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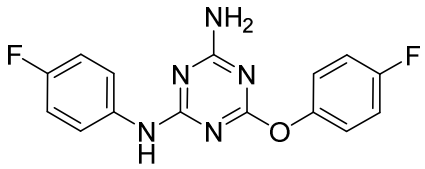
Novel lead compound for African trypanosomiasis



Compound **35**

IC_{50} (*T. bruc*) = 0.63 µM
IC_{50} (MRC5) = > 64 µM
% parent compound at 30 min in mouse microsomes: 80%
% parent compound at 30 min in human microsomes: 85%
Cmax after an oral dose of 50 mg/kg = 5.66 µM
$t_{1/2}$ after an oral dose of 50 mg/kg = 6.51 h

Optimization of the pharmacokinetic properties of potent anti-trypanosomal triazine derivatives

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ABSTRACT

Human African trypanosomiasis is causing thousands of deaths every year in the rural areas of sub-saharan Africa. There is a high unmet medical need since the approved drugs are poorly efficacious, show considerable toxicity and are not easy to administer. This work describes the optimization of the pharmacokinetic properties of a previously published family of triazine lead compounds. One compound (**35** (UAMC-03011)) with potent anti-trypanosomal activity and no cytotoxicity was selected for further study because of its good microsomal stability and high selectivity for *Trypanosoma brucei* over a panel including *Trypanosoma cruzi*, *L.eishmania infantum*, and *Plasmodium falciparum*. *In vivo* pharmacokinetic parameters were determined and the compound was studied in an acute *in vivo* mouse disease model. One of the important learnings of this study was that the rate of trypanocidal activity is an important parameter during the lead optimization process.

KEYWORDS

Trypanosoma brucei, triazine, phenotypic screening, microsomal stability, cidal efficacy

INTRODUCTION

Neglected tropical diseases have a significant impact on human health each year in countries with a less developed economic status and particularly suffer from a lack of research due to a limited return on investment. Among these disorders, African trypanosomiasis affects the rural areas of sub-Saharan Africa and affects either farm-animals named Nagana caused by *Trypanosoma brucei brucei* and human beings caused by *T. b. rhodesiense* and T. b. *gambiense* and share the same vector spreading the disease, *e.g.* the tse-tse fly. The current drugs are pentamidine and suramin for the first-stage, while melarsoprol, eflornithine and nifurtimox are used during the second stage when the parasite has spread to the central nervous system.[1] New and safer drugs that are easily administered are needed since the existing drugs show serious side effects and in some cases have fatal consequences.[2]

Triazine derivatives have been extensively investigated in our group. In the framework of a drug discovery programme for new-anti HIV microbicides, about 50 triazine and pyrimidine derivatives were synthesized and evaluated for anti-HIV-1 activity while one compound was further developed for prevention of sexual HIV transmission.[3-7] Although the antiprotozoal activity of anti-HIV-1 compounds had never been reported, the triazine core is known for its ability to act as substrate for the nucleoside P2 transporter[8, 9]. These compounds were therefore evaluated against *T. brucei* producing several interesting hits.[10, 11] During lead optimization, more than 100 monomers and dimers were evaluated for which it could be concluded that the triazine scaffold yields more potent compounds than the pyrimidine core. Compound **a** (6-(mesityloxy)-N^2-phenyl-1,3,5-triazine-2,4-diamine) showed sub-micromolar

activity on *T. brucei*, no cytotoxicity on MRC5 cells, and was not active against *T. cruzi*, *Leishmania infantum* and *Plasmodium falciparum*. Furthermore, the anti-HIV activity was considerably reduced, indicating selective anti-trypanosomal activity. However, due to a low *in vitro* metabolic stability (only 20% of parent compound left after 15 min upon incubation with mouse microsomal S9 fraction), *in vivo* activity could not be demonstrated in a mouse model of acute *T. brucei* infection. We report here on the design, synthesis and characterization of compounds with improved *in vivo* pharmacokinetic properties while maintaining the selective anti-trypanosomal activity.

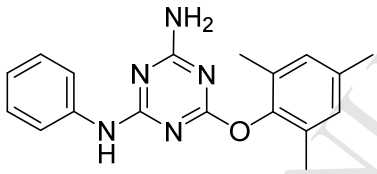


Figure 1. Previously reported compound **a** with antitrypanosomal activity.

RESULTS AND DISCUSSION

Synthesis of new *T. brucei* inhibitors

The design of the molecules was made based on the SAR obtained from the previously synthesized molecules.[10] Since the most likely metabolic hotspots are the benzylic methyl groups and the *para*-position of the phenyl ring, we introduced modifications such as halogen atoms to block aromatic hydroxylations.[12] Different linkers such as thioether or ether in both linker positions (X and Y) between the triazine ring and the aromatic moieties were also studied.

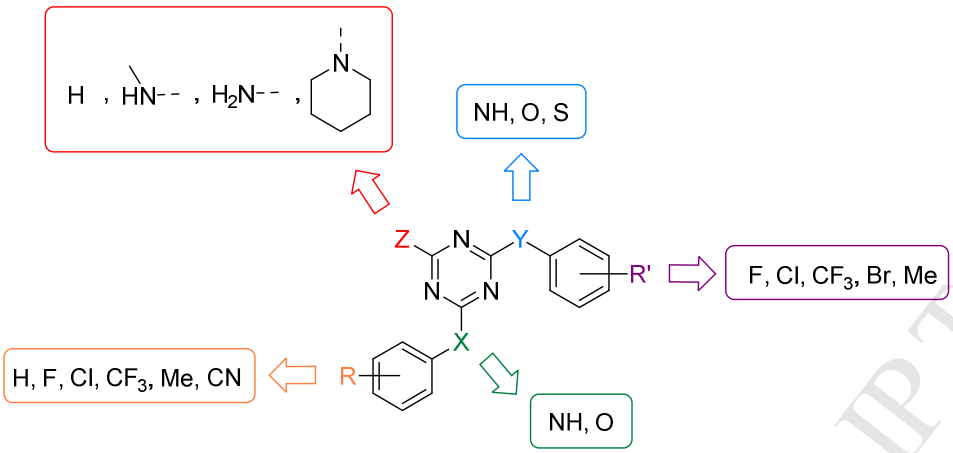
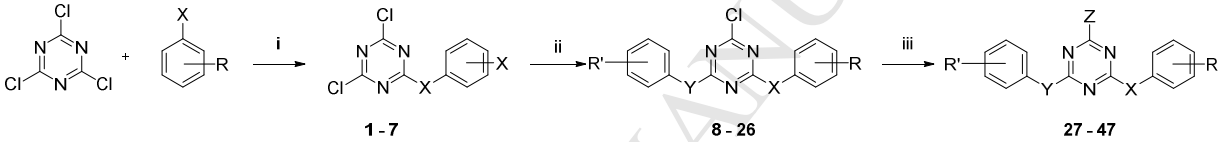


Figure 2. Different structural modifications based on compound **a**.

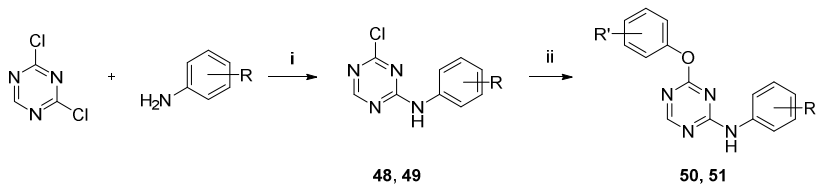
The synthesis was performed by subsequent nucleophilic aromatic substitutions. Different pathways were followed depending on the nature of the linker (Scheme 1).



Reaction conditions: i) When X = NH: DIPEA or K_2CO_3, aniline derivative, dioxane, 25 - 101°C, 40 min – 48 h. When X = O: $(NBu_4)HSO_4$, NaOH, phenol derivative, toluen/water (10:1), 0 – 25°C, 40 min – 24 h. ii) When Y = NH: DIPEA or K_2CO_3, aniline derivative, dioxane, 25 – 101°C, 45 min – 5 days. When Y = O: $(NBu_4)HSO_4$, NaOH, phenol derivative, toluen/water (10:1), 0 – 25°C, 5 mim – 5 days. When Y = S: DIPEA, dioxane, 0 °C, 1 h. iii) When Z = NH_2, NH_3 in methanol or dioxane, 25 – 101°C, 16 h – 8 days. When Z = NHMe, NH_2Me, DIPEA, dioxane, 101°C, 16 h. When Z = piperidinyl, piperidine, DIPEA, dioxane, 101 °C, 16 h.

Scheme 1. Synthetic pathway to obtain different trisubstituted triazine derivatives.

In addition, two disubstituted triazines were synthesized to study the importance of the free amino group on the potency of the compound (Scheme 2).

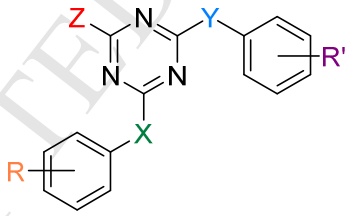


Reaction conditions i) DIPEA or K_2CO_3, aniline derivative, dioxane, 25°C, 5 min. ii) tetrabutylammoniumhydrogensulfate, sodium hydroxide, dioxane, 25°C, 30 min.

Scheme 2. Synthetic pathway to obtain different disubstituted triazine derivatives.

***In vitro* parasitology assays.** The compounds were evaluated on a panel including *T. brucei*, *T. cruzi*, *L. infantum* and *P. falciparum* (results presented in supporting information). Cytotoxicity on a human cell line (MRC-5) and on primary peritoneal mouse macrophages (PMM) was also tested. The activity of all the compounds against *T. brucei* and MRC-5 are shown in **Table 1**.

Table 1. In vitro antitrypanosomal activity and cytotoxicity of the final compounds and derivative b.



Compd.	X	Y	Z	R	R'	Antitrypanosomal activity and cytotoxicity IC_{50} (µM)	
						T. b. bruc.[a]	MRC5[b]
a	NH	O	NH_2	H	2,4,6-trimethyl	0.88	> 64
27	NH	O	NH_2	4-F	2,4,6-trimethyl	0.31	> 64
28	NH	O	piperidinyl	4-F	2,4,6-trimethyl	8.17	> 64
29	NH	O	NH_2	4-Cl	2,4,6-trimethyl	1.52	6.39
30	NH	O	NH_2	4-CF_3	2,4,6-trimethyl	6.94	6.5
31	NH	O	NH_2	3,5-diF	2,4,6-trimethyl	1.29	4.44
32	NH	O	NHMe	4-F	2,4,6-trimethyl	10.86	> 64
33	NH	O	NH_2	2,6-diF	2,4,6-trimethyl	> 64	> 64

34	O	O	NH_2	4-F	2,4,6-trimethyl	4.27	> 64
35	NH	O	NH_2	4-F	4-F	0.63	> 64
36	NH	O	NH_2	4-F	4-Me	0.39	> 64
37	NH	O	NH_2	H	4-Me	1.08	> 64
38	NH	O	NH_2	4-F	2-Cl,4-Me	0.51	> 64
39	NH	O	NH_2	4-CN	4-Me	0.96	> 64
40	NH	S	NH_2	4-F	4-F	>64	>64
41	NH	NH	NH_2	4-Me	2,6-diBr-4-Me	9.13	8.06
42	NH	NH	NH_2	4-F	2,6-diBr-4-Me	7.1	6.7
43	NH	NH	NH_2	4-F	4-F	2.77	> 64
44	NH	NH	NH_2	4-CN	4-F	39.83	> 64
45	NH	NH	NH_2	4-CN	4-Me	>64	>64
46	NH	NH	NH_2	4-CN	2-Cl,4-Me	7.7	> 64
47	NH	NH	NH_2	4-CN	2,6-diMe-4-F	7.87	23.74
50	NH	O	H	4-F	2,4,6-trimethyl	31.05	8.00
51	NH	O	H	H	2,4,6-trimethyl	32.46	17.76
Suramin[c]						0.03	

[a] *Each value is the mean of at least two independent determinations.* [b] *Cytotoxicity measurement using human lung fibroblast MRC-5 cells.* [c] *reference compound*

The structure-activity relationship follows the same pattern as the one previously found for this type of compounds.[10] The nature of the linker atom Y considerably influences the potency. If Y = O, the compounds are more potent than when Y = NH or Y = S (Compound **35** *vs* **40** and *vs* **43** or compound **39** *vs* **45**). When X = NH, the compound is more potent than when X = O (compound **27** *vs* **34**). The nature of Z also plays an important role in the potency. If Z = NH_2 the compound is 25-100 fold more potent than a piperidinyl, NHMe or H (compound **27** *vs* **28**, **32** and **50**, respectively).

Microsomal stability. The more potent compounds **27**, **35**, **36** and **38** were studied for *in vitro* metabolic stability with human and mouse microsomal fraction (S9) (**Figure 3**).

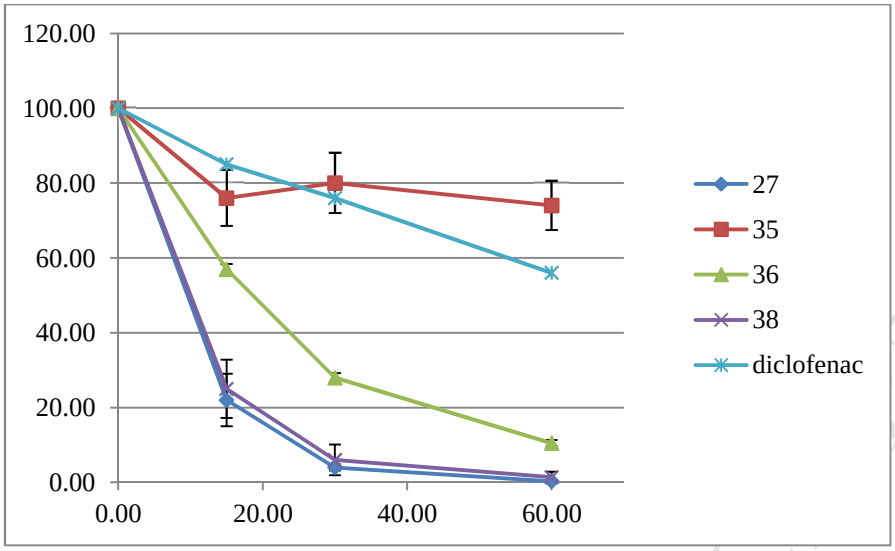


Figure 3. *In vitro* mouse microsomal stability of compounds **27**, **35**, **36** and **38**. Diclofenac was used as a reference compound.

As could be expected, the substituents on the two phenyl rings play an important role in the metabolic stability. Compound **35** (UAMC-03011) with a *para*-F in both phenyl rings is the most stable with 76% and 93% of remaining parent compound after 15 min upon incubation with mouse and human microsomes, respectively. All compounds had a (slightly) better metabolic stability compared to the starting lead compound **a**, which showed only 20% remaining parent compound after 15 min incubation with mouse microsomes. However, compounds **27**, **36** and **38** showed insufficient stability in mouse microsomes (remaining parent compound at 15 min of 22%, 57% and 25% respectively) to allow further study in mouse disease models. The plasma stability was also evaluated and compounds **27**, **35**, **36** and **38** remained fully stable after incubation in human plasma for 24 h at 37°C (Supporting information). Mouse plasma protein binding of **35** was 96% and plasma protein binding in the *T. brucei* culture medium was 50%, demonstrating a sufficiently high unbound fraction to exert anti-trypanosomal effect.

Pharmacokinetics

in view of its potent and selective anti-trypanosomal activity, and excellent microsomal and plasma stability, compound **35** was selected first for *in vivo* pharmacokinetic properties in mice and subsequent study in the acute *T. brucei* disease model. Compound **35** was administered orally to groups of mice using a single dose of either 50 mg/kg or 25 mg/kg. **Figure 4** shows the plasma concentrations of compound **35** *vs* time for the 50 mg/kg dose. Peak concentrations rise to about 10-fold the *in vitro* anti-trypanosomal IC_{50}, and then fall to about the IC_{50} of 0.63 µM at 24 h with a $T_{1/2}$ of about 6 h (**Table 2**).

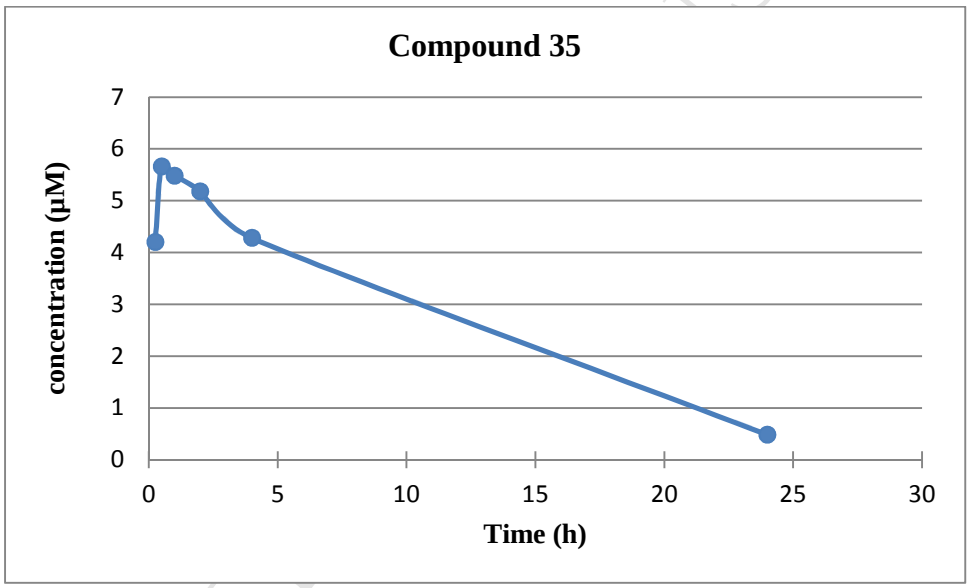


Figure 4. Concentration in blood of compound **35** after a single oral dose of 50 mg/kg in mice.

Table 2. Pharmacokinetic parameters of derivative **35**.

	T_{max} (h)	C_{max} (µM)	$T_{1/2}$ (h)	$AUC_{0->4}$ (ng.h/mL)	Vz (l)	Cl (mL/min)

50 mg/kg PO	0.5	5.66	6.51	28609	15.6	27.8

148

149

150 **Acute mouse model of *T. b. brucei***

151 The encouraging pharmacokinetic data and the high potency and selectivity of **35** motivated
152 to study this compound in an acute mouse disease model of *T. b. brucei*. The compound was
153 administered orally (PO) or injected intraperitoneally (IP) at doses differing from 10 to
154 50 mg/kg once (s.i.d.) or twice daily (b.i.d.) for 5 days after infection. Disappointingly, no *in*
155 *vivo* activity was demonstrated since the treated animals showed the same mean survival time
156 (MST < 7 days) as the vehicle-treated infected controls (**Table 3**).

157

158 **Table 3**. *In vivo* activities of compound **35** on the acute *T. b. brucei* model.

Compd.	dose (mg/kg)	freq.	MST	survivors on 14 dpi
Vehicle			7	0/3
Suramin	10	SID × 5 (IP)	> 7	3/3
35	25	BID × 5 (PO)	6	0/3
	50	BID × 5 (PO)	5.7	0/3
	50	SID × 5 (PO)	7	0/3
	10	SID × 5 (IP)	7	0/3

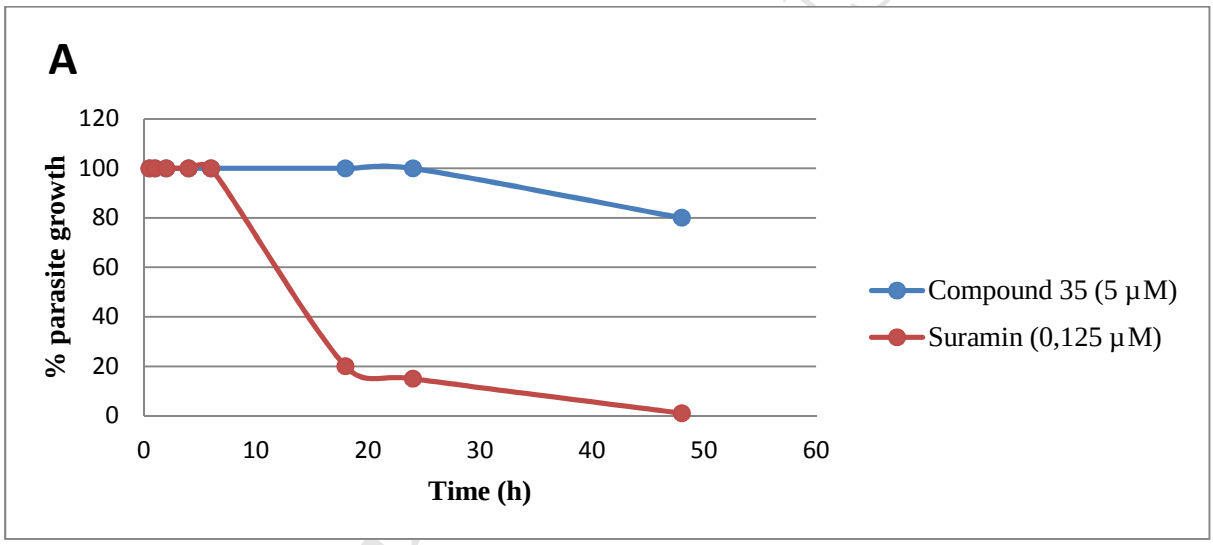
159

160

161 **Rate of trypanocidal activity of compound 35 compared to suramin**

162 To explain the lack of potency in the *in vivo* model for a compound of high potency and with
163 good pharmacokinetic properties, we decided to study the rate at which the compound is able

to kill the parasites.[13, 14] Based on the *in vivo* C_{max} that was around 10-fold the anti-trypanosomal IC_{50}, we investigated the parasite reduction over time using concentrations that were seven (**Figure 5A**) and forteen (**Figure 5B**) times higher than the IC_{50} of **35** and compared this with the reference compound suramin at concentrations which exceeded its IC_{50} in a similar way. Whereas suramin gave a very significant reduction after 18 h and a complete eradication after 48 h at both 7 and 14 times the IC_{50}, compound **35** only gave a very minor reduction of parasites after 48 h. It is clear that **35** is much slower acting than suramin.



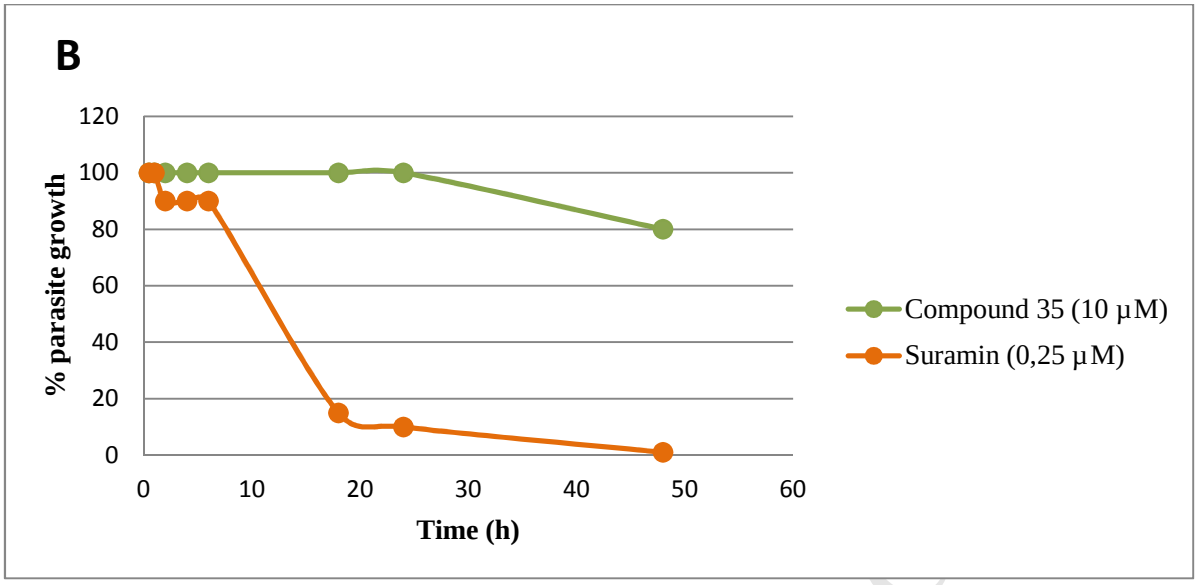


Figure 5. Graph A: % parasite growth vs time of compound **35** at 5 µM and suramin at 0.125 µM. Graph B: % parasite growth vs time of compound **35** at 10 µM and suramin at 0.25 µM.

CONCLUSIONS

A novel compound series was synthesized presenting a potent and selective anti-trypanosomal activity. By careful design considering the previously determined SAR and metabolic hotspots in the starting compound, we developed compound **35** (UAMC-03011) with a high *in vitro* microsomal stability and good pharmacokinetic properties while maintaining the excellent potency and selectivity. After oral administration at 50 mg/kg, **35** reached blood concentrations exceeding the IC_{50} by 10-fold and keeping the concentration in blood above or at the IC_{50} for 24 h until the next dose was administered. Despite these properties, compound **35** did not result in any activity in the acute mouse model of *T. b. brucei*, whereas the reference drug suramin at 100 mg/kg resulted in complete cure. Published pharmacokinetic data of suramin in rats treated with a single oral dose of 100 mg/kg showed a plasma concentration after 24 h of 2.3 µM, which is 76-fold its IC_{50}.[15]. Although rats and mice are not comparable, this indicates that the slightly higher plasma

concentrations of suramin compared to **35**, combined with its superior potency against *T. b. brucei* demonstrated by the 20-fold lower IC_{50} is probably responsible for the failure to demonstrate of *in vivo* activity of **35**. Furthermore, the rate of trypanocidal activity of **35** is much slower compared to suramin. In conclusion, the present study demonstrated that next to potency, selectivity, lack of toxicity and excellent pharmacokinetics, the rate of trypanocidal activity should be considered as an important parameter in lead selection and lead optimization.

EXPERIMENTAL PART

Chemistry. Reagents were purchased from commercial sources and without further purification. The products were purified with flash chromatography on a Flashmaster II (Jones chromatography) or on or IsoleraOne flash purification system from Biotage. Compounds were detected with UV light (254 nm). ^{1}H NMR spectra were obtained on a 400 MHz Bruker Avance DRX-400 and 400 MHz Bruker Avance III nanobay spectrometer with ultrashield. Typical spectral parameters: special width 16 ppm, pulse width 9 μs (57 °), data size 32 K. ^{13}C NMR experiments were carried out on the Bruker 400 MHz Bruker Avance DRX-400 and 400 MHz Bruker Avance III nanobay spectrometer with ultrashield operating at 100 MHz. The acquisition parameters: special width 16 ppm, pulse width 9 μs (57 °), data size 32 K. Chemical shifts are reported in values (ppm) relative to internal Me_4Si, and *J* values are reported in Hz. The UPLC (Ultra Performance liquid chromatography), used to quantify the purity of the products was an ACQUITY UPLC H-Class System with an TUV detector Waters coupled to a MS detector Waters QDa. An Acquity UPLC BEH C18 1.7μm (2.1 x 50 mm) column was used and as eluent a mixture of 0.1% FA in water, 0.1% FA in acetonitrile, water, acetonitrile. Final compounds were analyzed by high resolution mass. 10 μL of 10^{-5} M solution of each sample was injected using the CapLC system (Waters,

Manchester, UK) and electrosprayed using a standard electrospray source. Samples were injected with an interval of 5 min. Positive ion mode accurate mass spectra were acquired using a Q-TOF II instrument (Waters, Manchester, UK). The MS was calibrated prior to use with a 0.2% H_3PO_4 solution. The spectra were lock mass corrected using the know mass of the nearest H_3PO_4 cluster. All the compounds were obtained as amorphous solids. All the final compounds presented a purity at least 95% determined by UPLC and HNMR.

Synthesis of intermediates:

General procedure for Compounds **1** and **2**, **14 - 19**:

A solution of NaOH (1 equiv) in water (12 mL for 36.7 mmol) was added to a solution of phenol derivative (1 equiv), monoaryl-bischloro-triazine derivative (1 equiv) and tetrabutylammoniumhydrogensulfate (0.1 equiv) in toluene (120 mL for 36.7 mmol) at 0 °C. The reaction was stirred for a certain time at a fixed temperature specified in each case. The solvent was evaporated, dichloromethane (50 mL) and brine (50 mL) were added. The organic solvent was evaporated and the crude was used in the next step without further purification.

***2,4-Dichloro-6-(mesityloxy)-1,3,5-triazine* (*1*)**. Reagents: NaOH (1.45 g, 36.7 mmol), mesitol (5 g, 36.7 mmol), 2,4,6-trichloro-1,3,5-triazine (6.77 g, 36.7 mmol) and tetrabutylammoniumhydrogensulfate (1.25 g, 3.67 mmol). Reaction conditions: 2 h at 0 °C and 24 h at room temperature. Yield: 7.70 g, 65%. UPLC: purity 88%. m/z (ES) 284 [M], 286 [M + 2], 288 [M + 4].

2,4-Dichloro-6-(4-fluorophenoxy)-1,3,5-triazine (2). Reagents: NaOH (76 mg, 1.89 mmol), 4-fluorophenol (213 mg, 1.89 mmol), 2,4,6-trichloro-1,3,5-triazine (350 mg, 1.89 mmol) and tetrabutylammoniumhydrogensulfate (64.4 mg, 0.19 mmol). Reaction conditions: 2 h at 0 °C and 40 min at room temperature. Yield: 321.1 mg, 65%. UPLC: purity 70%. m/z (ES) 260 [M], 262 [M + 2], 264 [M + 4].

2-Chloro-4-(4-fluorophenoxy)-6-(mesityloxy)-1,3,5-triazine (14). Reagents: NaOH (23.07 mg, 0.57 mmol), mesitol (79 mg, 0.57 mmol), intermediate **2** (150 mg, 0.57 mmol) and tetrabutylammoniumhydrogensulfate (19.58 mg, 0.06 mmol). Reaction conditions: 5 min at 0 °C. Yield: 125 mg, 60%. UPLC: purity 50%. m/z (ES) 360 [M + 1], 362 [M + 3].

4-Chloro-6-(4-fluorophenoxy)-N-phenyl-1,3,5-triazin-2-amine (15). Reagents: NaOH (19.91 mg, 0.49 mmol), 4-fluorophenol (55.8 mg, 0.49 mmol), intermediate **3** (120 mg, 0.49 mmol) and tetrabutylammoniumhydrogensulfate (16.9 mg, 0.05 mmol). Reaction conditions: 5 min at 0 °C and after that, 1 h at room temperature. Yield: 102.7 mg, 65%. UPLC: purity 65%. m/z (ES) 317 [M + 1], 319 [M + 3].

4-Chloro-N-(4-fluorophenyl)-6-(p-tolyloxy)-1,3,5-triazin-2-amine (16). Reagents: NaOH (18.53 mg, 0.46 mmol), *p*-cresol (50.1 mg, 0.46 mmol), intermediate **3** (120 mg, 0.46 mmol) and tetrabutylammoniumhydrogensulfate (15.73 mg, 0.05 mmol). Reaction conditions: 5 min at 0 °C and after that 1 h at room temperature. Yield: 96.3 mg, 63%. UPLC: purity 63%. m/z (ES) 331 [M + 1], 333 [M + 3].

4-Chloro-N-phenyl-6-(p-tolyloxy)-1,3,5-triazin-2-amine (17). Reagents: NaOH (33.2 mg, 0.83 mmol), *p*-cresol (90 mg, 0.83 mmol), intermediate **4** (200 mg, 0.83 mmol) and tetrabutylammoniumhydrogensulfate (28.2 mg, 0.08 mmol). Reaction conditions: 5 min at 0 °C and after that 40 min at room temperature. Yield: 176 mg, 68%. UPLC: purity 68%. m/z (ES) 313 [M + 1], 315 [M + 3].

4-Chloro-6-(2-chloro-4-methylphenoxy)-N-(4-fluorophenyl)-1,3,5-triazin-2-amine (18). Reagents: NaOH (18.5 mg, 0.46 mmol), 2-chloro-4-methylphenol (66 mg, 0.46 mmol), intermediate **3** (120 mg, 0.46 mmol) and tetrabutylammoniumhydrogensulfate (15.7 mg, 0.04 mmol). Reaction conditions: 5 min at 0 °C and after that 1 h at room temperature. Yield: 109.8 mg, 68%.

4-((4-Chloro-6-(p-tolyloxy)-1,3,5-triazin-2-yl)amino)benzonitrile (19). Reagents: NaOH (29.6 mg, 0.74 mmol), *p*-cresol (0,08 ml, 0,74 mmol), intermediate **5** (250 mg, 0,74 mmol), and tetrabutylammonium hydrogensulfate (252 mg, 0,74 mmol). Reaction conditions: 5 min at 0 °C and after that 5 days at room temperature. Yield: 0,13 g, 52%. UPLC: purity 50%. m/z 338 [M], 340 [M + 2].

General procedures for compounds **3** – **13**, **21** - **26**:

A solution of the specified aniline (1 equiv), 2,4,6-trichloro-1,3,5-triazine (1 equiv) and a determined base (1 – 1.4 equiv) in dioxane (12 mL for 5.42 mmol) was stirred for a specified time at fixed temperature. The solvent was evaporated and DCM (50 mL) was added, the crude was extracted with a satured solution of $NaHCO_3$ (50 mL) and a satured solution of NaCl (50 mL). The organic phase was dried and the solvent was evaporated under vacuum. The product was purified by IsoleraOne using the eluents specified for each reaction.

4,6-Dichloro-N-(4-fluorophenyl)-1,3,5-triazin-2-amine (3). Reagents: 4-fluorobenzenamine (603 mg, 5.42 mmol), 2,4,6-trichloro-1,3,5-triazine (1 g, 5.42 mmol) and potassium carbonate (1.05 g, 7.59 mmol). Reaction conditions: 40 min at 25 °C. Purification: heptane/ ethyl acetate (0 – 100 ethyl acetate). Yield: 270 mg, 20%. UPLC: purity > 99%. m/z (ES) 259 [M], 261 [M + 2], 263 [M + 4].

4,6-Dichloro-N-phenyl-1,3,5-triazin-2-amine (4). Reagents: aniline (177 mg, 1.89 mmol), 2,4,6-trichloro-1,3,5-triazine (350 mg, 1.89 mmol) and potassium carbonate (367 mg, 2.66 mmol. Reaction conditions: 5 min at 25 °C. Purification: heptane/ ethyl acetate (0 – 100 ethyl acetate). Yield: 371.9 mg, 57%. UPLC: purity > 99%. m/z (ES) 241 [M], 243 [M + 2], 245 [M + 4].

4-((4,6-Dichloro-1,3,5-triazin-2-yl)amino)benzonitrile (5). Reagents: 2,4,6-trichloro-1,3,5-triazine (5 g, 27,1 mmol), 4-aminobenzonitrile (3,36 g, 28,45 mmol) and potassium carbonate (3,93 g, 28,5 mmol). Reaction conditions: 48 h at 25 °C and 2 h extra at 101 °C. Purification:

heptane/ ethyl acetate (0 – 100 ethyl acetate). Yield: 1,7 g, 23%. UPLC: purity 79%. m/z (ES) 266 [M], 268 [M + 2], 270 [M + 4].

4,6-Dichloro-N-(4-fluorophenyl)-1,3,5-triazin-2-amine (6). Reagents: *N*-ethyl-*N*-isopropylpropan-2-amine (2,26 ml, 13,01 mmol), 4-fluorobenzenamine (1,03 ml, 10,85 mmol) and 2,4,6-trichloro-1,3,5-triazine (2g, 10,85 mmol). Reaction conditions: 25 °C for 2 h. Purification: heptane/ ethyl acetate (0 – 100 ethyl acetate). Yield: 1,67 g, 60%. UPLC: purity > 99%. m/z (ES) 259 [M], 261 [M + 2], [M + 4].

4,6-Dichloro-N-(2,6-dibromo-4-methylphenyl)-1,3,5-triazin-2-amine (7). Reagents: 2,4,6-trichloro-1,3,5-triazine (1 g, 5,42 mmol), 2,6-dibromo-4-methylaniline (1,51 g, 5,69 mmol) and potassium carbonate (0,78 g, 5,70 mmol). Reaction conditions: 25 °C for 48 h. Purification: heptane/ ethyl acetate (0 – 100 ethyl acetate). Yield: 0,79 g, 34%. UPLC: purity: 84%. m/z (ES) [M - 2], [M + 2], [M + 4].

6-Chloro-N^2,N^4-bis(4-fluorophenyl)-1,3,5-triazine-2,4-diamine (8). Reagents: *N*-ethyl-*N*-isopropylpropan-2-amine (0,34 ml, 1,95 mmol), 4-fluorobenzenamine (0,31 ml, 3,25 mmol) and 2,4,6-trichloro-1,3,5-triazine (300 mg, 1,63 mmol). Reaction conditions: 25 °C for 24 h. Purification: heptane/ ethyl acetate (0 – 100 ethyl acetate). Yield: 224,5 mg, 41%. UPLC: purity: >99%. m/z (ES) 334 [M + 1], 336 [M + 3].

4-Chloro-N-(4-fluorophenyl)-6-(mesityloxy)-1,3,5-triazin-2-amine (9). Reagents: intermediate **1**, (2 g, 7.04 mmol), 4-fluoroaniline (0.78 g, 7.04 mmol), *N*-ethyl-*N*-isopropylpropan-2-amine (0.9 g, 7.04 mmol). Reaction conditions: 4 days at room temperature. Purification: hexane/ ethyl acetate (0 – 100 ethyl acetate). Yield: 1.3 g, 52%. UPLC: purity 88%. m/z (ES) 359 [M + 1], 361 [M + 3].

4-Chloro-N-(4-chlorophenyl)-6-(mesityloxy)-1,3,5-triazin-2-amine (10). Reagents: intermediate **1** (1 g, 3.5 mmol), 4-chloroaniline (0.45 g, 3.5 mmol), *N*-ethyl-*N*-isopropylpropan-2-amine (0.45 g, 3.5 mmol). Reaction conditions: 4 days at room

temperature. Purification: hexane/ ethyl acetate (0 – 100 ethyl acetate). Yield: 638.1 mg, 49%. UPLC: purity 99%. m/z (ES) 375 [M], 377 [M + 2], 379 [M + 4].

4-Chloro-6-(mesityloxy)-N-(4-(trifluoromethyl)phenyl)-1,3,5-triazin-2-amine (11). Reagents: intermediate **1** (500 mg, 1.76 mmol), 4-(trifluoromethyl)aniline (284 mg, 1.76 mmol), *N*-ethyl-*N*-isopropylpropan-2-amine (273 mg, 2.1 mmol). Reaction conditions: 45 min at 101 °C. Purification: hexane/ ethyl acetate (0 – 100 ethyl acetate). Yield: 409 mg, 57%. UPLC: purity > 99%. m/z (ES) 407 [M - 1], 309 [M + 1].

4-Chloro-N-(3,5-difluorophenyl)-6-(mesityloxy)-1,3,5-triazin-2-amine (12). Reagents: intermediate **1** (500 mg, 1.76 mmol), 3,5-difluoroaniline (227 mg, 1.76 mmol), *N*-ethyl-*N*-isopropylpropan-2-amine (273 mg, 2.1 mmol). Reaction conditions: 45 min at 101 °C. Purification: hexane/ ethyl acetate (0 – 100 ethyl acetate). Yield: 500.8 mg, 76%. UPLC: purity 85%. m/z (ES) 377 [M + 1], 379 [M + 3].

4-Chloro-N-(2,6-difluorophenyl)-6-(mesityloxy)-1,3,5-triazin-2-amine (13). Reagents: intermediate **1** (616 mg, 2.17 mmol), 2,6-difluoroaniline (280.2 mg, 2.17 mmol), *N*-ethyl-*N*-isopropylpropan-2-amine (280.2 mg, 2.17 mmol). Reaction conditions: 6 h at 101 °C. Purification: hexane/ ethyl acetate (0 – 100 ethyl acetate). Yield: 570 mg, 70%. UPLC: purity >99%. m/z (ES) 377 [M + 1], 379 [M + 3].

6-Chloro-N^2-(2,6-dibromo-4-methylphenyl)-N^4-(p-tolyl)-1,3,5-triazine-2,4-diamine (21). Reagents: intermediate **7** (300 mg, 0,61 mmol), *p*-toluidine (65,4 mg, 0,61 mmol), *N*-ethyl-*N*-isopropylpropan-2-amine (0,12 ml, 0,67 mmol). Reaction conditions: 19 h at 101 °C. Purification: heptane/ ethyl acetate (0 – 100 ethyl acetate). Yield: 216 mg, 73%. UPLC: purity > 99%. m/z (ES) [M - 2], [M + 2], [M + 4].

6-Chloro-N^2-(2,6-dibromo-4-methylphenyl)-N^4-(4-fluorophenyl)-1,3,5-triazine-2,4-diamine (22). Reagents: intermediate **6** (300 mg, 0,62 mmol), 4-fluoroaniline (0,06 ml, 0,62 mmol), *N*-ethyl-*N*-isopropylpropan-2-amine (0,11 ml, 0,62 mmol). Reaction conditions: 19 h at 101

°C. Purification: heptane/ ethyl acetate (0 – 100 ethyl acetate). Yield: 0,21 g, 69%. UPLC: purity > 99%. m/z (ES) 488 [M - 2], 487 [M], 489 [M + 2], 491 [M + 4].

4-((4-Chloro-6-((4-fluorophenyl)amino)-1,3,5-triazin-2-yl)amino)benzonitrile (23). Reagents: intermediate **5** (300 mg, 1,13 mmol), 4-fluoroaniline (0,11 ml, 1,13 mmol), *N*-ethyl-*N*-isopropylpropan-2-amine (0,21 ml, 1,24 mmol). Reaction conditions: 1 h at 101 °C. The organic crude was used in the next step without further purification (270.6 mg, 71%). UPLC: purity: 97%. m/z (ES) 341 [M + 1], 343 [M + 3].

4-((4-Chloro-6-(p-tolylamino)-1,3,5-triazin-2-yl)amino)benzonitrile (24). Reagents: intermediate **5** (300 mg, 1,13 mmol), *p*-toluidine (121 mg, 1,127 mmol), *N*-ethyl-*N*-isopropylpropan-2-amine (0,21 ml, 1,24 mmol) and. Reaction conditions: 1 h at 101 °C. The organic crude was used in the next step without further purification (342 mg, 90%). UPLC: purity: 90%. m/z (ES) 337[M + 1], 339 [M + 3].

4-((4-Chloro-6-((2-chloro-4-methylphenyl)amino)-1,3,5-triazin-2-yl)amino)benzonitrile (25). Reagents: intermediate **5** (250 mg, 0,74 mmol), 2-chloro-4-methylaniline (0,09 ml, 0,74 mmol), *N*-ethyl-*N*-isopropylpropan-2-amine (0,13 ml, 0,74 mmol). Reaction conditions: 22 h at 101 °C. The organic crude was used in the next step without further purification (0,3 g, 99% yield). UPLC: purity: 92%. m/z (ES) 371 [M], 373 [M + 2], 375 [M + 4].

4-((4-Chloro-6-((4-fluoro-2,6-dimethylphenyl)amino)-1,3,5-triazin-2-yl)amino)benzonitrile (26). Reagents: intermediate **5** (250 mg, 0,74 mmol), 4-fluoro-2,6-dimethylaniline (103 mg, 0,74 mmol), *N*-ethyl-*N*-isopropylpropan-2-amine (0,12 mL, 0,74 mmol). Reaction conditions: 4 h at 101 °C. The crude was used in the next reaction without further purification (0,29 g, 99%). UPLC: purity 91%. m/z (ES) 369 [M], 371 [M + 2].

4-Chloro-N-(4-fluorophenyl)-6-((4-fluorophenyl)thio)-1,3,5-triazin-2-amine (20). A solution of 4,6-dichloro-*N*-(4-fluorophenyl)-1,3,5-triazin-2-amine (300 mg, 1,16 mmol) in THF (4,2 mL) was cooled down to 0 °C. A second solution containing 4-fluorobenzenethiol

(135 mg, 1,05 mmol) and *N*-ethyl-*N*-isopropylpropan-2-amine (0,18 ml, 1,05 mmol) in THF (9 mL) was added dropwise to this first solution. The final solution was stirred for 1 h at 0 °C. The solution was concentrated in vacuo and the residue was redissolved in ethyl acetate (25 mL) and washed with water (25 mL), a saturated solution of $NaHCO_3$ (25 mL) and brine (25 mL).The crude was purified by IsoleraOne using heptane and ethyl acetate (0 – 100% ethyl acetate) as eluents (0,31 g, 56%). UPLC: purity: 42%. m/z (ES) 351 [M + 2].

4-Chloro-N-(4-fluorophenyl)-1,3,5-triazin-2-amine (48). A solution of 2,4-dichloro-1,3,5-triazine (300 mg, 2.0 mmol), 4-fluorobenzenamine (222 mg, 2.0 mmol) and *N*-ethyl-*N*-isopropylpropan-2-amine (284 mg, 2.2 mmol) in dioxane (4 mL) was stirred at room temperature during 5 min. Dichloromethane (50 mL) and water (50 mL) were added and the organic phase was dried over magnesium sulphate and the organic solvent was evaporated under vacuum. The crude was used in the next step without further purification (449 mg, 99%). UPLC: purity 99%. m/z (ES) 225 [M + 1], 227 [M + 3].

4-Chloro-N-phenyl-1,3,5-triazin-2-amine (49). A solution of 2,4-dichloro-1,3,5-triazine (300 mg, 2.0 mmol), aniline (186 mg, 2.0 mmol) and *N*-ethyl-*N*-isopropylpropan-2-amine (284 mg, 2.2 mmol) in dioxane (4 mL) was stirred at room temperature during 5 min. Dichloromethane (50 mL) and water (50 mL) were added and the organic phase was dried over magnesium sulphate and the organic solvent was evaporated under vacuum. The crude was used in the next step without further purification (413 mg, 99%). UPLC: purity 99%. m/z (ES) 207 [M + 1], 209 [M + 3].

Synthesis of final compounds:

General procedure for compounds **27 - 47**:

The triazine derivative (1 equiv) was dissolved in a solution of ammonia in a specified solvent with a determined normality. The solution was heated at a specified temperature for a

different time, given for each reaction. The solvent was evaporated and the crude was purified by IsoleraOne using different eluents for each reaction.

***N^2-(4-Fluorophenyl)-6-(mesityloxy)-1,3,5-triazine-2,4-diamine* (*27*)**: Reagents: 4-chloro-*N*-(4-fluorophenyl)-6-(mesityloxy)-1,3,5-triazin-2-amine (300 mg, 0.8 mmol) and ammonia 7 N in methanol (40 mL). Reaction conditions: 65 °C for 4 h. Purification: IsoleraOne using DCM/MeOH (0 – 10% MeOH). Yield: 171 mg, 60%. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 9.51 (s, 1H), 7.65 (s, 2H), 7.22 - 6.84 (m, 6H), 2.24 (s, 3H), 2.04 (s, 6H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 169.9, 168.4, 165.5, 157.41 (d, J = 239.1 Hz), 147.2, 136.2 (d, J = 2.3 Hz), 134.0, 129.7, 129.0, 121.4, 114.77 (d, J = 21.8 Hz), 20.3, 16.1. UPLC: purity > 99%. m/z (ES) 340.1 [M + 1]. HRMS: Calc: 339.15 Found: 340.1584 [M + 1].

***N^2-(4-Chlorophenyl)-6-(mesityloxy)-1,3,5-triazine-2,4-diamine* (*29*)**: Reagents: 4-chloro-*N*-(4-chlorophenyl)-6-(mesityloxy)-1,3,5-triazin-2-amine (300 mg, 0.8 mmol) and ammonia 7 N in methanol (40 mL). Reaction conditions: 65 °C for 16 h. Purification: IsoleraOne using DCM/MeOH (0 – 10% MeOH). Yield: 184.1 mg, 65%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.63 (s, 1H), 7.68 (s, 2H), 7.36 - 6.99 (m, 4H), 6.90 (s, 2H), 2.24 (s, 3H), 2.03 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 170.0, 168.4, 165.5, 147.2, 138.9, 134.0, 129.7, 129.0, 128.1, 125.5, 121.1, 20.3, 16.1. UPLC: purity > 99%. m/z (ES) 356.1 [M + 1], 358.1 [M + 3]. HRMS: Calc: 355.12 Found: 356.1278 [M + 1].

***6-(Mesityloxy)-N^2-(4-(trifluoromethyl)phenyl)-1,3,5-triazine-2,4-diamine* (*30*)**: Reagents: 4-chloro-6-(mesityloxy)-*N*-(4-(trifluoromethyl)phenyl)-1,3,5-triazin-2-amine (409 mg, 1.0 mmol) and ammonia 7 N in methanol (40 mL). Reaction conditions: 65 °C for 2 h. Purification: IsoleraOne using hexane/ethyl acetate (0 – 100% ethyl acetate). Yield: 209.7 mg, 54%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.89 (s, 1H), 7.85 (s, 2H), 7.50 (d, J = 7.9 Hz, 2H), 7.25 (d, J = 34.8 Hz, 2H), 6.92 (s, 2H), 2.25 (s, 3H), 2.04 (s, 6H). ^{13}C NMR (100 MHz,

DMSO-d_6) δ: 170.1, 168.5, 165.6, 147.2, 143.62 (c, J = 1.0 Hz), 134.1, 129.7, 129.0, 125.9 (c, J = 271.1 Hz), 125.5 (c, J = 3.6 Hz), 121.9 (c, J = 32.0 Hz), 119.3, 20.3, 16.1. UPLC: purity > 99%. m/z (ES) 390.2 [M + 1]. HRMS: Calc: 389.15 Found: 390.1542 [M + 1].

***N^2-(3,5-Difluorophenyl)-6-(mesityloxy)-1,3,5-triazine-2,4-diamine* (*31*)**: Reagents: 4-chloro-*N*-(3,5-difluorophenyl)-6-(mesityloxy)-1,3,5-triazin-2-amine (409 mg, 1.0 mmol) and ammonia 7 N in methanol (40 mL). Reaction conditions: 65 °C for 2 h. Purification: IsoleraOne using hexane/ethyl acetate (0 – 100% ethyl acetate). Yield: 173.2 mg, 36%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.85 (s, 1H), 7.77 - 7.01 (m, 4H), 6.90 (s, 2H), 6.70 (t, J = 8.9 Hz, 1H), 2.23 (s, 3H), 2.03 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 170.0, 168.4, 165.5, 162.31 (dd, J = 241.6, 15.7 Hz), 147.0, 142.6 (t, J = 14.2 Hz), 134.2, 129.5, 129.0, 102.0 (d, J = 29.3 Hz), 96.7 (t, J = 26.3 Hz), 20.3, 16.1. UPLC: purity > 99%. m/z (ES) 358.1 [M + 1]. HRMS: Calc: 357.14 Found: 358.1479 [M + 1].

***N^2-(2,6-Difluorophenyl)-6-(mesityloxy)-1,3,5-triazine-2,4-diamine* (*33*)**: Reagents: 4-chloro-*N*-(2,6-difluorophenyl)-6-(mesityloxy)-1,3,5-triazin-2-amine (500 mg, 1.3 mmol) and ammonia 7 N in methanol (40 mL). Reaction conditions: 65 °C for 16 h. Purification: IsoleraOne using hexane/ethyl acetate (0 – 100% ethyl acetate). Yield: 248 mg, 52%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.00 (s, 1H), 7.32 (tt, J = 7.9, 6.3 Hz, 1H), 7.13 (t, J = 8 Hz, 2H), 7.01 (d, J = 13.2 Hz, 2H), 6.87 (s, 2H), 2.22 (s, 3H), 2.03 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 170.2, 168.6, 167.1, 158.6 (d, J = 248.4 Hz), 147.1, 133.9, 129.6, 128.9, 127.7 (t, J = 5.5 Hz), 115.5 (t, J = 16.4 Hz), 111.8 (dd, J = 18.1, 5.4 Hz), 20.3, 16.1. UPLC: purity > 99%. m/z (ES) 358.2 [M + 1]. HRMS: Calc: 357.14 Found: 358.1485 [M + 1].

***4-(4-Fluorophenoxy)-6-(mesityloxy)-1,3,5-triazin-2-amine* (*34*)**: Reagents: 2-chloro-4-(4-fluorophenoxy)-6-(mesityloxy)-1,3,5-triazine (205 mg, 0.57 mmol) and ammonia 7 N in methanol (20 mL). Reaction conditions: 65 °C for 16 h. Purification: IsoleraOne using hexane/ethyl acetate (0 – 100% ethyl acetate). Yield: 38.1 mg, 20%. ^{1}H NMR (400 MHz,

$CDCl_3$) δ: 7.15 - 7.02 (m, 4H), 6.88 (d, *J* = 0.5 Hz, 2H), 2.29 (s, 3H), 2.11 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 172.8, 172.1, 169.5, 160.3 (d, *J* = 244.2 Hz), 147.8, 147.1, 135.5, 130.0, 129.3, 123.2 (d, *J* = 8.5 Hz), 116.1 (d, *J* = 23.5 Hz), 20.9, 16.5. UPLC: purity > 99%. m/z (ES) 341.1 [M + 1]. HRMS: Calc: 340.13 Found: 341.1412 [M + 1].

6-(4-Fluorophenoxy)-N^2-(4-fluorophenyl)-1,3,5-triazine-2,4-diamine **(*35*)**: Reagents: 4-chloro-6-(4-fluorophenoxy)-*N*-(4-fluorophenyl)-1,3,5-triazin-2-amine (167 mg, 0.5 mmol) and ammonia 7 N in methanol (20 mL). Reaction conditions: 65 °C for 16 h. Purification: IsoleraOne using hexane/ethyl acetate (0 – 100% ethyl acetate). Yield: 46.7 mg, 30%. ^{1}H NMR (400 MHz, MeOD-d_4) δ: 7.51 (s, 2H), 7.24 - 7.08 (m, 4H), 6.92 (s, 2H). ^{13}C NMR (100 MHz, MeOD-d_4) δ: 172.5, 169.9, 166.9, 161.5 (d, *J* = 242.3 Hz), 160.1 (d, *J* = 240.6 Hz), 149.8 (d, *J* = 2.8 Hz), 136.5 (d, *J* = 2.3 Hz), 124.8 (d, *J* = 8.5 Hz), 123.5 (d, *J* = 6.2 Hz), 116.9 (d, *J* = 23.7 Hz), 115.8 (d, *J* = 22.5 Hz). UPLC: purity > 99%. m/z (ES) 316.1 [M + 1]. HRMS: Calc: 315.09 Found: 316.1016 [M + 1].

N^2-(4-Fluorophenyl)-6-(p-tolyloxy)-1,3,5-triazine-2,4-diamine **(*36*)**: Reagents: 4-chloro-*N*-(4-fluorophenyl)-6-(*p*-tolyloxy)-1,3,5-triazin-2-amine (165 mg, 0.5 mmol) and ammonia 7 N in methanol (20 mL). Reaction conditions: 65 °C for 16 h. Purification: IsoleraOne using hexane/ethyl acetate (0 – 100% ethyl acetate). Yield: 49.6 mg, 32%. ^{1}H NMR (400 MHz, DMSO-d_4) δ: 9.49 (s, 1H), 7.65 (s, 2H), 7.21 (d, *J* = 8.3 Hz, 2H), 7.12 (s, 1H), 7.08 – 6.96 (m, 4H), 2.32 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 170.95, 168.29, 165.36, 157.45 (d, *J* = 242.1 Hz), 150.10, 136.07 (d, *J* = 2.3 Hz), 134.18, 129.78, 121.79, 121.59 (d, *J* = 7.5 Hz), 114.77 (d, *J* = 21.8 Hz), 20.40. UPLC: purity > 99%. m/z (ES) 312.1 [M + 1]. HRMS: Calc: 311.12 Found: 312.1257 [M + 1].

N^2-Phenyl-6-(p-tolyloxy)-1,3,5-triazine-2,4-diamine **(*37*)**: Reagents: 4-Chloro-*N*-phenyl-6-(*p*-tolyloxy)-1,3,5-triazin-2-amine (0.3 g, 0.8 mmol) and ammonia 7 N in methanol (20 mL). Reaction conditions: 65 °C for 16 h. Purification: IsoleraOne using hexane/ethyl acetate (0 –

100% ethyl acetate). Yield: 79.9 mg, 33%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.45 (s, 1H), 7.64 (s, 2H), 7.29 – 6.98 (m, 8H), 6.93 (t, *J* = 7.3 Hz, 1H), 2.32 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 170.9, 168.3, 165.5, 150.1, 139.7, 134.2, 129.8, 128.3, 122.0, 121.8, 119.9, 20.4. UPLC: purity > 99%. m/z (ES) 294.1 [M + 1]. HRMS: Calc: 293.13 Found: 394.1345 [M + 1].

6-(2-Chloro-4-methylphenoxy)-N^2-(4-fluorophenyl)-1,3,5-triazine-2,4-diamine **(*38*)**: Reagents: 4-chloro-6-(2-chloro-4-methylphenoxy)-*N*-(4-fluorophenyl)-1,3,5-triazin-2-amine (183 mg, 0.5 mmol) and ammonia 7 N in methanol (20 mL). Reaction conditions: 65 °C for 16 h. Purification: IsoleraOne using hexane/ethyl acetate (0 – 100% ethyl acetate). Yield: 56.8 mg, 33%. ^{1}H NMR (400 MHz, MeOD) δ 7.48 (s, 2H), 7.32 (s, 1H), 7.17 (dd, *J* = 8.3, 1.4 Hz, 1H), 7.12 (d, *J* = 8.2 Hz, 1H) 6.90 (s, 2H), 2.37 (s, 3H). ^{13}C NMR (100 MHz, MeOD-d_4) δ: 172.0, 169.9, 166.9, 160.1 (d, *J* = 240.6 Hz), 147.6, 138.3, 136.5 (d, *J* = 2.7 Hz), 131.5, 129.6, 128.0, 124.9, 123.4, 115.8 (d, *J* = 22.6 Hz), 20.7. UPLC: purity > 99%. m/z (ES) 346.1 [M + 1]. HRMS: Calc: 345.08 Found: 346.0874 [M + 1].

4-((4-Amino-6-(p-tolyloxy)-1,3,5-triazin-2-yl)amino)benzonitrile **(*39*)**: Reagents: 4-((4-chloro-6-(*p*-tolyloxy)-1,3,5-triazin-2-yl)amino)benzonitrile (130 mg, 0,4 mmol) and ammonia 0.5 N in dioxane (100 ml). Reaction conditions: 101 °C for 24 h. Purification: IsoleraOne using heptane/ethyl acetate (0 – 100% ethyl acetate). Yield: 9 mg, 7%. ^{1}H NMR (400 MHz, Acetone-d_6) δ: 9.02 (s, 1H), 7.95 (d, *J* = 7.3 Hz, 2H), 7.58 (d, *J* = 8.6 Hz, 2H), 7.26 (d, *J* = 8.3 Hz, 2H), 7.06 (d, *J* = 8.4 Hz, 2H), 6.65 (s, 2H), 2.39 (s, 3H). UPLC: purity: 97%. m/z (ES) 319.1 [M + 1].

N^2-(4-Fluorophenyl)-6-((4-fluorophenyl)thio)-1,3,5-triazine-2,4-diamine **(*40*)**: Reagents: 4-chloro-*N*-(4-fluorophenyl)-6-((4-fluorophenyl)thio)-1,3,5-triazin-2-amine (310.8 mg, 0,6 mmol) and ammonia 0.5 N in dioxane (50 ml). Reaction conditions: 101 °C for 17 h. Purification: IsoleraOne using heptane/ethyl acetate (0 – 100% ethyl acetate). Yield: 177.4

mg, 90%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.45 (s, 1H), 7.71 – 7.58 (m, 2H), 7.42 – 6.72 (m, 8H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 165.4, 162.9 (d, J = 244.9 Hz), 162.7, 157.3 (d, J = 233.7 Hz), 138.4, 135.9 (d, J = 2.4 Hz), 124.1 (d, J = 3.0 Hz), 121.2, 116.3 (d, J = 21.9 Hz), 114.6 (d, J = 23.8 Hz). UPLC: purity > 99%. m/z (ES) 332.1 [M + 1]. HRMS: Calc: 331.07 Found: 332.0779 [M + 1].

***N^2-(2,6-Dibromo-4-methylphenyl)-N4-(p-tolyl)-1,3,5-triazine-2,4,6-triamine* (41)**: Reagents: 6-chloro-N^2-(2,6-dibromo-4-methylphenyl)-N^4-(*p*-tolyl)-1,3,5-triazine-2,4-diamine (144.5 mg, 0,3 mmol) and ammonia 7 N in methanol (100 ml). Reaction conditions: 65 °C for 8 days. Purification: IsoleraOne using heptane/ethyl acetate (0 – 100% ethyl acetate). Yield: 71.7 mg, 52%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.95 – 8.75 (m, 1H), 8.75 – 8.50 (m, 1H), 7.78 – 7.21 (m, 4H), 7.18 – 6.69 (m, 2H), 6.43 (s, 2H), 2.39 – 2.28 (m, 3H), 2.28 – 2.12 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.1, 165.4, 139.7, 138.0, 134.4, 132.4, 129.8, 128.6, 125.4, 119.5, 20.4, 19.8. UPLC: purity > 99%. m/z (ES) 463.1 [M - 1], 465.1 [M + 1], 467.1 [M + 3]. HRMS: Calc: 463.98 Found: 462.9895 [M - 1].

***N^2-(2,6-Dibromo-4-methylphenyl)-N^4-(4-fluorophenyl)-1,3,5-triazine-2,4,6-triamine* (42)**: Reagents: 6-chloro-N^2-(2,6-dibromo-4-methylphenyl)-N^4-(4-fluorophenyl)-1,3,5-triazine-2,4-diamine (207.7 mg, 0,4 mmol) and ammonia 0.5 N in dioxane (75 ml). Reaction conditions: 101 °C for 5 days. Purification: IsoleraOne using DCM/methanol (0 – 10% methanol). Yield: 45 mg, 23%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.28 – 8.60 (m, 2H), 7.94 – 7.36 (m, 4H), 7.26 – 6.34 (m, 4H), 2.34 (s, 3H). ^{13}C NMR (100 MHz, Acetone) δ: 167.5, 165.8, 164.8, 157.9 (d, J = 238.8 Hz), 140.1, 136.7, 134.3, 132.6, 125.3, 121.1, 114.5 (d, J = 27.4 Hz), 19.5. UPLC: purity > 99%. m/z (ES) 467.0 [M - 1], 469.0 [M + 1], 471.0 [M + 3]. HRMS: Calc: 467.95 Found: 466.9628 [M - 1].

***N^2,N^4-bis(4-Fluorophenyl)-1,3,5-triazine-2,4,6-triamine* (43)**: Reagents: 6-chloro-N^2,N^4-bis(4-fluorophenyl)-1,3,5-triazine-2,4-diamine (224 mg, 0,7 mmol) and ammonia 7 N in

methanol (200 mL). Reaction conditions: 65 °C for 8 days. Purification: IsoleraOne using heptane/ethyl acetate (0 – 100% ethyl acetate). Yield: 52.3 mg, 25%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.10 (s, 2H), 7.76 (s, 4H), 7.12 - 7.03 (m, 4H), 6.61 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.7, 164.3, 157.3 (d, J = 238.1 Hz), 136.9, 121.5 (d, J = 5.5 Hz), 114.8 (d, J = 21.9 Hz). UPLC: purity > 99%. m/z (ES) 315.1 [M + 1]. HRMS: Calc: 314.11 Found: 315.1169 [M + 1].

4-((4-Amino-6-((4-fluorophenyl)amino)-1,3,5-triazin-2-yl)amino)benzonitrile **(*44*)**: Reagents: 4-((4-chloro-6-((4-fluorophenyl)amino)-1,3,5-triazin-2-yl)amino)benzonitrile (338.3 mg, 0,8 mmol) and ammonia 7 N in methanol (200 ml). Reaction conditions: 65 °C for 24 h. Purification: IsoleraOne using water/methanol (0 – 100% methanol). Yield: 26.1 mg, 10% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.59 (s, 1H), 9.24 (s, 1H), 8.02 (d, J = 8.7 Hz, 2H), 7.82 – 7.64 (m, 4H), 7.19 – 7.05 (m, 2H), 6.79 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.7, 164.3, 164.2, 157.4 (d, J = 237.6 Hz), 144.9, 136.3, 132.8, 132.7, 121.8 (d, J = 6.9 Hz), 119.6, 119.2, 114.8 (d, J = 22.0 Hz), 102.5. UPLC: purity > 99%. m/z (ES) 322.1 [M + 1]. HRMS: Calc: 321.11 Found: 322.1234 [M + 1].

4-((4-Amino-6-(p-tolylamino)-1,3,5-triazin-2-yl)amino)benzonitrile **(*45*)**: Reagents: 4-((4-chloro-6-(*p*-tolylamino)-1,3,5-triazin-2-yl)amino)benzonitrile (269,9 mg, 0,8 mmol) and ammonia 7 N in methanol (200 ml). Reaction conditions: 65 °C for 48 h. Purification: IsoleraOne using water/methanol (0 – 100% methanol). Yield: 14 mg, 6%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.59 (s, 1H), 9.13 (s, 1H), 8.03 (d, J = 8.6 Hz, 2H), 7.76 – 7.56 (m, 4H), 7.09 (d, J = 8.3 Hz, 2H), 6.77 (s, 2H), 2.26 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.7, 164.30, 164.2, 145.0, 137.4, 132.8, 128.8, 120.4, 119.6, 119.2, 102.5, 20.5. UPLC: purity > 99%. m/z (ES) 318.2 [M + 1]. HRMS: Calc: 317.14 Found: 318.1465 [M + 1].

4-((4-Amino-6-((2-chloro-4-methylphenyl)amino)-1,3,5-triazin-2-yl)amino)benzonitrile **(*46*)**: Reagents: 4-((4-chloro-6-((2-chloro-4-methylphenyl)amino)-1,3,5-triazin-2-

yl)amino)benzonitrile (300.3 mg, 0,7 mmol) and ammonia 0.5 N in dioxane (100 ml). Reaction conditions: 101 °C for 48 h. Purification: IsoleraOne using heptane/ethyl acetate (0 – 100% ethyl acetate). Yield: 164 mg, 63%. ^{1}H NMR (400 MHz, Acetone-d_6) δ: 8.82 (s, 1H,), 8.09 (s, 1H), 8.05 (d, J = 8.7 Hz, 2H), 7.63 – 7.60 (m, 3H), 7.29 (d, J = 1.2 Hz, 1H), 7.16 (d, J = 8.3 Hz, 1H), 6.36 (s, 2H), 2.33 (s, 3H). ^{13}C NMR (100 MHz, Acetone-d_6) δ: 168.6, 166.2, 165.8, 145.6, 135.5, 134.2, 133.5, 130.2, 128.7, 125.6, 120.3, 120.2, 119.9, 104.9, 20.5. UPLC: purity > 99 %. m/z (ES) 352.1 [M], 354.1 [M + 2]. HRMS: Calc: 351.10 Found: 352.1096 [M + 1].

4-((4-Amino-6-((4-fluoro-2,6-dimethylphenyl)amino)-1,3,5-triazin-2-yl)amino)benzonitrile **(*47*)**: Reagents: 4-((4-chloro-6-((4-fluoro-2,6-dimethylphenyl)amino)-1,3,5-triazin-2-yl)amino)benzonitrile (0,2972 g, 0,733 mmol) and ammonia 0.5 N in dioxane (100 ml). Reaction conditions: 101 °C for 6 days. Purification: IsoleraOne using heptane/ethyl acetate (0 – 100% ethyl acetate). Yield: 24.7 mg, 10%. ^{1}H NMR (400 MHz, MeOD-d_4) δ: 7.96 (t, J = 8.1 Hz, 1H), 7.66 – 7.57 (m, 2H), 7.37 (d, J = 8.6 Hz, 1H), 6.86 (dd, J = 15.2, 9.3 Hz, 2H), 2.22 (s, 5H). UPLC: purity: 95%. m/z (ES) 350.1 [M + 1]. HRMS: Calc: 345.15 Found: 350.1548 [M + 1].

N-(4-Fluorophenyl)-4-(mesityloxy)-6-(piperidin-1-yl)-1,3,5-triazin-2-amine **(*28*)**: 4-Chloro-*N*-(4-fluorophenyl)-6-(mesityloxy)-1,3,5-triazin-2-amine (300 mg, 0.8 mmol) was stirred with piperidine (85 mg, 1 mmol) and DIPEA (216 mg, 1.6 mmol) in dioxane (10 mL) at 101 °C overnight. After that, the solvent was evaporated under vacuum. The crude was purified by IsoleraOne using DCM and MeOH (0 – 10% methanol) as eluents obtaining the final compound (267.9 mg, 79%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.55 (s, 1H), 7.91 - 6.70 (m, 6H), 3.87 - 3.49 (m, 4H), 2.25 (s, 3H), 2.03 (s, 6H), 1.69 - 1.36 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 169.9, 165.5, 165.1, 157.4 (d, J = 231.7 Hz), 147.2, 136.1, 133.9, 129.6, 128.9,

121.4, 114.8 (d, *J* = 20.8 Hz), 43.8, 25.3, 24.2, 20.4, 16.2. UPLC: purity >99%. m/z (ES) 408.2 [M + 1]. HRMS: Calc: 407.21 Found: 408.2200 [M + 1].

***N^2-(4-Fluorophenyl)-6-(mesityloxy)-N^4-methyl-1,3,5-triazine-2,4-diamine* (*32*)**: 4-Chloro-*N*-(4-fluorophenyl)-6-(mesityloxy)-1,3,5-triazin-2-amine (300 mg, 0.8 mmol) was stirred with a solution 2 M of methanamine in THF (31.2 mg, 1 mmol) and DIPEA (216 mg, 1.6 mmol) in dioxane (10 mL) at reflux temperature for 16 h. After that, the solvent was evaporated under vacuum. The crude was purified by IsoleraOne using hexane and ethyl acetate (0 – 100 ethyl acetate) as eluents obtaining the final compound (162.1 mg, 22%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.64 – 9.41 (m, 1H), 7.94 – 7.39 (m, 17H), 7.26 – 6.80 (m, 4H), 2.76 (d, *J* = 4.6 Hz, 3H), 2.25 (d, *J* = 4.5 Hz, 3H), 2.04 (d, *J* = 4.6 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 169.4, 167.4, 164.7, 157.4 (d, *J* = 237.9 Hz), 147.2, 136.2, 134.0, 129.7, 128.9, 121.3 (d, *J* = 6.9 Hz), 114.8 (d, *J* = 17.7 Hz), 27.5, 20.3, 16.1. UPLC: purity > 99%. m/z (ES) 354.2 [M + 1]. HRMS: Calc: 357.17 Found: 358.1505 [M + 1].

***N*-(4-Fluorophenyl)-4-(mesityloxy)-1,3,5-triazin-2-amine (*50*)**: To a solution of 2,4,6-trimethylphenol (272 mg, 2 mmol), 4-chloro-*N*-(4-fluorophenyl)-1,3,5-triazin-2-amine (449 mg, 2 mmol) and tetrabutylammoniumhydrogensulfate (67.9 mg, 0.2 mmol) in Toluene (6.5 mL); a solution of NaOH (80 mg, 2 mmol) in water (0.6 mL) was added at 0 °C. The solution was stirred at 0 °C for 30 min. The solvent was evaporated, and the crude was purified by IsoleraOne using hexane and ethyl acetate (0 – 100 ethyl acetate) as eluents obtaining the final product (132.6 mg, 20%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.58 – 10.11 (m, 1H), 8.52 (s, 1H), 7.77 – 7.38 (m, 2H), 7.24 – 6.86 (m, 4H), 2.28 (s, 3H), 2.02 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 169.8, 168.3, 164.8, 158.1 (d, *J* = 243.2 Hz), 146.9, 134.8, 134.6, 129.5, 129.1, 121.6, 115.0 (d, *J* = 24.2 Hz), 20.3, 16.0. UPLC: purity > 99%. m/z (ES) 325.2 [M + 1]. HRMS: Calc: 324.14 Found: 325.1459 [M + 1].

***4-(Mesityloxy)-N-phenyl-1,3,5-triazin-2-amine* (*51*)**: To a solution of 2,4,6-trimethylphenol (272 mg, 2 mmol), 4-chloro-*N*-phenyl-1,3,5-triazin-2-amine (413 mg, 2 mmol) and tetrabutylammoniumhydrogensulfate (67.9 mg, 0.2 mmol) in Toluene (6.5 mL); a solution of NaOH (80 mg, 2 mmol) in water (0.6 mL) was added at 0 °C. The solution was stirred at 0 °C for 30 min. The solvent was evaporated, and the crude was purified by IsoleraOne using hexane and ethyl acetate (0 – 100 ethyl acetate) as eluents obtaining the final product (69.7 mg, 11%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.57 – 10.08 (m, 1H), 8.53 (s, 1H), 7.81 – 6.88 (m, 7H), 2.28 (s, 3H), 2.03 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 169.6, 168.5, 164.9, 146.9, 138.4, 134.6, 129.5, 129.1, 128.5, 123.2, 119.9, 39.5, 20.4, 16.0. UPLC: purity > 99%. m/z (ES) 307.2 [M + 1]. HRMS: Calc: 306.15 Found: 307.1546 [M + 1].

ASSOCIATED CONTENT

Full panel of parasitology screens for the final compounds, antiprotozoal *in vitro* and *in vivo* assays such as cytotoxicity, *T. cruzi*, *L. infantum*, *P. falciparum*, microsomal and plasma stability assays and results. Protocols to evaluate the pharmacokinetics of compound **35** and the pharmacokinetic parameters. Protocol to evaluate the rate of action is also included.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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REFERENCES

1. Buscher, P., et al., *Human African trypanosomiasis.* Lancet, 2017. **390**(10110): p. 2397-2409.
2. Eperon, G., et al., *Treatment options for second-stage gambiense human African trypanosomiasis.* Expert Rev Anti Infect Ther, 2014. **12**(11): p. 1407-17.
3. Venkatraj, M., et al., *Synthesis, evaluation and structure-activity relationships of triazine dimers as novel antiviral agents.* Bioorg Med Chem Lett, 2012. **22**(23): p. 7174-8.
4. Arien, K.K., et al., *Diaryltriazine non-nucleoside reverse transcriptase inhibitors are potent candidates for pre-exposure prophylaxis in the prevention of sexual HIV transmission.* J Antimicrob Chemother, 2013. **68**(9): p. 2038-47.
5. Grammen, C., et al., *Development and in vitro evaluation of a vaginal microbicide gel formulation for UAMC01398, a novel diaryltriazine NNRTI against HIV-1.* Antiviral Res, 2014. **101**: p. 113-21.
6. Grammen, C., et al., *Development and characterization of a solid dispersion film for the vaginal application of the anti-HIV microbicide UAMC01398.* Int J Pharm, 2014. **475**(1-2): p. 238-44.
7. Arien, K.K., et al., *Resistance and cross-resistance profile of the diaryltriazine NNRTI and candidate microbicide UAMC01398.* J Antimicrob Chemother, 2016. **71**(5): p. 1159-68.
8. Collar, C.J., et al., *Predictive computational models of substrate binding by a nucleoside transporter.* J Biol Chem, 2009. **284**(49): p. 34028-35.
9. de Koning, H.P. and S.M. Jarvis, *Adenosine transporters in bloodstream forms of Trypanosoma brucei brucei: substrate recognition motifs and affinity for trypanocidal drugs.* Mol Pharmacol, 1999. **56**(6): p. 1162-70.
10. Venkatraj, M., et al., *From human immunodeficiency virus non-nucleoside reverse transcriptase inhibitors to potent and selective antitrypanosomal compounds.* Bioorg Med Chem, 2014. **22**(19): p. 5241-8.
11. Venkatraj, M., et al., *Novel triazine dimers with potent antitrypanosomal activity.* Eur J Med Chem, 2018. **143**: p. 306-319.
12. Ullrich, R. and M. Hofrichter, *Enzymatic hydroxylation of aromatic compounds.* Cell Mol Life Sci, 2007. **64**(3): p. 271-93.

13. Diaz, R., et al., *Identification and characterization of hundreds of potent and selective inhibitors of Trypanosoma brucei growth from a kinase-targeted library screening campaign.* PLoS Negl Trop Dis, 2014. **8**(10): p. e3253.
14. Jacobs, R.T., et al., *SCYX-7158, an orally-active benzoxaborole for the treatment of stage 2 human African trypanosomiasis.* PLoS Negl Trop Dis, 2011. **5**(6): p. e1151.
15. Ogden, A., M.G. Wientjes, and J.L. Au, *Suramin as a chemosensitizer: oral pharmacokinetics in rats.* Pharm Res, 2004. **21**(11): p. 2058-63.

HIGHLIGHTS

-Structural optimization of a series of triazine derivatives to improve metabolic stability and to maintain potent anti-trypanosomal activity.

-Four compounds were selected for human and mouse microsomal stability, based on their potency against *T. brucei*.

-Based on the excellent *in vitro* metabolic stability and potency, one compound was selected for *in vivo* evaluation of pharmacokinetic properties and potency in a mouse model of acute *T. brucei* infection.

-The disappointing *in vivo* potency is related to the low rate of trypanocidal activity. We recommend the rate of trypanocidal activity as an important parameter during lead selection and lead optimization.