Pharmacological Profile of the Thyroid Hormone Receptor Antagonist NH3 in Rats

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ABSTRACT

NH3 is a thyroid hormone receptor (TR) antagonist that inhibits binding of thyroid hormones to their receptor and that inhibits cofactor recruitment. It was active in a tadpole tail resorption assay, with partial agonist activity at high concentrations. We determined the effect of NH3 on the cholesterol-lowering, thyroid stimulating hormone (TSH)-lowering, and tachycardic action of thyroid hormone (T3) in rats. Cholesterol-fed, euthyroid rats were treated for 7 days with NH3, and a dose response (46.2–27,700 nmol/kg/day) was determined. We also determined the effect of two doses of T3 on the NH3 dose-response curve. NH3 decreased heart rate modestly starting at 46.2 nmol/kg/day, but the effect was lost at >2920 nmol/kg/day. At 27,700 nmol/kg/day, tachycardia was seen, suggesting partial agonist activity. NH3 increased plasma cholesterol to a maximum of 27% at 462 nmol/kg/day. At higher doses, cholesterol was reduced, suggesting partial agonist activity. Plasma TSH was increased from 46.2 to 462 nmol/kg/day NH3, but at higher doses, this effect was lost, and partial agonist effects were apparent. T3 at 15.4 and 46.2 nmol/kg/day increased heart rate, reduced cholesterol, and reduced plasma TSH. NH3 inhibited the cholesterol-lowering, TSH-lowering and tachycardic effects of 15.4 nmol/kg/day T3, but much of the effect was lost at >924 nmol/kg/day doses. NH3 had no effect on the cholesterol-lowering action of 46.2 nmol/kg/day T3, but it inhibited the tachycardic and TSH suppressant effects up to the 924 nmol/kg/day dose. Single doses of 462 and 27,700 nmol/kg caused no TR inhibitory effects. In conclusion, NH3 has TR antagonist properties on T3-responsive parameters, but it has partial agonist properties at higher doses.

Resolution of the three-dimensional structure of TR ligand binding domain has allowed the development of detailed structure-activity relationships for TR agonists and antagonists (Green et al., 1988; Renaud et al., 1995; Ribeiro et al., 1998; Yen, 2001; Dow et al., 2003; Hangeland et al., 2004). TRs have two known subtypes, TRα and TRβ, that are generated from different genes (Forrest and Vennström, 2000; Yen, 2001), with TRα regulating heart rate and most of the metabolic rate effects of T3, and with TRβ mediating cholesterol and TSH suppression (Johansson et al., 1998; Wikström et al., 1998; Grover et al., 2003). Recent studies show that development of TR subtype-selective agonists is possible, and these are useful tools for dissecting TR function (Chiellini et al., 1998; Trost et al., 2000; Scanlan et al., 2001; Dow et al., 2003). Structure-activity relationships have also been generated with the purpose of developing TR antagonists, although few are useful in vivo (Carlsson et al., 2002; Malm, 2004a).

Development of TR antagonists is of interest, but few exist without having a plethora of other activities. Such is the case for amiodarone and its analogs. Amiodarone is an iodinated benzofuran that is a class III antiarrhythmic agent. It is though few are useful in vivo (Carlsson et al., 2002; Malm, 2004a). Development of TR antagonists is of interest, but few exist without having a plethora of other activities. Such is the case for amiodarone and its analogs. Amiodarone is an iodinated benzofuran that is a class III antiarrhythmic agent. It is

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The hypothesis was based on the understanding that modification of a nuclear hormone ligand can be predicted by the placement of molecular extensions that disrupt folding of the carboxyl-terminal helix 12, preventing coactivator recruitment (Arnold et al., 2005; Nguyen et al., 2005). Therefore, NH3 competitively binds to TR, but it also inhibits cofactor recruiting, explaining its antagonist activity. Although it showed full antagonist activity in vitro, NH3 showed partial agonist activity at high concentrations in the tadpole tail resorption assay that was not predicted by cell-based assays of transactivation. The possibility that the complete chromatin assembly on the transfected plasmids, as would be seen in vivo, was not incorporated into the assay was hypothesized in addition to the hypothesis that there was the generation of an active agonist metabolite from the parent compound. This compound was synthesized previously, and it was found to be a partial agonist at TRs in vitro (Nguyen et al., 2002). Therefore, these data still leave open the question of the activity of NH3 in mammalian systems in vivo. Little is known about the pharmacokinetics of NH3, and it is unknown whether this compound will be an effective blocker when given orally to mammals.

The goal of this study was to determine the TR antagonist profile of NH3 in rats using the well-characterized effects of thyroid hormones on cholesterol, heart rate, and TSH suppression. We chose these parameters because cholesterol is a thyroid hormone-dependent parameter (Grover et al., 2003). The rats were maintained on this diet for 2 weeks after which the drug treatments were begun as described above. Animals were treated with either 15.4 or 46.2 nmol/kg/day T3 alone or in combination with 46.2 to 2920 nmol/kg/day NH3 via oral gavage for 7 days with n = 6 per group. Therefore, three families of curves were generated for each parameter with a dose response to NH3 with 0, 15.4, or 46.2 nmol/kg/day T3. The vehicle group used for this portion was the same group used for the first part of the study, although T3 was not combined with the 27,700 nmol/kg/day dose of NH3. Once again, on the seventh day, the animals were given their last dose or doses. Two hours later, the animals were anesthetized with 30 mg/kg pentobarbital i.p., and then the heart rate was assessed using the lead II ECG. Blood was collected as described above, and serum cholesterol and TSH were determined. It should be noted that for proper comparisons, we use molar doses. The doses chosen are benchmarked to the 1 μg/kg/day T3 dose, which is 1.54 nmol/kg/day.

Materials and Methods

Male euthyroid Sprague-Dawley rats (250–300 g) were used for all of the studies, and they were fed and watered ad libitum. This study was approved by the institutional animal care and use committee of Eurofins Scientific-Product Safety Laboratories (Dayton, NJ). The rats were switched from their normal chow to that containing 1.5% cholesterol and 0.5% cholic acid to increase low-density lipoprotein cholesterol to >90% of the total plasma cholesterol. This was necessary because rats transport cholesterol predominantly in the high-density lipoprotein form, and cholesterol feeding of rats is a well characterized and accepted protocol to enhance this TRβ-sensitive parameter (Grover et al., 2003). The rats were kept on this diet for 2 weeks after which the studies were begun. The rats were maintained on the high-cholesterol diet throughout the study. For drug treatments, the animals were given either NH3 or T3 for 7 days to achieve a steady-state response because each parameter (cholesterol, heart rate, and TSH) responds with a different time course. Therefore, 7 days was allowed so that all of these parameters had achieved steady state based on historical knowledge of this model (Grover et al., 2003). Heart rate is the slowest to respond to thyroid hormone modulators, often taking 3 to 5 days to see effects.

NH3 Studies. After 2 weeks of cholesterol feeding, the rats were treated via oral gavage with vehicle (10% m-pyrol, 5% ethanol, 5% cremaphor (Sigma Chemical Co., St. Louis, MO), and 80% water) or NH3 at doses of 46.2, 154, 462, 924, 2920 or 27,700 nmol/kg/day daily for 7 days (n = 6/group). The highest dose was added at the end of the study to show whether NH3 had partial agonist activity. We have historically found that 7 days of treatment is ideal for alteration of the thyroid hormone-dependent parameters of interest in this study, namely, heart rate, cholesterol levels, and TSH levels (Grover et al., 2003). On the 7th day, the animals were given their last dose, and 1 h after this last dosing, the animals were anesthetized with 30 mg/kg pentobarbital i.p., and the heart rate was determined using the lead II ECG. The animals were then bled through the vena cava, and blood was collected and serum was obtained. The serum cholesterol (enzymatic assay; Hitachi 747100; IDEXX, Inc., North Grafton, MA) and TSH values (radioimmunoassay; IDEXX, Inc.) were then determined as described previously (Grover et al., 2003).

Effect of T3 on NH3 Dose-Response Curves. The next series of studies were designed to determine the effect of two doses of T3 on the dose-response curve of NH3. Rats were cholesterol-fed for 2 weeks after which the drug treatments were begun as described above. Animals were treated with either 15.4 or 46.2 nmol/kg/day T3 alone or in combination with 46.2 to 2920 nmol/kg/day NH3 via oral gavage for 7 days with n = 6 per group. Therefore, three families of curves were generated for each parameter with a dose response to NH3 with 0, 15.4, or 46.2 nmol/kg/day T3. The vehicle group used for this portion was the same group used for the first part of the study, although T3 was not combined with the 27,700 nmol/kg/day dose of NH3. Once again, on the seventh day, the animals were given their last dose or doses. Two hours later, the animals were anesthetized with 30 mg/kg pentobarbital i.p., and then the heart rate was assessed using the lead II ECG. Blood was collected as described above, and serum cholesterol and TSH were determined. It should be noted that for proper comparisons, we use molar doses. The doses chosen are benchmarked to the 1 μg/kg/day T3 dose, which is 1.54 nmol/kg.

Single Dose NH3 Studies. Another study was performed to determine whether a single medium dose (462 nmol/kg) or high dose (27,700 nmol/kg) could produce TR blocking or partial agonist effects. Male Sprague-Dawley rats (250–300 g) were cholesterol-fed as described above for 2 weeks. A serum sample was withdrawn via the retoorbital route before drug and 2 and 24 h after the low or high dose. Serum TSH and cholesterol levels were determined from these serum samples. ECG analysis showed no effect on heart rate with this single dose, which is not surprising.

Statistical Analysis. Statistical differences between groups were determined using factorial ANOVA and Newman-Keuls post hoc test. All data are presented as the mean ± S.E.M.

Results

Studies with 7 Days of Dosing of NH3. A dose response to NH3 was determined to assess TR blocking activity in euthyroid rats. In vehicle-treated animals, all measured values (heart rate, TSH, and cholesterol) were within the expected range as shown in Table 1. The serum cholesterol values were high as would be expected with high cholesterol feeding (Grover et al., 2003). Baseline values for all of the NH3 groups were similar to vehicle group values. The NH3 dose-response data are shown in Figs. 2 to 4, and they are shown as the percentage of change from vehicle-treated group values. Cholesterol was significantly increased by the 154 to 924 nmol/kg/day NH3 doses compared with vehicle-treated control animals showing TR inhibitory activity (Fig.
2). At >924 nmol/kg/day NH3, plasma cholesterol was significantly reduced, suggesting agonist activity. Heart rate was reduced up to the 924 nmol/kg/day dose, showing TR blockade. This effect was lost at 2920 nmol/kg/day and at 27,700 nmol/kg/day NH3; significant tachycardia was noted, once again suggesting partial agonist activity (Fig. 3). Significant bradycardia was first noted at the 462 nmol/kg/day dose of NH3. The tachycardia noted at the highest dose was statistically different compared with vehicle-treated animals, although this was a relatively modest effect. TSH was increased by NH3 between 46.2 and 924 nmol/kg/day, but this effect was lost at higher doses, and at the highest dose (27,700 nmol/kg/day), significant TSH suppression was observed, showing agonist activity (Fig. 4).

**Effect of T3 on the Dose-Response Curves for NH3.** As expected, T3 at 15.4 and 46.2 nmol/kg/day alone significantly reduced cholesterol, reduced TSH, and increased heart rate (Table 1). The reduction of cholesterol and TSH seen for these two doses of T3 was close to being maximally reduced based on previous studies with this model (Grover et al., 2003). NH3 reduced the cholesterol-suppressive effect of 15.4 nmol/kg/day T3, although much of this effect was lost at >924 nmol/kg/day doses of NH3 (Fig. 5). The maximal blocking effect seen was for the lowest dose of NH3 (46.2 nmol/kg/day). The TR blocking effect of NH3 on cholesterol suppression was completely surmounted by 46.2 nmol/kg/day T3. T3 shifted the NH3 dose-response curve for cholesterol down and to the right in a dose-dependent manner. Virtually all of the cholesterol data points for both doses of T3 in combination with NH3 were significantly lower compared with the data for NH3 alone.

The tachycardic effect of T3 was inhibited by NH3, although this effect was lost at the 2920 nmol/kg/day dose (Fig. 6). The blocking effect of NH3 was reduced at the higher dose of T3, although unlike cholesterol, heart rate did not return completely to the values for T3 alone. Therefore, the TR blocking effect of NH3 on cholesterol uptake is more readily surmountable by T3 than it was for heart rate. T3 shifted the NH3 dose-response curve upward and to the left, although the effect was not clearly dose-dependent. Virtually all of the heart rate data points for both doses of T3 in combination with NH3 were significantly higher compared with the data for NH3 alone.

The TSH suppressive effect of T3 was inhibited by NH3, with maximal inhibition occurring at 462 nmol/kg/day NH3 (Fig. 7). The TSH suppressive effect of T3 was lost at the 2920 nmol/kg/day dose of NH3. T3 shifted the NH3 dose-response curve for TSH downward, and this was not dose-dependent, at least for the two doses of T3 used in this study. Both doses

**Table 1**

Values for plasma cholesterol, plasma TSH, and heart rate after 7 days of T3 treatment in cholesterol-fed rats

<table>
<thead>
<tr>
<th></th>
<th>Plasma Cholesterol</th>
<th>Heart Rate</th>
<th>Plasma TSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>258 ± 23</td>
<td>368 ± 15</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>15.4 nmol/kg/day T3</td>
<td>94 ± 4*</td>
<td>411 ± 12*</td>
<td>2.3 ± 0.2*</td>
</tr>
<tr>
<td>46.2 nmol/kg/day T3</td>
<td>56 ± 6*</td>
<td>425 ± 10*</td>
<td>2.1 ± 0.2*</td>
</tr>
</tbody>
</table>

* p < 0.05, significance from its respective vehicle-treated group value.
of T₃ significantly reduced TSH compared with NH₃ when given alone. The doses of T₃ given seemed to be maximally TSH-suppressing doses.

**Single Dose Studies with NH₃.** We determined whether NH₃ exerted TR blocking effects after a single dose on TSH and cholesterol levels. As shown in Table 2, NH₃ had no significant effect on TSH at 2 or 24 h after dosing at the low and high dose. Both the high and low doses significantly reduced serum cholesterol at 24 h after single dosing suggesting TR agonist activity. These data argue in favor of the metabolite theory for partial agonist activity, because no TSH increase was seen and increased release of endogenous thyroid hormones seems unlikely. The fact that the liver (increased hepatic low-density lipoprotein receptor) effects were seen so quickly suggests a liver metabolite that is rapidly formed, although this is speculative.

**Discussion**

There has been an increased interest in development of new TR modulators (Malm, 2004a,b). Selective TRβ agonists such as GC-1 and KB-141 represent new tools for dissecting the various functions of TR subtypes as well as the function of these receptors in disease states (Chiellini et al., 1998; Grover et al., 2003, 2004; Webb, 2004). Exciting potential for such selective agonists for treatment of metabolic syndrome also suggests the possibility of clinical as well as research utility (Grover et al., 2003; Malm, 2004b). Development of TR modulators is somewhat more challenging than developing agents that interact with cell surface receptors since TRs work by activating or repressing gene expression; binding assays for TR modulators are only the beginning of understanding how (or if) TR modulators function and even cell-based assays may not always predict activity in vivo.

Development of antagonists has been more difficult than agonists, although many TR antagonists have been reported (Carlsson et al., 2002; Lim et al., 2002). Unfortunately little work has been done in vivo to confirm this activity. Unpublished data from our laboratory showed that several of the antagonists reported in the literature in vitro are not active as antagonists in vivo and that most act as TR agonists. This seems likely to be due, in some cases, to rapid in vivo drug metabolism to agonist metabolites or to the inability of cell-based screening methods to accurately predict activity in vivo. TRs act as transcription factors by binding to thyroid hormone response elements, usually in combination with retinoid X receptors, allowing for multiple activities in many tissues (Yen, 2001). This activity is further modified by the activity of coactivators or corepressors, and the complexity of their interactions is partially what makes prediction of the activity of TR modulators in vivo difficult (Harvey and Williams, 2002; Moore et al., 2004). Nevertheless, development

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**TABLE 2**

Effect of 1 day of treatment of NH₃ on serum cholesterol and TSH in cholesterol-fed rats

<table>
<thead>
<tr>
<th>Dose of NH₃</th>
<th>TSH Predrug</th>
<th>2 h</th>
<th>24 h</th>
<th>Cholesterol Predrug</th>
<th>2 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>462 nmol/kg NH₃</td>
<td>4.8 ± 1.0</td>
<td>4.0 ± 0.8</td>
<td>3.6 ± 0.3</td>
<td>316 ± 18</td>
<td>312 ± 20</td>
<td>259 ± 11*</td>
</tr>
<tr>
<td>27,700 nmol/kg NH₃</td>
<td>4.8 ± 0.9</td>
<td>4.8 ± 0.9</td>
<td>4.8 ± 0.9</td>
<td>337 ± 25</td>
<td>319 ± 14</td>
<td>253 ± 9*</td>
</tr>
</tbody>
</table>

*p < 0.05, compared with its respective paired predrug value.
of TR antagonists with in vivo activity would be welcome not only as research tools but also as potential antiarrhythmic agents. Development of TRα1-selective antagonists might be of particular interest in this regard.

NH3 is a TR inhibitor (Lim et al., 2002; Nguyen et al., 2002) in vitro and in vivo in amphibians, although some partial agonist properties were seen at higher doses. In rats, NH3 did show TR inhibitory activity, particularly at the low and intermediate doses used. We also observed an apparent partial agonist activity for NH3 for cholesterol, heart rate, and TSH at high doses. These three parameters are directly modulated by TRα1 with cholesterol- and TSH lowering being TRβ1- and TRβ2-mediated, respectively, and tachycardia being TRα1-mediated effects of TR activation (Johansson et al., 1998; Grover et al., 2003). NH3 is not TR subtype-selective in vitro, and it does not seem to be selective in rats because both TRα1- and TRβ3-mediated parameters were inhibited in the present study.

The blocking effect of NH3 alone showed a profile of blockade that increased up to the 924 nmol/kg/day doses and a loss of these effects at higher concentrations. Indeed, when the dose was pushed up to 27,700 nmol/kg/day, NH3 behaved as an agonist with TSH suppression, cholesterol reduction, and tachycardia being observed. Interestingly, the “crossover” points from an antagonist dose to agonist dose were remarkably similar for all three of the parameters measured, and this occurred at or above the 924 nmol/kg/day dose of NH3. This is especially interesting despite the differing time course for the onset of action of thyroid hormone modulators on TSH (minutes), cholesterol (hours), and heart rate (days).

At the present time, we do not know whether there is true partial agonist activity or whether a metabolite with agonist properties is being generated. If the proposed nitro reduction metabolism to the aniline metabolite does occur, then the dose-dependent exposure to the aniline metabolite could be the basis for the observed partial agonism at high NH3 doses. It is also possible that increasing TSH might also increase circulating thyroid hormones, and at higher concentrations, the higher levels of T4 and T3 could surmount the NH3 antagonist effects. Although we cannot completely rule this out, the degree of TSH increase seemed to “plateau” between 154 and 924 nmol/kg/day NH3, and we feel that this represents a maximal blocking effect, and the loss of apparent blocking efficacy due to enhanced production of TSH seems to be doubtful. Under the conditions (high doses) where NH3 produced agonist effects, TSH was also reduced so it is difficult to see how TSH could have caused increased endogenous thyroid hormone production, although we did not measure T4 or T3 levels in these studies.

The TR blocking effects of NH3 were obviated by T33 and this seemed to be dose-dependent for cholesterol and heart rate, but not for TSH. At 46.2 nmol/kg/day, T3 completely surmounted the TR blocking effect of NH3 on cholesterol. At the present time, we do not know why T3 is better able to surmount the blocking effect of NH3 on cholesterol, but we speculate that T3 readily penetrates the liver; therefore, the loss of blocking effects is more apparent (Grover et al., 2003). In previous studies, we showed that the cholesterol-lowering potency of T3 is higher than its potency for increasing heart rate or metabolic rate, and this parallels its significant accumulation in liver relative to other tissues (Grover et al., 2003). The cholesterol data are also interesting because partial agonist effects are seen at 924 nmol/kg/day NH3, but when combined with 15.4 nmol/kg/day T3, the effects were not additive. The degree of cholesterol reduction for T3 alone is approximately 60%, and it is approximately 25% with NH3 alone, but when combined, the percentage of reduction is around 45%. Because there is no TSH suppression with NH3 alone at the 924 nmol/kg/day dose, the cholesterol lowering is unlikely due to increased circulating endogenous thyroid hormones, but the perplexing question is why is there not an additive agonist effect? Does the presence of the NH3 affect the binding or interaction of T3 even when NH3 is in the “agonist” mode? This would imply these two compounds are interacting with TR in two different ways, at least with respect to cholesterol lowering.

The single dose studies showed that NH3 showed no TR blocking effects with just one dose. We chose a low dose that showed TR blocking effects with 7-day dosing and a high dose that showed TR agonist effects after 7 days of dosing. Although NH3 had no effect on TSH at either dose, it lowered cholesterol to equivalent levels at both doses, despite the great difference in the doses. Currently, we do not know the mechanism for this effect. A liver metabolite that has potent agonist properties may explain this activity, but this hypothesis remains to be proven. Certainly, TSH depression is not always necessary to see the agonist effects of NH3. It is clear that at least several days of treatment are necessary for TR antagonist effects to become apparent. It may simply take this long for the changes in gene regulation to become apparent, but at this point, we can only speculate. If the cholesterol reduction seen within 24 h for both doses is a “partial agonist” effect, this effect is rapidly apparent with single dosing, unlike the TR blocker effects. Any future studies using NH3 must take such complicated pharmacology into account.

The results of this study show that although NH3 does exert TR blocking activity in rats, the degree of blockade and loss of blockade are dose-dependent for some parameters and not dose-dependent for TSH. The TR blocking effect of NH3 is surmountable, although it is difficult in the present study to say more (i.e., competitive, noncompetitive, etc.). The use of this compound as a TR blocker tool for dissecting TR function must be used with caution, and the proper dose must be used and documented as a dose capable of blocking TR activation. Finally, the results of these studies further demonstrate that cell-based assays for TR-induced transactivation are not perfect predictors of in vivo activity, particularly for dose-dependent in vivo partial agonism.

References

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