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Article

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# Design and Synthesis of a Series of Melamine-based Nitroheterocycles with Activity against Trypanosomatid Parasites

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The parasites that give rise to human African trypanosomiasis (HAT) are auxotrophs for various nutrients from the human host, including purines. They have specialist nucleoside transporters to import these metabolites. In addition to uptake of purine nucleobases and purine nucleosides, one of these transporters, the P2 transporter, can carry melamine derivatives; these derivatives are not substrates for the corresponding mammalian transporters. In this paper, we report the coupling of the melamine moiety to selected nitro heterocycles with the aim of selectively delivering these compounds to the parasites. Some compounds prepared have similar in vitro trypanocidal activities as melarsoprol, the principal drug used against late-stage HAT, with 50% growth inhibitory concentrations in the submicromolar range. Selected compounds were also evaluated in vivo in rodent models infected with *Trypanosoma brucei brucei* and *T. brucei rhodesiense* and showed pronounced activity and in two cases were curative without overt signs of toxicity. Compounds were also tested against other trypanosomatid pathogens, *Leishmania donovani* and *Trypanosoma cruzi*, and significant activity in vitro was noted for *T. cruzi* against which various nitro heterocycles are already registered for use.

## Introduction

Parasitic trypanosomatids cause a number of important diseases, including human African trypanosomiasis (HAT), Chagas disease, and the leishmaniases. HAT is caused by the protozoan parasites *Trypanosoma brucei gambiense* and *T. brucei rhodesiense* and is endemic in sub-Saharan Africa, where it is a major health problem.[1] The current drugs used for the treatment of the infection are unsatisfactory, due to poor blood−brain barrier permeability, toxicity, and increasing problems due to resistance.[2,3] Chagas disease is caused by *Trypanosoma cruzi*, and no drugs are currently registered that are totally active against these parasites, particularly at the chronic stage of infection.[4] The leishmaniases represent a spectrum of diseases which are caused by several species of leishmania[5] and for which chemotherapy is also difficult.[6]

When present in the human host, the parasites require nutrients from the host. To achieve this, *T. brucei* encodes a number of transporters for uptake of essential nutrients, which are expressed in the clinically relevant bloodstream form of the parasite. One of the nutrient classes that the parasites sequester from the host are the purines. Carter and Fairlamb[7] first described an unusual purine transporter from *T. brucei* that has been designated the P2 transporter. This purine transporter shows significant differences to the corresponding mammalian transporters. The parasites also contain a more general purine uptake system, designated the P1 transporter, which in fact comprises the activities of a number of different transporters.[8] The normal substrates for the P2 transporter are adenosine and adenine. However, in addition to this, other motifs such as melamines and benzamidines (Figure 1) have been shown to be substrates for this transporter, which differs from the case of mammalian transporters.[2,7] Results of structure−activity relationship studies have indicated the following requirements for uptake through the P2 transporter: an amidine moiety, an aromatic ring, and an electronegative heteroatom. These features can be seen in substrates for the P2 transporter shown in Figure 1.[2,9] Melarsoprol and pentamidine have been shown to be substrates of the P2 transporter, and this is one of the mechanisms by which these trypanocidal agents are concentrated in the parasite.[3] Loss or mutation of the P2 transporter has been implicated in resistance to melarsoprol and also some diamidines in laboratory studies.[2,3,10] It is possible that the increased incidence of treatment failure in the field[11] is related to the emergence of drug resistance in this setting too.

Recently it has been shown that the situation is more complex than originally thought. Loss of the P2 transporter only causes a small (50%) decrease in susceptibility of parasites to melarsoprol, although this small decrease may be sufficient to explain the clinical resistance of this drug. The small decrease in susceptibility to melarsoprol of the parasite indicates that there are additional modes of uptake of melarsoprol into the parasite, and it has been shown that there are other transporters involved in uptake of melamine and ben-

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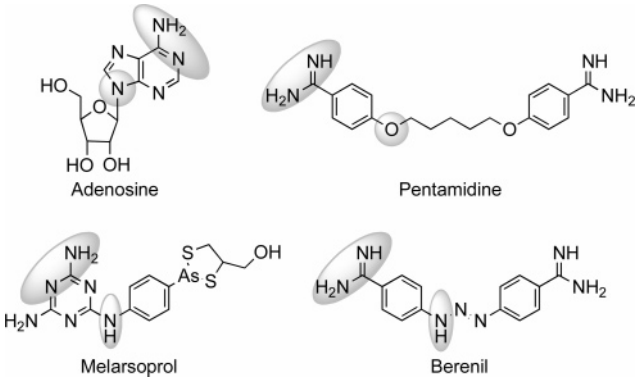



**Figure 1.** Substrates of the P2 transporter. The amidine and electronegative heteroatom are highlighted on each structure.

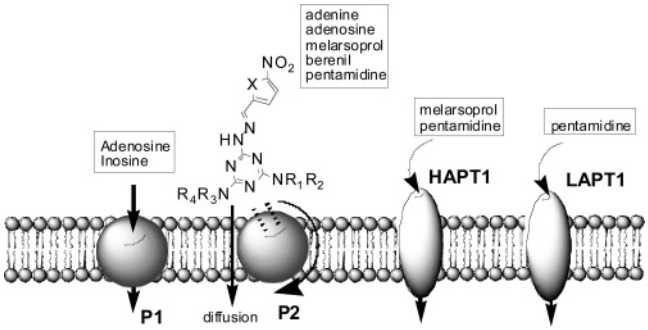



**Figure 2.** The known routes of uptake of diamines and melaminophenyl arsenicals into *T. brucei*. The P2 transporter is also represented interacting with a melamine-nitrofuran compound (**6a**), whose transport through the membrane is probably facilitated by the transporter and passive diffusion.

zamidine motifs into parasites (Figure 2).[9,12] In particular at least two other transporters have been identified for pentamidine uptake, designated "HAPT1" (high-affinity pentamidine transporter) and "LAPT1" (low-affinity pentamidine transporter). The transporter HAPT1 is possibly also responsible for uptake of melarsoprol. The normal physiological roles of LAPT1 and HAPT1 are certainly unknown. It is possible that other transporters are also involved in uptake of these motifs as well; for example, another purine nucleoside transporter, an *S*-adenosylmethionine transporter, has been reported.[13] However it is clear that these motifs are selectively taken up into parasites.

The presence of the P2 transporter with its particular substrate specificity presents an opportunity in the field of drug design. This could be achieved by attaching trypanocidal compounds to motifs that are substrates of the P2 transporter.[14,15] Then both the P2 recognition motif and trypanocidal agent should be selectively taken up into the parasite. To study this, we have investigated the coupling of the P2 recognition motif, melamine, to the polyamine analogues. Polyamine analogues are toxic to *T. brucei*.[16,17] We have reported the coupling of polyamine analogues to the melamine moiety and prepared compounds that showed potent trypanocidal activity and good selectivity on a cellular level.[14] However, the compounds turned out to be toxic in animal models. Therefore, we have selected an alternate trypanocidal moiety for attachment to the melamine and benzamidine delivery moiety, nitroaromatics. Nitroaromatic compounds have been shown to have potent activity against a variety of microbes. Of particular

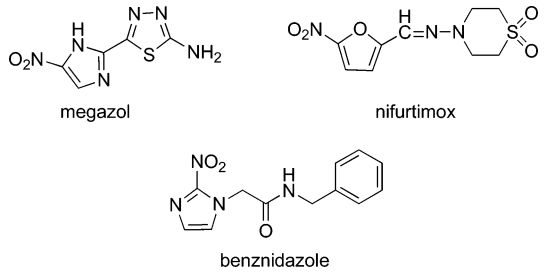
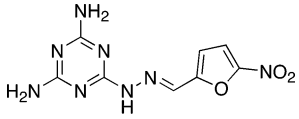



**Figure 3.** Structure of some known nitro heterocycles having activity against *T. brucei* and or *T. cruzi*.



**6a**

**Figure 4.** The structure of the lead compound.

relevance, nifurtimox and benznidazole (Figure 3) are nitro heterocycles used for treatment of Chagas disease, which is caused by the related organism *T. cruzi*. In addition, nifurtimox is being considered as a potential treatment for melarsoprol refractory African trypanosomiasis.[18,19] A key problem with nitroaromatics is that some, but not all, compounds in this class are mutagenic.[20,21] We decided to couple nitroaromatics to the melamine motif, with the aim of selectively delivering these compounds to the parasites. Rapid and selective delivery of these compounds to the parasites should give selective accumulation in the parasite and minimize side effects, possibly allowing reduced doses. We have recently described the activity of some compounds in which we have linked a nitro heterocycle to a melamine delivery motif.[12] In this current paper we report the preparation of these initial compounds and some new compounds plus a detailed analysis of the structure–activity relationships.

As nitro heterocycles are the only drugs currently registered against Chagas disease, we considered it of interest to test the new compounds against *T. cruzi*. *Leishmania* parasites too share substantial similarity to the other trypanosomatids belonging to the same taxonomic order and here too new drugs are required, thus we also tested for activity against *Leishmania*.

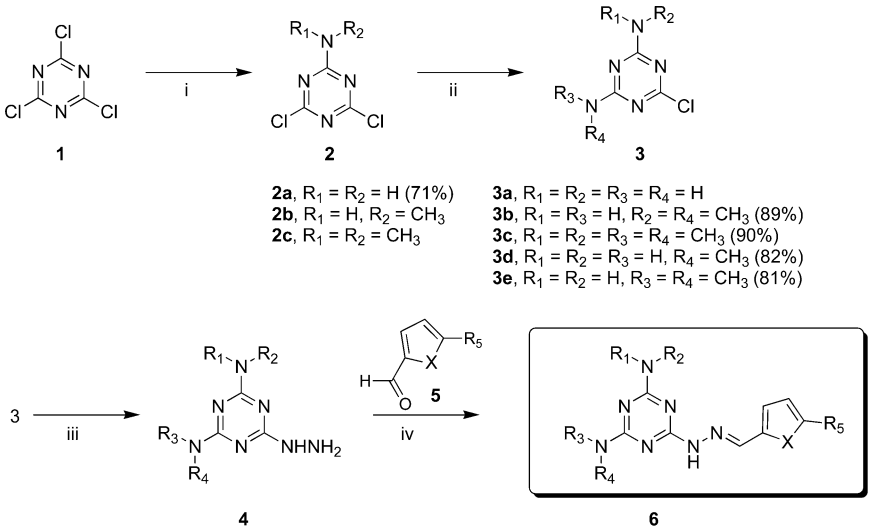
## Chemistry

The general structure of our lead compound, **6a**, is shown in Figure 4.[12] In this compound the melamine motif is linked to the nitro heterocycle via a hydrazone.

We proposed to investigate the following structure–activity relationships: (i) addition of methyl substitutents to the melamine, to alter the lipophilic properties of the molecules; (ii) replacement of the oxygen in the nitro heterocycle with sulfur, to modify the electronic properties of the nitro heterocycle and lipophilic properties of the molecule; (iii) investigation of the necessity of the nitro group, by replacement of the nitro group with a hydrogen or another electron-withdrawing group.

The triazines described were synthesized starting from cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) (**1**) and different nucleophiles. The chlorine atoms of cyanuric chloride can be replaced successively by substituted or nonsubstituted amino groups. The nucleophiles

**Scheme 1**[a]



**2a**, $R_1 = R_2 = H$ (71%)
**2b**, $R_1 = H$, $R_2 = CH_3$
**2c**, $R_1 = R_2 = CH_3$

**3a**, $R_1 = R_2 = R_3 = R_4 = H$
**3b**, $R_1 = R_3 = H$, $R_2 = R_4 = CH_3$ (89%)
**3c**, $R_1 = R_2 = R_3 = R_4 = CH_3$ (90%)
**3d**, $R_1 = R_2 = R_3 = H$, $R_4 = CH_3$ (82%)
**3e**, $R_1 = R_2 = H$, $R_3 = R_4 = CH_3$ (81%)

**4a**, $R_1 = R_2 = R_3 = R_4 = H$ ( 72%)
**4b**, $R_1 = R_3 = H$, $R_2 = R_4 = CH_3$ (96%)
**4c**, $R_1 = R_2 = R_3 = R_4 = CH_3$ (66%)
**4d**, $R_1 = R_2 = R_3 = H$, $R_4 = CH_3$ (30%)
**4e**, $R_1 = R_2 = H$, $R_3 = R_4 = CH_3$ (64%)

**6a**, $R_1 = R_2 = R_3 = R_4 = H$, $R_5 = NO_2$, X = O (36%)
**6b**, $R_1 = R_3 = H$, $R_2 = R_4 = CH_3$, $R_5 = NO_2$, X = O (71%)
**6c**, $R_1 = R_2 = R_3 = R_4 = CH_3$, $R_5 = NO_2$, X = O (58%)
**6d**, $R_1 = R_2 = R_3 = H$, $R_4 = CH_3$, $R_5 = NO_2$, X = O (51%)
**6e**, $R_1 = R_2 = H$, $R_3 = R_4 = CH_3$, $R_5 = NO_2$, X = O (80%)
**6f**, $R_1 = R_2 = R_3 = R_4 = H$, $R_5 = H$, X = O (58%)
**6g**, $R_1 = R_2 = R_3 = R_4 = H$, $R_5 = CN$, X = O (13%)
**6h**, $R_1 = R_2 = R_3 = R_4 = H$, $R_5 = NO_2$, X = S (25%)
**6i**, $R_1 = R_2 = R_3 = R_4 = H$, $R_5 = H$, X = S (38%)

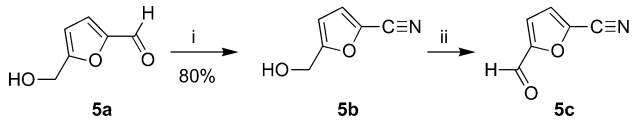
[a] (i) $R_1R_2NH$, acetone/$H_2O$, rt; (ii) $R_2R_3NH$, 2 N NaOH, acetone/$H_2O$, rt; (iii) $NH_2NH_2$, $H_2O$, reflux; (iv) aryl aldehyde, MeOH, rt. The yields are shown in brackets.

can selectively displace the different chlorines by controlling the reaction temperature.[22] In general, the first chlorine can be displaced while the temperature is maintained below 0 °C, the second between 25 and 50 °C, and the third substitution at 67 °C.[23] Another important factor that has to be considered for the preparation of the different derivatives is the nature of the reactive group and the order of entry of the group. When different amino groups were introduced, the less reactive amine was introduced before the more reactive one.

The reactions, in most cases, were carried out in aqueous suspensions, since the products precipitate from solution, simplifying their isolation.[24] To increase the reactivity and the yield, the cyanuric chloride was previously dissolved in acetone and then poured into ice−water to get a very fine suspension. The direct reaction of cyanuric chloride with ammonia, methylamine, and dimethylamine gave the 2-substituted-4, 6-dichloro-1,3,5-triazines (**2a**−**c**) (Scheme 1).[25,26]

The 2,4-disubstituted-6-chloro-1,3,5-triazines (**3a**−**e**) were obtained by reaction of a further amine with the 2-substituted-4,6-dichloro-1,3,5-triazine in the presence of base (NaOH or $NaHCO_3$). The displacement of the last chlorine by the hydrazine was achieved by further increasing the temperature to 85 °C, affording the hydrazine derivatives (**4**) in good yields.[27] The first set of hydrazones (**6a**−**i**) were prepared by reaction of the hydrazine derivatives (**4**) with 5-nitro-2-furaldeyde, affording the corresponding 4,6-disubstituted-2-(5-nitrofurfurylidenehydrazino)-1,3,5-triazines (**6a**−**e**). The products had a low solubility in most organic solvents, except DMSO. However purification was achieved by recrystallization from methanolic and ethanolic water solutions.

**Scheme 2**[a]



[a] (i) $I_2$, $NH_3$, THF/$H_2O$, rt; (ii) oxalyl chloride, DMSO, $NEt_3$ $CH_2Cl_2$, −78 °C.

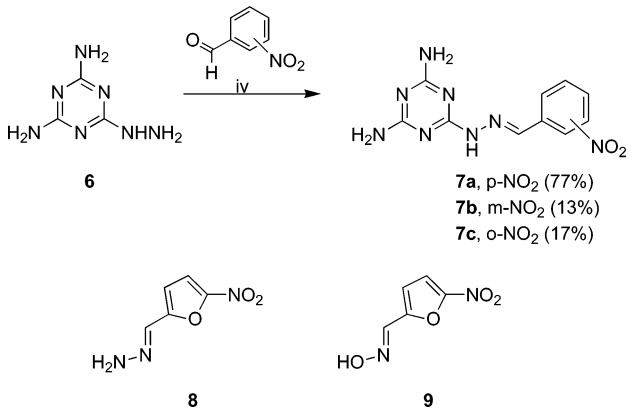
Amended data is presented for compound **6b** to that originally presented;[12] this compound was remade and repurified.

With the purpose of improving the solubility and evaluating the pharmacological importance of the redox potentials of the heteroaromatic rings, the hydrazine (**4a**) was coupled with 5-nitro-2-thiophenecarboxaldehyde to prepare the thiophene analogue (**6h**).

To investigate the pharmacological role of the nitro group in these compounds, compounds without a nitro group were prepared. Replacement of the nitro with a hydrogen was achieved by coupling the hydrazine **4a** with 2-furaldehyde or 2-thiophenecarboxaldehyde to give the related hydrazones (**6f**, **6i**). The solubility observed for all these compounds was slightly improved and the recrystallization was achieved using the conditions described for the previous hydrazones.

We were also interested to replace the nitro with a cyano group, as this is electron-withdrawing, like the nitro. To do this, we required the 5-cyano-substituted 2-furaldehyde. This compound was synthesized as showed in Scheme 2. 5-Hydroxymethyl-2-furaldehyde (**5a**) was used as starting material. The aldehyde function was successfully converted in nitrile by using iodine in aqueous ammonia. The reaction seems to proceed via oxidation of aldimine with iodine to give an N-iodo aldimine intermediate, which eliminates an HI

**Scheme 3**[a]




[a] (iv) Methanol, rt.

molecule in ammonia solution to afford the nitrile product (**5b**) in good yield (80%).[28] The hydroxyl group was then oxidized to the aldehyde **5c** via Swern oxidation.[29]

The three isomers of nitrobenzaldehyde (**7a**−**c**) have also been used to synthesize hydrazones where the heterocyclic ring has been replaced with a nonheterocyclic structure.

## Biology

The activity of the compounds was evaluated in a variety of different models.

**Affinity for the P2 Transporter.** Compounds were evaluated for their ability to interact with the P2 aminopurine transporter of *T. brucei* (Table 1). This was achieved by measuring their ability to antagonize the uptake of radiolabeled adenosine.[10] In addition to uptake through the P2 transporter, the other main route of adenosine uptake is through the P1 system. To differentiate transport through the P1 and P2 systems, the experiments were conducted in the presence of a large excess of inosine, which saturates the P1 transporter. Thus, adenosine uptake will only occur through the P2 transporter. It is important to note that this experiment does not actually measure uptake of compounds through the P2 transporter, but it does give a measure of the affinity of compounds for the P2 transporter.

The results suggest that compounds without a melamine group have relatively low affinity for the P2 transporter (compound **9** has $IC_{50} = 404\ \mu M$), as would be anticipated. Also compound **6c** in which all the nitrogens on the melamine ring were methylated showed poor affinity ($IC_{50} = 129\ \mu M$). The other compounds assayed showed $IC_{50}$ values all within about a log range of each other that were of a similar order as melarsoprol. Changes to the nitrofuran ring gave very small changes to the apparent affinity for the P2 transporter: thus, removal of the nitro group (compare **6a/6f** and **6h/6i**) gave virtually no change in affinity, while replacement of the oxygen with a sulfur gave a small increase in affinity (compare **6a/6h** and **6f/6i**). This suggests that the primary determinant of affinity for the P2 transporter is the melamine ring and the nitro heterocycle has a limited effect on affinity. This is consistent with previous reports in the literature.[2−15]

**Activities against *T. brucei* in Vitro.** Compounds were tested against several *T. brucei* lines in vitro. The *T. brucei brucei* AT1 wild type and a TbAT1 knockout line were compared to study the effects of the P2 transporter on the activity of the compounds (the TbAT1 gene encodes the P2 transporter). A strain of *T. brucei rhodesiense*, the causative agent of acute HAT, was also studied. Compounds were also assayed against mammalian L6 cells as a measure of cellular toxicity toward mammalian cells.

Very similar results were seen with compounds against both the wild type and P2 knockout *T. brucei brucei*. There was a slight reduction in activity for the knockout line, on the order of 2-fold less active, for many compounds. This was observed for melarsoprol as well as our compounds. This reduction in activity is not very significant and suggests that there are routes other than the P2 transporter for uptake of these compounds. Melarsoprol was the most active compound against *T. brucei brucei* ($IC_{50} = 53$ nM). However, some of the nitro heterocycles prepared showed only slightly reduced activity against *T. brucei brucei*, notably **6a**, **6c**, **6d**, **6h** ($IC_{50} = 230$, 200, 130, and 850, respectively).

Compounds were more potent against the *T. brucei rhodesiense* line studied here than against the *T. brucei brucei* line, although very similar trends were seen within the different species. These probably reflect a range of activities against different strains of the *T. brucei* trypanosomes in general rather than a particular difference between the nonhuman pathogenic *T. brucei brucei* line and the human infectious *T. brucei rhodesiense* line. Thus, melarsoprol had an $IC_{50}$ against *T. brucei rhodesiense* of 6 nM, while **6c** had an $IC_{50}$ of 3 nM. A significant number of our compounds had $IC_{50}$ values in the nanomolar range (**6a**, **6b**, **6c**, **6d**, **6e**, and **6h**). Compound **6c** was the most active compound in our series in cellular assays.

In comparing all activities the following structure−activity relationships can be observed:

(1) Compounds without a melamine structure (**8** and **9**) showed weak activity ($IC_{50} = 2.3\ \mu M$) and also weak selectivity compared to the mammalian L6 cells.

(2) Compounds with a nitrofuran ring joined to a melamine ring showed potent in vitro activity (**6a**−**e**).

(3) Replacement of the oxygen with a sulfur led to a 10-fold loss in activity and a small increase in toxicity to L6 cells.

(4) Compounds in which the nitro group was replaced by a hydrogen had significant loss in activity (compare **6a/6f** and **6h/6I**).

(5) Replacement of the nitro group with another electron-withdrawing group, the nitrile group, led to loss of activity (**6g**), implying that more than an electron-withdrawing effect is necessary for activity.

(6) Complete removal of the nitro heterocycle caused loss of activity (**4a**).

(7) Replacement of the nitrofuran with a nitrophenol also led to loss of activity (**7a**−**c**).

(8) A number of the nitro heterocycles were markedly less toxic against mammalian cells than melarsoprol; of particular note are **6a**, **6d**, and **6e**.

**Activities against *Trypanosoma cruzi* and *Leishmania donovani*.** An interesting point noted from the data on in vitro activities against *T. brucei brucei* was the fact that compounds retained activity despite the loss of the P2 transporter. This indicates that routes

**Table 1.** In Vitro Activities of Compounds against the P2 Transporter, Bloodstream *T. brucei*, and Mammalian Cells as a Measure of Toxicity[d]

| | | | $IC_{50}$, $\mu$m | | | |
|---|---|---|---|---|---|---|
| compd | MW | P2 uptake[a] | *T. brucei brucei* AT1 wild type | *T. brucei brucei* AT1 knockout[b] | *T. brucei rhodesiense* | L6 cells[c] |
| **4a** | 141.1 | 11.9 | >200 | >200 | ND | ND |
| **6a** | 264.2 | 22.9 | 0.23 | 0.38 | 0.025 | 183 |
| **6b** | 292.2 | ND | ND | ND | 0.010 | 44.1 |
| **6c** | 320.3 | 129 | 0.2 | 0.3 | 0.003 | 18.7 |
| **6d** | 278.2 | ND | 0.13 | 0.06 | 0.018 | 48.9 |
| **6e** | 292.2 | ND | 5.36 | 3.09 | 0.053 | 109.5 |
| **6f** | 216.2 | 15.9 | 16.5 | 29.3 | 12.9 | >400 |
| **6g** | 244.2 | ND | ND | ND | 46.27 | ND |
| **6h** | 280.2 | 1.9 | 0.85 | 1.52 | 0.24 | 11.8 |
| **6i** | 235.3 | 4.9 | 89 | 170 | 10.2 | 78.2 |
| **7a** | 274.2 | ND | >75 | >75 | 29.9 | ND |
| **7b** | 274.2 | ND | >75 | >75 | 52.88 | ND |
| **7c** | 274.2 | ND | >75 | >75 | 36.47 | ND |
| **8** | 155.1 | 404 | 23.5 | 13.5 | 2.3 | 20 |
| **9** | 156.1 | 300 | 1.18 | 1.26 | 0.68 | 40 |
| melarsoprol | | 1.2 | 0.053 | 0.12 | 0.006 | 7.8 |
| nifurtimox | | ND | 5.6 | ND | 1.5 | 68 |

[a] Inhibition of adenosine uptake by the P2 transporter in *T. brucei brucei* 427. [b] *T. brucei brucei* AT1 knockout is a mutant with a non functional P2 transporter. [c] L-6 cells are rat skeletal myoblasts and are used as a measure of cytotoxicity to mammalian cells. ND, not determined. [d] Data for compounds **4a**, **6a**, **6c**, **6f**, **6h**, **6i** has been reported previously,[12] but is included here for comparative purposes. Amended data for **6b** is presented.

**Table 2.** In Vitro Activities of Compounds against Intracellular Amastigotes of *T. cruzi* and *L. donovani*[a]

| Compound | MW | *T. cruzi* ($IC_{50}$ [$\mu$M]) | *Leishmania donovani* ($IC_{50}$ [$\mu$M]) |
|---|---|---|---|
| **4a** | 141.1 | ND | ND |
| **6a** | 264.2 | 2.1 | >12.5 |
| **6b** | 292.2 | 0.24 | 3.14 |
| **6c** | 320.3 | 0.38 | tox |
| **6d** | 278.2 | 0.39 | 0.75 |
| **6e** | 292.2 | 0.29 | tox |
| **6f** | 216.2 | >400 | tox |
| **6g** | 244.2 | 122 | ND |
| **6h** | 280.2 | 2.6 | tox |
| **6i** | 235.3 | 85.84 | tox |
| **7a** | 274.2 | 91.54 | ND |
| **7b** | 274.2 | 328 | ND |
| **7c** | 274.2 | 328 | ND |
| **8** | 156.1 | ND | tox |
| **9** | 156.1 | ND | tox |
| Melarsoprol | | 0.006 | 7.8 |
| Nifurtimox | | 1.5 | 68 |

[a] Standards: For *T. cruzi*, Benznidazole, $IC_{50}$ = 1.435 $\mu$M; *L. donovani*: Miltefosine, $IC_{50}$ = 1.16 $\mu$M: tox − toxic to macrophages thus precluding measurement of leishmanicidal activity.

other than the *T. brucei* specific transporter clearly exist for these compounds. This prompted us to also test for activity against the related pathogens *T. cruzi* and *L. donovani*.

As shown in Table 2 activities against *T. cruzi* were observed for compounds **6b**−**e**, with activities in the submicromolar range. None of the compounds showed sub-micromolar activities in vitro against *L. donovani*, except for compound **6d**, with an $IC_{50}$ of 0.75 $\mu$M.

**In Vivo Activity in Rodent Models of Infection.** Selected compounds were evaluated in several rodent models of *T. brucei* group infection. Compounds **6a**, **6b**, **6d**, and **6e** were examined in rodent models infected with *T. brucei brucei* STIB 795 at a dose of 20 mg/kg for 4 days ip (days 3−6). The mice are considered cured when no infection was found at 60 days. Compounds **6a** and **6d** were able to cure the STIB 795 *T. brucei brucei* model mice, where four mice of four were all cured. No overt signs of toxicity were observed in these mice. Compounds **6b** and **6e** were not curative in vivo at a dose of 20 mg/kg.

**Table 3.** In Vivo Activities of Compounds against *T. brucei brucei* Model and *T. b. rhodesiense* Model[a]

| | | *T. brucei brucei* (STIB 795 model) | | *T. b. rhodesiense* (STIB 900 model) | |
|---|---|---|---|---|---|
| Compound | Dose mg/Kg | Cured/ Infected | Survival average (days) | Cured/ Infected | Survival average (days) |
| **6a**[b] | 4 × 20 i.p. | 4/4 | >60 | 1/4 | 35.25 |
| **6c**[b] | 4 × 20 i.p. | ND | ND | 0/4 | 7.25 |
| **6b** | 4 × 20 i.p. | 0/4 | 5.25 | ND | ND |
| **6d** | 4 × 20 i.p. | 4/4 | >60 | 2/4 | >38.5 |
| **6e** | 4 × 20 i.p. | 0/4 | 18.3 | ND | ND |
| Control (avrg) | | 0/4 | 7 | 0/4 | 6.5 |
| Melarsoprol | 4 × 1 i.p. | 2/4 | >60 | 0/4 | 21.25 |
| | 4 × 8 i.p. | ND | ND | 4/4 | >60 |
| Pentamidine | 4 × 5 i.p. | 4/4 | >60 | ND | ND |
| | 4 × 20 | ND | ND | 0/4 | 42.75 |

[a] The control represents the average of the controls for each experiments performed. [b] These data was presented previously,[12] but is included here for comparative purposes.

The compounds were also tested with *T. brucei rhodesiense* STIB 900 model in rodents, which is a more stringent test. For example, pentamidine, one of the drugs currently used for early stage HAT, was not able to cure this model at this dose. However, this model responds to melarsoprol, a drug currently in use for the treatment of the late stage. In the STIB 900 model, parasites appear to leave the vasculature system early so that drugs must penetrate extravascular compartments to effect radical cure. Therefore, this in vivo test represents a good model for evaluating likely outcomes of drugs in late-stage models.

As already described in our previous work,[12] compound **6a** was given intraperitoneally at 20 mg/kg for 4 days and cured only one mouse of four. However, the compound caused a significant increase in life span of the mice to 35 days compare to the 7 days for the untreated control animals. Pentamidine did not cure any of the four mice but increased the life span to 43 days.

Some compounds were also investigated for their in vivo activities in mice model infected with *T. cruzi*.

**Table 4.** In Vivo Activities against a Rodent Model of Chagas Disease for Some Selected Compounds

| Compound | Dose mg/Kg | No of doses | Route | Survival Time | % inhibition |
|---|---|---|---|---|---|
| **6c** | 15 | 5 | i.p. | 12.8 | N. D. |
| **6a** | 25 | 5 | i.p. | 13 | N. D. |
| **6d** | 50 | 5 | i.p. | 13 | 27.75 |
| **6e** | 50 | 5 | i.p. | 14 | 44.95 |
| Control | - | | | 13 | 0.00 |
| Benznidazole | 45 | 5 | p.o. | >30 | 100.00 |

Although none of the compounds evaluated was able to cure the model mice, compounds **6d** and **6e** were able to reduce parasitaemia, the latter by 45%. Compound **6d** was investigated in a rodent model of leishmaniasis (*L. donovani*-infected mice). The compound had a moderate effect. When dosed at 40 mg/kg intraperitoneally for 5 days, there was 28% reduction in parasitaemia, compared to the control. With the standard drug, pentostam, there was a 62% reduction in parasitaemia compared to the control when dosed at 15 mg/kg for 5 days.

**Mode of Action Studies.** We have previously reported mode of action studies[12] and have repeated these studies now also including **6d** and the nitroimidazole benznidazole, too. Two possible mechanisms of action of the compounds are damage to DNA and oxidative damage.

First, to investigate damage to DNA, experiments were undertaken with a *T. brucei* mutant deficient in a DNA repair enzyme (RAD51). This mutant is compromised in its ability to repair double-stranded DNA breaks; thus, agents that induce such damage are more active against these mutants than against wild-type cells. Megazol (see Figure 3 for structure) is a heterocycle that exerts its mode of action by damage to DNA. Thus the RAD51$^{-/-}$ line was more susceptible than wild type to megazol[30] (Figure 3). However, compound **6a** showed similar activity against both the wild-type and RAD51$^{-/-}$, indicating that the mode of action does not involve damage to DNA in the same manner as megazol, although other types of DNA damage cannot be ruled out. Benznidazole has markedly lower activity against *T. brucei* in vitro than other compounds and shows no additional activity in the RAD51 deficient cell line. Compound **6d** shows a very similar profile to **6a**.

Nifurtimox (Figure 3) is another nitro heterocycle that is currently registered for treatment of Chagas disease and is being investigated for the treatment of melarsoprol refractory HAT. Nifurtimox is believed to act via oxidative stress.[31,32] Thus, culturing the *T. brucei* in the presence of *N*-acetylcysteine, which reduces free radical damage due to oxidative stress, antagonizes the mode of action of nifurtimox. This effect was not seen in the case of **6a** nor **6d**, suggesting that the main mode of action is not due to accumulation of high levels of reactive oxygen species within compartments that are targeted by *N*-acetylcysteine. More subtle effects associated with oxidative damage, however, cannot be ruled out.

## Discussion

In this paper, we report results on a group of trypanocidal nitro heterocycles identified during work aiming to induce selective toxicity through selective uptake of compounds into *T. brucei* using nucleoside transporters. The compounds prepared were investigated for their ability to antagonize uptake of radiolabeled adenosine through the P2 transporter. This assay does not give proof that the compounds are internalized through the transporter, but does indicate that many of the compounds showed good affinity for the P2 transporter, as they compete with adenosine in binding to the transporter. However, there was no correlation between affinity for the P2 transporter and activity in vitro against intact parasites. In addition to this, there are no remarkable changes in the sensitivity when the in vitro activities against the *T. brucei brucei* model are compared with the activities against the *T. brucei brucei* knockout line (i.e., deficient in P2 transporter activity). Therefore, it is not possible to directly correlate the affinity of the P2 transporter and the trypanocidal activity. Passive diffusion or other transporters may be involved in the uptake of the compounds.[33] However, compound **8**, which lacked a melamine group and had poor affinity to the P2 transporter, showed weak activity against the parasites, suggesting that the melamine group is required for uptake, or else in interactions with enzymes involved in either activation or directly in the mode of action of the compounds. Future research should focus on learning more about roles of the melamine moiety.

Interestingly, **6c** showed poor affinity for the P2 transporter but marked in vitro activity against *T. brucei brucei* and *T. brucei rhodesiense*. In compound **6c**, all the hydrogen atoms on the amino group have been replaced by methyl groups. The lack of affinity of this compound for the P2 transporter could possibly be explained by a requirement for substrates of the P2 transporter to have an H-bond donor. Compound **6c** showed much higher solubility in a variety of solvents than other compounds, and this may allow rapid passive diffusion, which may account for high activity against the parasites. It is clear that the melamine moiety (with at least some NH bonds) is selectively concentrated within the parasite, but the route of uptake is not exclusively through the P2 transporter. This is important, since loss of the P2 transporter can induce resistance to drugs that enter cells exclusively via this route. The fact that other routes of uptake exist for the melamine nitroheterocycles means that resistance resulting from simple loss of the P2 transporter should not be an issue.

Some of the compounds prepared showed potent activity against *T. brucei rhodesiense*, of the same order as melarsoprol (**6a**, $IC_{50}$ = 25 nM; **6b**, $IC_{50}$ = 10 nM **6c**, $IC_{50}$ = 3 nM; **6d**, $IC_{50}$ = 18 nM; **6e**, $IC_{50}$ = 53 nM; **6h**, $IC_{50}$ = 240 nM, melarsoprol, $IC_{50}$ = 6 nM). In addition, some of these compounds showed lower toxicity against mammalian (L6) cells than melarsoprol, although further work is required to show that these compounds would have better selectivity/therapeutic indices. Compound **6a** gave an $IC_{50}$ value of 0.025 $\mu$M against *T. brucei rhodesiense*, which is 60-fold better than nifurtimox (1.5 $\mu$M) that is clinically in use against Chagas' disease[34] and in trial against melarsoprol refractory trypanosomiasis.[18,19]

We decided to investigate the roles of both the nitro group and the furan ring. The nitro group is a strong

**Table 5.** Activities[a] of Compounds against Wild Type, Wild-type with NAC and RAD51$^{-/-}$ Mutant *T. brucei*

| Compound | **6d** | **6a** | megazol | nifurtimox | benznidazole |
|---|---|---|---|---|---|
| Wild-type (427) | 0.08 ± 0.05 | 0.08 ± 0.03 | 0.12 ± 0.05 | 4.1 ± 1.7 | 116.3 ± 7.5 |
| Wild-type (427) + NAC | 0.09 ± 0.04 | 0.12 ± 0.05 | 0.13 ± 0.06 | 11.4 ± 5.2 | 116.3 ± 13.6 |
| RAD51$^{-/-}$ mutant | 0.07 ± 0.03 | 0.10 ± 0.02 | 0.04 ± 0.04 | 4.3 ± 1.6 | 114.2 ± 32 |

[a] Values are $IC_{50}$ ($\mu$M).

electron-withdrawing group and also can be involved in the generation of free radicals. Replacement of the nitro with a hydrogen (**6f**) or by another electron-withdrawing group such as the nitrile (**6g**) led to an inactive compound, indicating the importance of the nitro group itself. Replacement of the nitrofuran with a nitrophenyl gave significant loss of activity. Even replacement of the oxygen with sulfur (**6h**) gave reduced activity, suggesting that perhaps the redox potential of the nitrofuran is important for the mode of action of these compounds.

Nitro heterocycles often work through free-radical mechanisms, so genotoxicity represents a main issue in the development of new trypanocidal nitro heterocyclic compounds. Megazol, a nitro heterocyclic compounds, was found to be mutagenic in mammalian cell tests[35] and also positive in the Ames test.[36] It has been found that the trypanocidal activity of megazol was related to its ability to induce mutations in DNA; trypanosomes deficient in their own DNA repair enzymes (the RAD51$^{-/-}$ line) are hypersensitive to this compound. However, the RAD51$^{-/-}$ line showed susceptibility similar to that of wild-type parasites to compound **6a**. This suggests that **6a** has a different mode of action to megazol. Another nitro heterocycle, nifurtimox (which is also positive in the Ames test[37]), appears to work by oxidative stress, as can be seen by antagonizing the compound with *N*-acetylcysteine. Compound **6a** was not antagonized by *N*-acetylcysteine, indicating that its mode of action is not by oxidative stress. These findings may indicate that the compounds do not work by damage to DNA or oxidative stress, both of which are implicated in positive Ames tests. Further studies are continuing in this area.

In addition to in vitro activity, several compounds retained trypanocidal effect in mice. Compounds **6a** and **6d** were able to cure mice infected with *T. brucei brucei* at a dose of 20 mg/kg for 4 days. It is noteworthy that addition of methyl groups to the melamine $NH_2$ groups reduces activity both in vitro and in vivo. Addition of one methyl group to one of the amino groups (**6d**) yields a compound with slightly better activity in vitro and activity is retained in vivo. However, addition of an extra methyl to the same nitrogen (**6e**) or a methyl to the other amino (**6b**) led to loss of activity in vivo at the dose tested. The fact that trypanocidal activity in each case was barely altered by the presence of the P2 transporter indicates that the relative activity of these compounds is not related to their ability to interact with that transporter. It is noteworthy that the most active compound in vitro, **6c**, was inactive in vivo at the dose tested, highlighting the role of pharmacokinetic issues, beyond simple antiparasite activity, on in vivo trypanocidal capability.

Compound **6a** was also tested with the more stringent *T. brucei rhodesiense* STIB 900; using the same treatment schedule, it cured only one of four animals infected with a mean survival of 35 days compare to the untreated controls that had a mean survival of 8 days.

The discovery that the nitro heterocycles were active against *T. brucei* regardless of the presence of the P2 transporter prompted us to test the compounds against other pathogenic trypanosomatids that do not possess this transporter. Considerable activity was noted against amastigote *T. cruzi* in vitro within mammalian cells. Several compounds were several-fold more active than the registered drug, nifurtimox, in vitro. However, all failed to cure mice in vivo, although some compounds also led to a reduction in parasitaemia in vivo, highlighting the lead status of these new molecules for use in Chagas therapy.

In the case of *Leishmania*, none of the compounds were particularly active against the intracellular amastigotes, except for compound **6d**. A modest activity was found for this compound in a rodent model of disease.

## Conclusion

We have prepared some melamine-nitrofuran conjugates that have potent activity against *T. brucei rhodesiense* on the same order of magnitude as melarsoprol and that are significantly more active than nifurtimox, which is currently undergoing trials for HAT. Two of the compounds were able to cure an animal model of trypanosomiasis (*T. brucei brucei* STIB795) and one of the compounds was able to have a significant effect on the course of another model of infection (*T. brucei rhodesiense* STIB900). The fact that the mode of action of **6a** does not appear to involve DNA damage in trypanosomes might indicate that it will be not mutagenic in tests in mammalian cells, thus greatly improving the chances that this compound or its derivatives can proceed to clinical trials against trypanosomiasis. These compounds represent exciting new leads for further evaluation for HAT. Moreover, some compounds also showed considerable activity against *T. cruzi* in vitro and some reduction in parasitaemia in vivo, indicating that this class of molecule should also be considered as a lead in developing novel drugs against Chagas disease.

## Experimental Section

**P2 Transporter Affinity Measurements.** Parasites purified from blood were stored on ice in Carter's buffered saline solution.[7] Transport assays used the centrifugation-through-oil technique, which is routinely used in analyses.[9,14,15,33,38] Radiolabeled adenosine (0.5 $\mu$M) uptake via P2 was measured in the presence of 1 mM inosine, which blocks the P1 transporter.[7] Compounds were assayed for affinity for the P2 transporter by using labeled adenosine fixed at 0.5 $\mu$M and a range of inhibitor concentrations. $IC_{50}$ values were calculated using the Grafit 4.0 Software (Erithacus) by plotting inhibitory value against concentration of inhibitor.

**In Vitro Activities against *T. brucei brucei and T. brucei rhodesiense* and Cytotoxicity.** The activity of compounds was determined for *T. brucei rhodesiense* trypomastigotes of STIB900. This stock was isolated in 1982 from a human patient in Tanzania. Minimum essential medium (50 $\mu$L) supplemented with 2-mercaptoethanol and 15% heat-

inactivated horse serum[39] was added to each well of a 96-well microtiter plate. Serial drug dilutions were prepared covering a range from 90 to 0.123 $\mu$g/mL. Then, 50 $\mu$L of a trypanosome suspension was added to each well, and the plate incubated at 37 °C under a 5%$CO_2$ atmosphere for 72 h. Alamar Blue (10 $\mu$L) was then added to each well and incubation continued for a further 2−4 h. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Corp., Sunnyvale, CA) by using an excitation wavelength of 536 nm and an emission wavelength of 588 nm.[40] Fluorescence development was expressed as a percentage of the control, and the 50% inhibitory concentration ($IC_{50}$) values were determined. Cytotoxicity was assessed by using the same assay and rat skeletal myoblasts (L-6 cells).

To investigate whether transport of these compounds through the P2 transporter is necessary for activity, compounds were assayed against the *T. brucei brucei* trypomastigotes using either the wild type or P2 knockout mutants (*TbAT1*$^{-/-}$).[41] The Alamar Blue assay[40] was also used to determine $IC_{50}$ values. To determine whether DNA damage was associated with trypanocidal activity, the Alamar Blue assay was also used to determine $IC_{50}$ values against the *RAD51*$^{-/-}$ deletion mutant.[42]

**In Vitro Activities against *Trypanosoma cruzi*.** Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well/100 $\mu$L in RPMI 1640 medium with 10% FBS and 2 mM L-glutamine. After 24 h, 5000 trypomastigotes of *T. cruzi* (Tulahuen strain C2C4 containing the $\beta$-galactosidase (Lac Z) gene) were added in 100 $\mu$L per well with 2× of a serial drug dilution. The plates were incubated at 37 °C in 5% $CO_2$ for 4 days. Then the substrate CPRG/Nonidet was added to the wells. The color reaction, which developed during the following 2−4 h, was read photometrically at 540 nm. From the sigmoidal inhibition curve $IC_{50}$ values were calculated.

**Activities against *L. donovani*.** Mouse peritoneal macrophages were seeded in RPMI 1640 medium with 10% heat-inactivated FBS into Lab-tek 16 chamber slides. After 24 h *L. donovani* amastigotes were added at a ratio of 3:1 (amastigotes to macrophages). The medium containing free amastigotes was replaced by fresh medium 4 h later. The next day the medium was replaced by fresh medium containing different drug concentrations. The slides were incubated at 27 °C under a 5% $CO_2$ atmosphere for 96 h. When the medium was removed, the slides were fixed with methanol and stained with Giemsa. The ratio of infected to noninfected macrophages was determined microscopically, expressed as percentage of the control, and the $IC_{50}$ value was calculated by linear regression.

**In Vivo Biological Activity against *T. brucei*.** Female NMRI mice weighting 22−25 g were infected with cryopreserved stabilates of *T. brucei brucei* STIB 795 (derivate of strain 427[43]) or *T. brucei rhodesiense* STIB 900. Each mouse was infected intraperitoneally with (2−4) × $10^4$ bloodstream forms. Melarsoprol (Arsobal; Aventis) acted as standard drug and was diluted with sterile distilled water to an appropriate concentration. Groups of four mice were treated on days 3, 4, 5, and 6 intraperitoneally with 20 mg/kg. A control group remained untreated. The parasitemia of all animals was checked on day 7 and 10 postinfection and every second day thereafter until 60 days. Death of animals was recorded to calculate the mean survival time. Surviving and aparasitemic mice were considered cured at 60 days and then euthanized.

**In Vivo Activity for *T. cruzi*.** Female BALB/c mice weighing 20 g (Charles Rivers Ltd, UK) were infected intraperitoneally with 2 × $10^4$ trypomastigotes in 0.2 mL, harvested from the blood of a passage mouse. Infected mice were randomly sorted into groups of five. After 5 days tail blood was examined for patency of infection: the number of trypomastigotes in 10 microscope field was noted. Dosing commenced for five consecutive days. Tail blood was examined 2 days after the end of treatment and at 7 day intervals on any surviving mice.

**Chemistry. General.** Chemicals were purchased from Aldrich and Fluka and were used without further purification. Dry solvents were generally purchased from Fluka in sure-seal bottles and stored over molecular sieves. Qualitative thin-layer chromatography (TLC) was performed on precoated aluminum sheets silica gel $60F_{254}$ from Merck. Melting points were determined with a Gallenkamp melting point apparatus and are not corrected. $^1$H and $^{13}$C NMR data were recorded on a Bruker Avance DPX 300 MHz NMR spectrometer, with tetramethylsilane as the internal standard and deuterated solvents purchased from Goss unless stated otherwise. Infrared (IR) spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer. Mass Spectra were recorded at a Platform II mass spectrometer (Micromass) from Fisons. Ionization was achieved in the positive electrospray mode using a mixture of acetonitrile/water (1:1) or MeOH (HPLC grade) as mobile phase. High-resolution mass spectra were recorded by the National Mass Spectrometry Service Centre in Swansea with a MAT 900 XLT high resolution double focusing mass spectrometer from Finnigan using the same ionization procedure. Combustion analyses were performed by the analytical and chemical consultancy services MEDAC Ltd. The triazine derivatives, as observed in other work,[44] often present a problem in analysis. The problem was mainly found to be for the microanalysis of the nitrogen content.

**5-Nitro-2-furaldehyde (4,6-Diamino)-[1,3,5]-triazin-2-ylhydrazone (6a).** A mixture of **4a** (149.50 mg, 1.06 mmol) and 5-nitrofuraldehyde (150.00 mg, 1.06 mmol) was dissolved in methanol (5 mL). The suspension was left stirring overnight at room temperature. The reaction mixture was filtered and the yellow precipitate was washed with methanol and dried under vacuum at 40 °C. The crude product was recrystallized from water to get 100.5 mg of a pure yellow solid. Yield: 100.5 mg, 36%. mp: >350 °C. IR: $\nu_{N-H}$ 3313.9 cm$^{-1}$, $\nu_{C=N}$ 1631.9 cm$^{-1}$, $\nu_{C-NO_2}$ 1537.8 cm$^{-1}$, $\nu_{C-N}$ 809.3. LRMS ($ES^+$): *m/z* 265 (M + H$^+$, 100%), 287 (M + Na$^+$, 80%). HRMS ($ES^+$): calcd for $(C_8H_9N_8O_3)^+$ 265.0792, found 265.0799. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 6.51 (bs, 4H), 7.05 (d, $J$ = 3.84) 7.78 (d, $J$ = 3.84 Hz), 8.00 (s, 1H), 11.25 (s, 1H). $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ 114.2, 115.6, 129.4, 151.8, 153.2, 164.9, 167.7.

**5-Nitro-2-furaldehyde ($N^4$,$N^6$-Dimethyl-4,6-diamino)-[1,3,5]-triazin-2-ylhydrazone (6b).** Compounds **4b** (180 mg, 1.06 mmol) and 5-nitrofuraldehyde (142.5 mg, 1.06 mmol) were suspended in MeOH (10 mL). The suspension was left stirring overnight. The reaction was reduced under vacuum. The yellow solid was then recrystallized from $H_2O$/MeOH (50/50 mL). Yield: 220 mg, 71%. LRMS ($ES^+$): *m/z* 293.1 ((M + H)$^+$, 100%), 315.2 (M + Na$^+$, 20%). HRMS: calcd mass for $(C_{10}H_{13}N_8O_3)^+$ 293.1105, found 293.1104. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ (mixture of the two geometric isomers) 3.36 (m, 6H), 7.05 (bs, 2H) 7.23 (bs, 1H), 7.78 (m, 1H), 11.06 (s, 1H), 11.20 (s, 1H). $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ (mixture of the two geometric isomers) 27.4, 27.6*, 113.9, 115.6, 129.0, 129.4*, 151.8, 153.3, 164.1, 166.4, 166.6*. Anal. ($C_{10}H_{12}N_8O_3$·0.4$H_2O$) C, H, N.

**5-Nitro-2-furaldehyde ($N^4$,$N^4$,$N^6$,$N^6$-Tetramethyl-4,6-diamino)-[1,3,5]-triazin-2-ylhydrazone (6c).** A mixture of **4c** (201 mg, 1.01 mmol) and 5-nitro-2-furaldehyde (143 mg, 1.02 mmol) was dissolved in 10 mL of ethanol. The reaction mixture was allowed to stir overnight at room temperature and the solvent was evaporated under vacuum. The residue was purified by flash column chromatography (gradient from 1 to 5% MeOH/DCM). Compound **6c** was isolated as a clear white solid. Yield: 187 mg, 58%. HRMS: calcd mass for $(C_{12}H_{17}N_8O_3)^+$ 321.1424, found 321.1421. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 3.21 (s, 12H), 7.04 (m, 1H), 7.44 (m, 1H), 7.92 (s, 1H), 9.05 (bs, 1H). $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ 36.5, 111.1, 114.2, 129.4, 153.5, 164.1, 165.8.

**5-Nitro-2-furaldehyde ($N^4$-Methyl-4,6-diamino)-[1,3,5]-triazin-2-ylhydrazone (6d).** 5-nitrofuraldehyde (90 mg, 0.58 mmol) and **3d** (81.82 mg, 0.58 mmol) were mixed together and suspended in MeOH (5 mL), and the suspension was left stirring overnight. The mixture was reduced under vacuum, giving 120 mg of brown solid. The solid was recrystallized from

$H_2O$:MeOH (35 mL:20 mL), giving a dark yellow solid. Yield: 83 mg, 51%. Mp: 261−263 °C. LRMS ($ES^+$): *m/e* 279.2 ($M + H^+$, 50%), 301.1 ($M + Na^+$, 100%). HRMS: calcd mass for $(C_9H_{11}N_8O_3)^+$ 279.0949, found 279.0951. $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ 2.77 (s, 3H), 6.77 (bs, 3H), 7.07 (s, 1H), 7.78 (d, $J$ = 4.02), 8.01 (s, 1H), 11.10 (s, 1H). $^{13}C$ NMR (75 MHz, DMSO-$d_6$): $\delta$ 27.6, 114.0, 115.6, 129.5, 151.8, 153.3, 166.9. Anal. ($C_9H_{10}N_8O_3 \cdot 0.4H_2O \cdot 0.02HCl$) C, H, Cl; calcd: N, 38.4. Found: N, 37.9.

**5-Nitro-2-furaldehyde (*N*,$^4$*N*$^4$-Dimethyl-4,6-diamino)-[1,3,5]-triazin-2-ylhydrazone (6e).** 5-Nitrofuraldehyde (246 mg, 99%, 1.45 mmol) and **3e** (204.56 mg, 1.45 mmol) were suspended in MeOH (5 mL) and left stirring overnight. The mixture was reduced under vacuum, giving 120 mg of brown solid. The solid was recrystallized from $H_2O$:MeOH (30 mL: 20 mL), giving a light brown solid. Yield: 336 mg, 80%. Mp: 264−266 °C. LRMS ($ES^+$): *m/z* 293.1 ($(M + H)^+$, 20%), 315.1 ($M + Na^+$, 100%). HRMS: calcd mass for $(C_{10}H_{13}N_8O_3)^+$ 293.1105, found 293.1110. $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ 3.11 (s, 6H), 6.75 (br s, 3H), 7.11 (d, 1H, $J$ = 3.84), 7.82 (d, $J$ = 3.84), 8.08 (s, 1H), 11.21 (s, 1H). $^{13}C$ NMR (75 MHz, DMSO-$d_6$): $\delta$ 36.1, 113.8, 115.6, 129.4, 151.8, 153.4, 164.5, 166.0, 167.5. Anal. ($C_{10}H_{12}N_8O_3 \cdot 0.02HCl$) C, H, N, Cl.

**2-Furaldehyde (4,6-Diamino)-[1,3,5]-triazin-2-ylhydrazone (6f).** A mixture of **4a** (150 mg, 1.06 mmol) and 2-furaldehyde (102.88 mg, 1.06 mmol) was suspended in MeOH (5 mL). The suspension was left stirring overnight at room temperature. The mixture was filtered and the brown solid was washed with methanol and then dried under vacuum at 40 °C. The crude product was recrystallized from $H_2O$/MeOH (5%) to give a pure brown solid. Yield: 133 mg, 58%. Mp: 281−283 °C. LRMS ($ES^+$): *m/z* 219 $(M + H)^+$, 241.9 $(M + Na)^+$. HRMS: calcd mass for $(C_8H_{10}N_7O)^+$ 220.0941, found 220.0941. $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ 6.38 (bs, 4H), 6.57 (m, 1H), 6.69 (d, $J$ = 3.29 Hz, 1H), 7.76 (m, 1H), 7.94 (m, 1H), 10.56 (s, 1H). $^{13}C$ NMR (75 MHz, DMSO-$d_6$): $\delta$ 111.5, 112.2, 131.8, 144.3, 150.5, 165.0, 167.7.

**5-Cyano-2-furaldehyde (4,6-Diamino)-[1,3,5]-triazin-2-ylhydrazone (6g).** Compounds **4a** (98.7 mg, 0.70 mmol) and crude **5c** (85 mg, 0.70 mmol) were mixed together and suspended with MeOH (3 mL), and the suspension was left stirring overnight. The mixture was reduced under vacuum, giving a light brown solid. The solid was recrystallized from $H_2O$:MeOH (10 mL:15 mL), giving a light brown solid. Yield: 22 mg, 13%. Mp: >350 °C. IR: $\nu_{CN}$ 2229.7 $cm^{-1}$. LRMS ($ES^+$): *m/z* 245 ($(M + H)^+$, 100). HRMS: calcd mass for $(C_9H_9N_8O)^+$ 245.0894, found 245.0893. $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ 6.52 (bs, 4H), 6.96 (d, $J$ = 3.76 Hz, 1H) 7.71 (d, $J$ = 3.76 Hz, 1H), 8.04 (s, 1H), 10.95 (s, 1H). $^{13}C$ NMR (75 MHz, DMSO-$d_6$): $\delta$ 111.8, 112.3, 124.7, 125.5, 129.7, 155.3, 165.0, 167,7.

**5-Nitrothiophene-2-carbaldehyde (4,6-Diamino)-[1,3,5]-triazin-2-ylhydrazone (6h).** A mixture of **4a** (150 mg, 1.06 mmol) and 5-nitro-2-thiophenecarboxaldehyde (166.58 mg, 1.06 mmol) was suspended in methanol (5 mL). The suspension was left stirring for 2 days at room temperature. The reaction mixture was filtered and the brown precipitate was washed with MeOH and then dried under vacuum at 40 °C. The crude product was recrystallized from water and ethanol (5:1) to give a pure brown solid. Yield: 75.7 mg, 25%. Mp: >350 °C. LRMS ($ES^+$): *m/z* 281 ($(M + H)^+$, 100%), 303 ($(M + Na)^+$, 10%). HRMS ($ES^+$): calcd mass for $(C_8H_9N_8O_2S)^+$ 281.0569, found 281.0572. $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ 6.50 (br s, 4H), 7.36 (d, $J$ = 4.29 Hz, 1H), 8.08 (d, $J$ = 4.29 Hz, 1H), 8.25 (s, 1H), 11.10 (s, 1H). $^{13}C$ NMR (75 MHz, DMSO-$d_6$): $\delta$ 127.6, 131.1, 134.9, 148.7, 149.7, 164.8, 167.6.

**Thiophene-2-carbaldehyde (4,6-Diamino)-[1,3,5]-triazin-2-ylhydrazone (6i).** A mixture of **4a** (150 mg, 1.06 mmol) and 2-thiophenecarboxaldehyde (121.31 mg, 1.06 mmol) was suspended in methanol (5 mL). The suspension was left stirring overnight at room temperature. The mixture was filtered and the brown solid was washed with methanol and dried under vacuum at 40 °C. The crude product was recrystallized from water−ethanol (10%) to give a pure brown-yellow solid. Yield: 95 mg, 38%. Mp: 309−310 °C. LRMS ($ES^+$): *m/z* 236 ($(M + H)^+$, 100%). HRMS ($ES^+$): calcd mass for $(C_8H_{10}N_7S)^+$ 236.0713, found 236.0717. $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ 6.38 (br s, 4H), 7.07 (m, 1H), 7.25 (m, 1H), 7.53 (m, 1H), 8.25 (s, 1H), 10.53 (s, 1H). $^{13}C$ NMR (75 MHz, DMSO-$d_6$): $\delta$ 127.6, 127.9, 129.1, 137.3, 140.2, 164.9, 167.6. Anal. ($C_8H_9N_7S \cdot 0.7H_2O$) C, H, N, S.

**4-Nitrobenzaldehyde (4,6-diamino)-[1,3,5]-triazin-2-ylhydrazone (7a).** Compounds **4a** (328.23 mg, 2.32 mmol) and **iv-a** (357.75 mg, 98%, 2.32 mmol) were suspended in MeOH (5 mL), and the suspension was left stirring overnight. The mixture was reduced under vacuum, giving a yellow solid. The solid was recrystallized from $H_2O$:MeOH (120 mL:200 mL), giving a yellow solid. Yield: 493 mg, 77%. Mp: 321−322 °C. LRMS (EI): *m/z* 274 ($M^+$, 100%). HRMS: calcd mass for $(C_{10}H_{11}N_8O_2)^+$ 275.0999, found 275.1004. $^1H$ NMR (300 MHz, DMSO-$d_6$) $\delta$ 6.50 (br s, 4H), 7.86 (d, 2H, $J$ = 8.78), 8.18 (s, 1H), 8.28 (d, $J$ = 8.78 Hz, 1H), 10.98 (s, 1H). $^{13}C$ NMR (75 MHz, DMSO-$d_6$) $\delta$ 124.4, 127.3, 139.3, 142.0, 147.3, 165.1, 167.7. Anal. ($C_{10}H_{10}N_8O_2 \cdot 1.1H_2O \cdot 0.03HCl$) C, N, Cl calcd: H, 4.2. Found: H, 3.4.

**3-Nitrobenzaldehyde (4,6-Diamino)-[1,3,5]-triazin-2-ylhydrazone (7b).** Compounds **4a** (331.6 mg, 2.35 mmol) and **iv-b** (358.71 mg, 98%, 2.35 mmol) were suspended in MeOH (5 mL), and the suspension was left stirring overnight. The mixture was reduced under vacuum, giving a green solid. The solid was recrystallized from $H_2O$:MeOH (170 mL:50 mL), giving a light green solid. Yield: 83 mg, 13%. Mp: > 345 °C. LRMS (EI): *m/z* 274 ($M^+$, 100%). HRMS: calcd mass for $(C_{10}H_{11}N_8O_2)^+$ 275.0999, found 275.0999. $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ 6.50 (bs, 4H), 7.72 (m, 1H), 8.01 (d, 1H), 8.20 (m, 2H), 8.47 (s, 1H), 10.85 (s, 1H). $^{13}C$ NMR (75 MHz, DMSO-$d_6$): $\delta$ 120.0, 123.3, 130.7, 133.3, 137.4, 139.4, 148.6, 165.2, 167,7.

**2-Nitrobenzaldehyde (4,6-Diamino)-[1,3,5]-triazin-2-ylhydrazone (7c).** Compounds **4a** (328.23 mg, 2.32 mmol) and **iv-c** (357.75 mg, 98%, 2.32 mmol) were suspended in MeOH (5 mL), and the suspension was left stirring overnight. The mixture was reduced under vacuum, giving a yellow solid. The solid was recrystallized from $H_2O$:MeOH (120 mL:80 mL,) giving a yellow solid. Yield: 107 mg, 17%. Mp: 288−291 °C. LRMS (EI): *m/z* 274 ($M^+$, 100%). HRMS: calcd mass for $(C_{10}H_{11}N_8O_2)^+$ 275.0999, found 275.1001. $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ 6.45 (bs, 4H), 7.62 (m, 1H), 7.78 (m, 1H), 8.09 (m, 2H), 8.48 (m, 1H), 10.98 (s, 1H). $^{13}C$ NMR (75 MHz, DMSO-$d_6$): $\delta$ 124.9, 127.8, 129.8, 129.9, 133.7, 136.5, 147.9, 165.2, 167,7.

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**Supporting Information Available:** Experimental procedures and spectral data for compounds **2**−**5** and microanalytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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