-3 cells was previously exploited in the mammalian two-hybrid assays involving this subgroup of receptors (Perlmann & Jansson 1995).

We observed that coexpression of VP16-NOR-1 and VP16-Nur77 effectively and significantly trans-activated GAL-TRAP220 by ~5- and 15-fold, respectively (Fig. 5B), in concordance with our GST-pulldown studies (Wansa et al. 2002, 2003).

To determine the regions of NOR-1 and Nur77 that mediated the in vivo interactions with TRAP220, we
performed mammalian two-hybrid experiments in which the N and C-terminal regions of NOR-1 and Nur77 (for example, NOR-1-AB, NOR-1-DE, Nur77-AB or Nur77-DE) were linked to the transactivation domain of the herpes simplex virus, VP16. We observed that VP16-NOR-1-AB and VP16-Nur77-AB effectively and significantly transactivated GAL-TRAP220 activity by ~5-fold. In contrast, the C-terminal DE regions of these receptors, in an atypical manner, failed to complement the activity of TRAP220 (Figs 5C and 5D). This is consistent with the AF-1-dependent nature of NOR-1 activity.

However, we did not observe increased NOR-1-mediated recruitment of TRAP220 in the presence of 6-MP (Fig. 6A). To further determine whether 6-MP could potentiate TRAP220-induced NOR-1-mediated transcription we utilized and further exploited the GAL4 hybrid assay (Fig. 6B). As demonstrated in Figure 4A, NOR-1 activity was stimulated in the presence of TRAP220, in this reverse assay TRAP220 activity was increased by NOR-1 co-expression by ~3-fold (Fig. 6C). As observed initially, TRAP220 activity was stimulated by 6-MP (Figs 1B and 6C), however NOR-1 expression did not increase the inducibility of TRAP220 activity by 6-MP. Although the absolute level of activation (relative to the GAL4 DBD) was clearly increased.

We progressed to further utilize this assay to delimit the region of TRAP220 that mediates interaction with NOR-1 and Nur77 employing the mammalian two-hybrid assay. Interestingly, the region between amino acids 1–800 of TRAP220, which mediates transcriptional activation by 6-MP, is also necessary to support the cellular interaction with NOR-1 and Nur77. We observed that VP16-NOR-1-FL and VP16-Nur77-FL significantly transactivated GAL-TRAP220-aa1–800 activity by ~3-fold and ~11-fold, respectively (Fig. 7). The regions of TRAP220 that were previously shown to not be modulated by 6-MP (Fig. 2B) did not interact with NOR-1 and Nur77, thus demonstrating the importance of the region between amino acids 1–800 of

Figure 4 TRAP220 potentiates the activity of NOR-1 (A) A diagrammatic representation of the GAL4 hybrid assay is shown. This assay was used to determine the effect of SRC-2/GRIP-1-TRAP220 expression on NOR-1-mediated transactivation of gene expression. pCMV-GAL-NOR-1-FL (0.16 µg) was co-transfected with the reporter plasmid G5E1b-LUC (1 µg) into C2C12 proliferating myoblasts in the presence and absence of the co-transfected expression plasmid encoding pcDNA-SRC-2 or pcDNA-TRAP220 (2 µg per 3 wells). Fold activation is expressed relative to luciferase activity obtained after cotransfection of the pCMV-GAL4 DBD alone arbitrarily set at 1. The mean luciferase fold activation and s.d. values (bars) were derived from a minimum of 2 independent triplicate experiments. (C) A diagrammatic representation of the reporter gene assay. (D) SGS-NOR-1-FL (0.16 µg) pcDNA-TRAP220 (0.1, 0.3 and 1 µg per 3 wells) were co-transfected into C2C12 proliferating myoblasts together with the reporter gene NurRE-5-tk-Luc in the presence and absence of 100 µM 6-MP. Fold activation is expressed relative to luciferase activity obtained after cotransfection of pSG5 alone, arbitrarily set at 1. The mean luciferase fold activation values and standard deviations (bars) were derived from a minimum of 2–3 independent triplicate experiments.

Journal of Molecular Endocrinology (2005) 34, 835–848
TRAP220 for modulation by 6-MP, as well as mediating interactions with NOR-1 and Nur77 (Fig. 7).

These mammalian two-hybrid experiments clearly demonstrate that NOR-1 and Nur77 significantly interact with TRAP220 in a cellular context in an AF-1/AB region-dependent manner. This is concordant with the in vitro GST-pulldown assays that showed that the AB regions of NOR-1 and Nur77 mediate coactivator recruitment, and that the LBD interacts poorly with coactivators (Wansa et al. 2002, 2003). Moreover, we demonstrated that a TRAP220 deletion mutant containing only the NR-interacting regions (LXXLL motifs) did not interact with NOR-1 and Nur77. This is in contrast to other NRs, where coactivators interact with NRs namely through key interactions with residues in the LBD domain that form part of a charged clamp that accommodates the coactivator LXXLL α-helix motifs within a hydrophobic cleft in the LBD, conversely NOR-1 and Nur77 were shown to have unusually hydrophilic coactivator binding interfaces, thus accounting for the inability of NOR-1 and Nur77 LBDs to efficiently recruit TRAP220 (data not shown).

Discussion

In this study we have provided evidence that the purine anti-metabolite 6-MP, which has anti-proliferative, anti-cancer and anti-inflammatory properties, stimulated the activity of the coactivator TRAP220. The 6-MP-mediated activation of NOR-1 and TRAP220 is observed at an EC50 of 33–50µM, and increases in a dose-dependent manner. We could not obtain saturating concentrations of 6-MP on NOR-1 and TRAP220 activation because at 6-MP concentrations of >100µM we observed a dose-dependent increase of >2-fold in the activity of the basal GAL4 DBD. This is consistent with the observations from Ordentlich and colleagues (Ordentlich et al. 2003). The range of 6-MP concentrations required for inhibition of purine biosynthesis varies greatly from cell to cell, with sensitive cell lines...
such as human lymphoblastic Molt F4 requiring 0.5–10µM and some cell lines being resistant at concentrations above 100µM (Ordentlich et al. 2003). Therefore, most biological effects of 6-MP observed to date occur in the micro molar range, and activation of NOR-1 and TRAP220 is concordant with the clinical utilization of this drug.

Transcriptional activation of NRs involves the stepwise recruitment and binding of coactivators to target gene promoters (McKenna & O’Malley 2002b). Initial recruitment of SRCs, p300/CBP, PCAF and CARM1 by ligand-activated NRs leads to subsequent

Figure 6 6-MP does not potentiate stimulation by TRAP220 in NOR-1-mediated transcription. GAL-TRAP220-aa1–1000, GAL-TRAP220-aa1–800 (0.33µg), VP16-NOR-1-FL and VP16-Nur77-FL (0.33µg) were cotransfected with the reporter plasmid G5E1b-LUC (1µg) in JEG-3 cells. Fold activation is expressed relative to luciferase activity obtained after cotransfection of the SV40-GAL4 DBD and the SV40-VP16AD plasmids alone in the absence of 6-MP, arbitrarily set at 1. (B) A diagrammatic representation of the GAL4 hybrid assay is shown (C) GAL-TRAP220-aa1–1000 (0.16µg) and SG5-NOR-1-FL (2µg per 3 wells) were co-transfected into C2C12 proliferating myoblasts together with the reporter gene G5E1b-LUC (1µg) in the presence and absence of 50µM of 6-MP. Fold activation is expressed relative to luciferase activity obtained after cotransfection of the GAL4 DBD and SG5 alone in the absence of 6-MP, arbitrarily set at 1. The mean luciferase fold activation and s.d. values (bars) were derived from a minimum of 2–3 independent triplicate experiments.
transgenic mice (Kuang et al. 1999). TRAP220 has also been shown to regulate apoptosis through direct regulation of the tumour suppressor p53 (Frade et al. 2002) Interestingly, 6-MP targets the AB region of the NR4A1–3 subfamily and regulates the receptors’ activity, furthermore TRAP220 interacts with the AB region of NOR-1 and Nur77 and stimulates NOR-1-mediated transcription. Whereas many of the steps involved in 6-MP-mediated cytotoxicity have been elucidated, it is possible that these effects can be enhanced or suppressed by transcriptional regulation of the NR4A1–3 subfamily.

6-MP is metabolized into the active derivative, 6-thioinosine mono-phosphate (6-TIMP), by hypoxanthine-guanine phosphoribosyltransferase (HG-PRT), a key enzyme involved in the purine salvage pathway. The 6-thio-IMP can be metabolized into 6-thioguanosine 5’ monophosphate, which is then incorporated into DNA and RNA as 6-thio-guanosine triphosphate (6-thio-GTP, 6-TGN) resulting in eventual cytotoxicity and cell death (Cara et al. 2004). Incorporation of 6-TGNs into nucleic acids is considered the main mechanism of 6-MP cytotoxicity. 6-TIMP can be methylated by thiopurine methyltransferase (TPMT) to methyl thiopurine monophosphate (methyl-TIMP), a potent inhibitor of de novo purine biosynthesis which depletes the intracellular ATP pools (Cara et al. 2004).

Most relevant to the observation presented in this study is the finding that 6-methyl-mercaptopurine riboside and 6-TGN are specific and effective inhibitors of protein kinase N, which has been implicated in nerve growth factor signalling (Volonte & Greene 1992, Figure 7).
Volonte et al. 1993). 6-methyl-mecaptopurine riboside can also regulate tyrosine kinase signalling in angiogenesis (Presta et al. 1999). Furthermore, it has been shown that azathioprine and its metabolite 6-MP induced apoptosis of T cells in patients with Crohn’s disease and control patients. Apoptosis induction required costimulation with CD28 and was mediated by a specific blockade of Rac1 activation through binding 6-TGN to Rac1 instead of GTP. The activation of Rac1 target genes such as mitogen-activated protein kinase/extracellular signal regulated kinase (MEK), NF-kappaB and bcl-x(L) was suppressed by azathioprine, leading to a mitochondrial pathway of apoptosis (Tiede et al. 2003).

The precedent set by these findings suggests that 6-MP may also regulate as yet unidentified activities in the cell that may specifically influence TRAP220 activity. Indeed, stimulation of CBP transcriptional coactivation by MAPK (Janknecht & Nordheim 1996, Liu et al. 1998), of p300-mediated transcription by mitogen-activated/extracellular response kinase kinase (MEKK1) (See et al. 2001, Gusterson et al. 2002), and SRC-1 function by ERK1 and ERK2 (Rowan et al. 2000) points to phosphorylation as a positive regulatory modification in coactivator activity. Furthermore, Ca^{2+}/Calmodulin-dependent protein kinase IV (CaM-KIV) enhances Nur77 transcription in cotransfections in synergy with the CaMKIV responsive coactivator activating signal cointegrator-2 (ASC-2) (Sohn et al. 2001). Interestingly, TRAP220 contains PKC, PKA and MAPK sites that were shown to be phosphorylated by these kinases. The effect of MAPK activation on TRAP220 mediated PPARγ transcription suggests that this kinase signalling pathway positively influences TRAP220 function (Misra et al. 2002). Interestingly, the region of TRAP220 (aa1–800) that mediates transactivation in response to 6-MP contains PKC and PKA phosphorylation sites, however mutation of these sites did not compromise the ability of 6-MP to activate TRAP220.

Apoptosis represents an effective way to eliminate cancerous cells and a variety of evidence suggests both the NR4A1–3 subfamily of nuclear receptors and TRAP220 to be implicated in cancer and apoptosis (Li et al. 1997, Zhu et al. 1999, Kang et al. 2000, Li et al. 2000, Ohkubo et al. 2000, Frade et al. 2002, Liu et al. 2002, Wu et al. 2002, Zhang 2002, Wada et al. 2004). Therefore, we speculate that there exists a cell signalling cascade that is sensitive to either levels of purine nucleotides or to regulation by 6-MP and that this pathway induces NR4A1–3 modulation. We suggest that 6-MP could act in concert with cellular signal transduction pathways to modulate NR4A-mediated transcription. In this regard, TRAP220 could serve to integrate the signalling cascade with NR4A-mediated transcription in response to 6-MP. Characterization of these signalling cascades has utility in the design of pharmacological tools for the selective therapeutic regulation of the NR4A subgroup in treatment of Parkinson’s disease, atherogenesis, Alzheimer’s disease, rheumatoid arthritis and cancer.

References

Acevedo ML & Kraus WL 2003 Mediator and p300/CREB-steroid receptor coactivator complexes have distinct roles, but function synergistically, during estrogen receptor alpha-dependent transcription with chromatin templates. *Molecular and Cellular Biology* 23 335–348.


Chen SL, Dowhan DH, Hosking BM & Muscat GE 1998 The steroid receptor coactivator, GRIP-1, is necessary for MEF2C-dependent gene expression and skeletal muscle differentiation. *Genes and Development* 12 1209–1228.


Maira M, Martens C, Philips A & Drouin J 1999 Heterodimerization between members of the Nur subfamily of orphan nuclear receptors as a novel mechanism for gene activation Molecular and Cellular Biology 19 7549—7557.
Maltais A, Filion G & Labelle Y 2002 The AF2 domain of the orphan nuclear receptor TEC is essential for the transcriptional activity of the oncogenic fusion protein EWS/TEC. Cancer Letters 183 87–94.
Schimmel JJ, Crews L, Roffler-Tarlov S & Chikaraisi DM 1999 4.5 kb of the rat tyrosine hydroxylase 5’ flanking sequence directs tissue specific expression during development and contains
consensus sites for multiple transcription factors. *Brain Research and Molecular Brain Research* 74:1–14.


Wansa KD, Harris JM, Yan G, Ordentlich P & Muscat GE 2003 The AF-1 domain of the orphan nuclear receptor NOR-1 mediates trans-activation, coactivator recruitment, and activation by the purine anti-metabolite 6-mercaptopurine. *Journal of Biological Chemistry* 278:24776–24790.


Received in final form 27 December 2004
Accepted 19 January 2005