Activities of Quinoxaline, Nitroquinoxaline and [1,2,4]Triazolo[4,3-a]quinoxaline Analogs of MMV007204 against *Schistosoma mansoni*

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ABSTRACT:

The reliance on one drug, praziquantel, to treat the parasitic disease schistosomiasis in millions of people a year shows the need to further develop a pipeline of new drugs to treat this disease. Recently, an antimalarial quinoxaline derivative (MMV007204) from the Medicines for Malaria Venture (MMV) Malaria Box demonstrated promise against *Schistosoma mansoni*. In this study, 47 synthesized compounds containing quinoxaline moieties were first assayed against the larval stage of this parasite, newly transformed schistosomula (NTS); of these, 16 killed over 70% NTS at 10 μM. Further testing against NTS and adult *S. mansoni* yielded three compounds with 50% inhibitory

concentrations (IC_{50}s) of ≤ 0.31 μM against adult *S. mansoni* and selectivity indices of ≥ 8.9. Administration of these compounds as a single oral dose of 400 mg/kg of body weight to *S. mansoni*-infected mice yielded only moderate worm burden reduction (WBR) (9.3% – 46.3%). The discrepancy between these compounds' good *in vitro* activities and their poor *in vivo* activities indicates that optimization of their pharmacokinetic properties may yield compounds with greater bioavailabilities and better antischistosomiasis activities *in vivo*.

INTRODUCTION

Schistosomiasis is a parasitic disease affecting over 200 million people, mostly in the developing world, caused by parasites of the *Schistosoma* genus, primarily *S. mansoni*, *S. haematobium*, and *S. japonicum*. Though this disease is responsible for a considerable health burden (1), its treatment thus far has relied on only one drug, the tetrahydroisoquinoline praziquantel. While this drug has proven safe, inexpensive at scale and efficacious, the sheer scale of its use as an antiparasitic suggests that drug resistance may eventually become a concern (2-4).

For this reason, the development of new drugs effective against the parasite is a clear and pressing need. One of us has looked to antimalarial drugs for possible new leads (5), and screened the Medicines for Malaria Venture (MMV) Malaria Box (6) of 400 commercially available compounds for antischistosomal activity against both newly transformed schistosomula (NTS) and adult worms (7). In that work, two of the most active compounds, both *in vitro* and *in vivo* in a mouse model, were the N,N'-diarylurea

1 (MMV665852) and the 2,3-dianilinoquinoxaline **2** (MMV007204) (Figure 1). Further exploration of the former led to the development of several analogs, including N-phenylbenzamides and N-arylphenylcarbamates, with excellent *in vitro* activity against *S. mansoni* but only moderate effect in a mouse model (8).

In this work, we have developed analogs of the latter of those lead compounds, dianilinoquinoxaline **2**. Quinoxaline compounds have shown promising anticancer (9-11), antiprotozoal (12) and antimycobacterial activities (13, 14). Among this set are several 6-nitroquinoxaline analogs; nitroaromatic antiparasitic compounds have shown activity against malaria (15), giardiasis (16, 17), trypanosomiasis (18, 19), amoebiasis (20), and trichomoniasis (21). Nitroquinoxaline compounds in particular have demonstrated potent activity against Gram-positive bacteria (22).

We have also prepared and tested a small set of other compounds that include a quinoxaline moiety within a more complex polyheterocyclic system, including a series of [1,2,4]triazolo[4,3-a]quinoxalines. Similar triazolopyrazines have shown antimalarial potential in work done in the Open Source Malaria program. (https://openwetware.org/wiki/OpenSourceMalaria:Triazolopyrazine_(TP)_Series) Moreover, in previous studies, triazolopyrazines have exhibited a range of biological activities (11, 23, 24), including broad antimicrobial activity (25).

MATERIALS AND METHODS

Synthesis. Disubstituted quinoxaline and nitroquinoxaline compounds were synthesized from the nucleophilic aromatic substitution (S_NAr) reactions of 2,3-dichloroquinoxaline (**3-23**) and 6-nitro-2,3-dichloroquinoxaline (**24-32**), respectively (Figure 2). While substitution reactions with aliphatic amines generally proceeded smoothly at moderate temperatures, those involving anilines required more robust heating. When 2-(dimethylamino)ethylamine was used as the amine nucleophile, the major product isolated was 1-methyl-1,2,3,4-tetrahydropyrazino[2,3-b]quinoxaline (**18**), the unexpected result of an intramolecular S_NAr reaction by the tertiary amine followed by demethylation. Two nitroquinoxaline products, **29** and **30**, were subjected to nitro reduction and acetylation to give the analogous acetamides **33** and **34**.

Tetracyclic compounds **35** and **36** were synthesized by the condensation of *o*-phenylenediamine with isatin and ninhydrin, respectively; (26) the latter was then subjected to palladium catalyzed transfer hydrogenolysis (27) to yield 11H-indeno[1,2-b]quinoxaline (**37**) (Figure 3). [1,2,4]triazolo[4,3-a]quinoxalines (**38-49**) were synthesized from 2-hydrazino-3-chloroquinoxaline by acid-mediated condensation with an aldehyde or orthoester, followed by nucleophilic aromatic substitution at the 4-position with a secondary amine heterocycle (24).

Experimental details and ^{1}H NMR spectral characterization data for all synthesized compounds can be found in the Supporting Information.

Drugs and culture media. Compounds were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). The culture media were prepared from medium 199 (NTS testing) or RPMI 1640 (adult testing) (Life Technologies) with L-glutamine (Sigma-Aldrich), 5% heat-inactivated fetal calf serum (FCS), 1% penicillin, and streptomycin mix, which were purchased from LuBioScience.

Mice and parasites. Animal studies were carried out following Swiss national and cantonal regulations on animal welfare at the Swiss Tropical and Public Health Institute (Basel, Switzerland (Swiss TPH) (permission no. 2070). The *S. mansoni* life cycle (Liberian strain) is maintained at Swiss TPH. For the *in vitro* and *in vivo* studies female mice (NMRI strain; age 3 weeks; weight ca. 20-22 g) were purchased from Charles River, Germany. Mice were kept under environmentally controlled conditions (temperature ~25 °C; humidity ~70 %; 12 h light and 12 h dark cycle) with free access to water and rodent diet, and acclimatized for 1 week before infection.

Newly transformed schistosomula (NTS) drug assay. *S. mansoni* cercariae were gathered from infected snails and mechanically transformed to newly transformed schistosomula (NTS). 30-40 NTS/well were incubated with 0.01-10 µM of the drugs for 72 h at 37°C, 5 % CO_2 in a final well volume of 200-250 µl. Compounds were tested in triplicate and the highest concentration of DMSO (<1%) served as control. Evaluation was done by microscopic readout (Carl Zeiss, Germany, magnification 80x) using a viability scale as described recently (3 = motile, no changes to morphology or transparency; 2 = reduced motility and/or some damage to tegument noted, as well as

reduced transparency and granularity; 1 = severe reduction of motility and/or damage to tegument observed, with high opacity and high granularity; 0 = dead) (28).

Adult *S. mansoni* drug assay. Adult schistosomes were collected by mechanical picking from the hepatic portal system and mesenteric veins 49 day post-infection with 100 *S. mansoni* cercariae. Worms were incubated with 0.1 and 1 µM of the compounds for 72 h. Wells with 1% DMSO served as negative controls. Phenotypes were monitored under an inverted microscope and viability scores calculated (28). Each compound was tested twice in duplicate. To calculate IC_{50} values, viability scores were converted into effect scores using CompuSyn2® (ComboSyn Inc., 2007).

Rat skeletal myoblast L6 cytotoxicity. Rat skeletal myoblast L6 cells were seeded in 96 well plates (2 x 103 cells/ well) using supplemented RPMI 1640 medium as described above. Following adhesion of the cells for 24 h at 37 °C and 5% CO_2, the IC_{50} of the compounds was determined using concentrations of 0.12, 0.37, 1.11, 3.33, 10, 30, and 90 µM. Podophyllotoxin served as positive control. After 70 h post-incubation, 10 µL resazurin dye (Sigma) was added and the plates incubated for another 2 h. Analysis was done at 72 h using a SpectraMax M2 (Molecular Devices) plate reader with an excitation wavelength of 530 nm and emission wavelength of 590 nm.

Calculation of physicochemical properties. The *in silico* prediction tool ALOGPS 2.1 was used to calculate log P (clogP) and log S values for all compounds ((29); http://www.vcclab.org).

***In vivo* studies.** Mice were infected with 100 *S. mansoni* cercariae subcutaneously. Single oral doses of 400 mg/kg of the three lead compounds were administered to groups of four mice 49 days (adult infection) post-infection, respectively. A 400 mg/kg dose is often used as starting dose in *S. mansoni in vivo* experiments (30), as it is the efficacious dose of praziquantel in *S. mansoni* infected mice. Untreated mice (31) served as controls. Mice were euthanized using CO_2 three weeks post treatment, and worms were picked, sexed and counted and the worm burden reduction was calculated. A Kruskal Wallis test was employed to determine statistical significance.

RESULTS

***In vitro* activity against NTS.** 47 compounds were tested at 10 µM for activity against NTS. Tetracycles **35-37** and triazoloquinoxalines **38-49** showed marginal activity (<35%) at this concentration after 72 h (data in Supporting Information). Among the other quinoxaline test compounds, the best activity was found with dianilinoquinoxalines, with 16 of these showing an activity of more than 70% after 72 h (Table 1). At a lower, 1 µM concentration, nine of those sixteen compounds revealed an activity above 70% against NTS after 72 h. The most active compounds, nitroquinoxalines **29** and **30,** affected NTS with an activity of over 70% at 0.1 µM, and showed low activity (21.9% for each) even at 0.01 µM, the lowest concentration tested. For comparison, praziquantel shows an IC_{50} of 2.2 µM against NTS (30).

***In vitro* activity against adult *S. mansoni*.** The 16 quinoxaline compounds that showed good *in* vitro activity against NTS at 10 µM were also generally active against adult *S. mansoni* at the same concentration, with 15 of the 16 showing an activity of at least 70% against the worms after 72 h (Table 1). Of these, 8 compounds were active (>70%) at 1 µM. The three most active compounds, **27**, **29**, and **30**, showed also moderate activity against adult worms at 0.1 µM (16-41% after 72 h). The IC_{50} values against adult worms for these compounds were all under 0.3 µM, and were comparable to that of praziquantel (0.1 µM) (30).

Calculated physicochemical properties and solubility. Log P and log S values were calculated for all 47 compounds in this study. In this set, the 19 compounds that showed the greatest antiparasitic activity against NTS all had clog P values over 4.98, essentially the "Lipinski limit" (32), and low calculated aqueous solubilities, ranging from 1.8 to 25.7 µM (clog S -5.74 to -4.59) (Table 1). Although these clog P values contravene Lipinski's "rule of five" (32), the use of those heuristics in antiparasitic drug development has been cautioned against (33).

Antischistosomal selectivity. Nitroquinoxaline compounds **24**-**32** were tested for cytotoxicity against an L6 rat skeletal muscle cell line. Compounds that showed good activity against adult worms (>70% at 10 µM) were moderately cytotoxic to L6 cells (IC_{50}'s of 1.7-27.5 µM) (Table 2). Among our three most active compounds, **29** showed the highest antischistosomal selectivity (44.6), significantly higher than those of **27** and **30** (18.3 and 8.9, respectively), due largely to the compounds' differential in cytotoxicity.

***In vivo* studies.** The three most active compounds progressed to *in vivo* studies, where they were tested in mice harboring adult *S. mansoni*. Compound **29** was the most active compound, with a worm burden reduction (WBR) of 46.4% ($P < 0.05$) at 400 mg/kg (Table 2). This is roughly half of the antiparasitic activity of praziquantel at the same dosage (94%) (34). WBR values for compounds **27** and **30** were considerably lower (9.3% and 12.5%, respectively).

DISCUSSION

The overwhelming dependence on praziquantel to treat schistosomiasis worldwide demonstrates the potential danger that praziquantel resistance poses. There is therefore a clear need for the development of antischistosomal drugs with novel pharmacophores and modes of action. The dianilinoquinoxaline **2** was identified in previous work as a promising lead for further antischistomiasis drug development (8).

In this follow-up study we have synthesized 47 analogs of this lead compound by varying both the amine/aniline substituents on the quinoxaline scaffold and the scaffold itself. Eleven of these analogs (**3-13)** were dianilinoquinoxalines like **2**, while ten others (**14**-**23**) were synthesized from 2,3-dichloroquinoxaline and non-aniline amines; comparing these two groups, the aniline-containing quinoxalines showed better antischistosomal activity than the second group did. Nitroquinoxaline analogs (**24**-**32**) similar to the first, more active group were also synthesized, as were a series of triazoloquinoxalines (**38**-**49**) and a small set of tetracyclic compounds (**35-37**) incorporating the quinoxaline moiety.

All compounds were first tested *in vitro* on newly transformed schistosomula (NTS). Test compounds with aniline substituents (**3**-**13**, **24**-**34**) generally showed strong antiparasitic activity against both NTS and adult worms at a concentration of 1 µM; the remainder of our set showed only weak activity against NTS at 10 µM. The most active were nitroquinoxalines **27**, **29** and **30**, which demonstrated sub-micromolar IC_{50} values against adult worms, comparable to the published values for our lead compound **2** and for praziquantel itself (7). Notably, compound **30** is similar to the diarylurea lead compound **1** in that they both carry two 3,4-dichloroaniline moieties.

The addition of a nitro group to the quinoxaline scaffold increased the activity against NTS in a few cases (**4** vs. **29**, **5** vs. **30**), but this effect was not consistent across our set of analogs. Like other nitroaromatic antiparasitic compounds, such as nitazoxanide and metronidazole, this added activity upon nitration may be due to the targeting of parasitic redox systems (16, 35).

Unfortunately, the highest WBR achieved here, with nitroquinoxaline **29**, was only half of that measured for praziquantel; two similar compounds with very good *in vitro* activity, **27** and **30**, showed very little WBR at the same concentration. The *in vitro*/*in vivo* discrepancy found here may be due to rapid metabolic reduction of these nitro compound to anilines (36). However, acetamidoquinoxalines **33** and **34**, which would also ostensibly be metabolized to anilines within the parasite, showed markedly less activity against both NTS and adult worms than their corresponding non-acetamido

analogues in our compound set (**4**, **5**, **29** and **30**). More generally, the poor *in vivo* activity of these compounds may simply be due to poor pharmacokinetic properties – that is, high lipophilicities (clogP > 5) and low aqueous solubilities. Further structural optimization may be able to improve the bioavailabilities of these compounds and improve their activities *in vivo*.

In conclusion, several analogs of the antimalarial quinoxaline MMV007204 were synthesized and shown to have high activities against NTS and adult *S. mansoni* worms in *in vitro* experiments. While the *in vivo* activities of these compounds proved to be moderate at best, further development of more hydrophilic derivatives may provide more active compounds.

Acknowledgements:

S.L.D, M.J.H., K.R.E., C.J.B. and G.E.F. were supported in this work by the Lawrence University Excellence in Science Fund. S.L.D. wishes to thank Judith Humphries for helpful conversation, and Maxwell Stahl and the undergraduate students in Lawrence University's Organic Chemistry II classes in 2018 and 2019 for their contributions to our synthetic efforts. J.K. is grateful to the Swiss National Science Foundation for financial support (no. 320030_175585).

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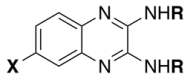

	X	R	% dead after 72 h NTS 1 µM	NTS 0.1 µM	NTS 0.01 µM	Adult worms 1 µM	Adult worms 0.1 µM	clog P	clog S
3	H	3-Cl-Ph	100 (0)	0 (0)	-	82.8 (9.8)	41.0 (5.6)	6.01	-5.41
4	H	4-Cl-Ph	100 (0)	12.5 (5.9)	-	79.3 (4.9)	27.3 (2.8)	6.01	-5.44
5	H	3,4-diCl-Ph	100 (0)	12.5 (0)	-	88.5 (10.9)	37.1 (5.6)	7.09	-5.6
6	H	3,5-diCl-Ph	100 (0)	18.8 (2.9)	-	84.6 (5.4)	37.1 (0)	7.1	-5.64
7	H	3-4-Ph	100 (0)	25.0 (0)	-	71.1 (2.7)	19.4 (2.8)	5.1	-4.94
8	H	3-CH_3O-Ph	86.0 (2.8)	8.33 (0)	-	34.4 (0)	-	5.06	-4.76
9	H	4-CH_3O-Ph	80.0 (5.7)	6.25 (8.8)	-	39.6 (2.4)	-	5.1	-4.78
26	NO_2	3-CF_3-Ph	44.2 (8.2)	-	-	60.7 (0)	-	5.79	-5.21
27	NO_2	3-CH_3O-Ph	24.9 (2.7)	-	-	82.1 (0)	24.4 (2.9)	4.98	-4.77
28	NO_2	4-Br-Ph	59.6 (2.7)	-	-	67.8 (5.1)	-	5.76	-5.54
29	NO_2	4-Cl-Ph	75 (2.7)	73.1 (5.4)	21.9 (2.8)	92.9 (0)	16.3 (2.9)	5.88	-5.51
30	NO_2	3,4-diCl-Ph	80.8 (5.4)	78.8 (2.7)	21.9 (8.5)	78.5 (10.1)	40.8 (8.7)	6.65	-5.74
31	NO_2	3,4-di(CH_3O)-Ph	32.6 (2.7)	-	-	†	-	5.06	-4.61
32	NO_2	3,5-di(CH_3O)-Ph	34.6 (5.4)	-	-	66 (2.5)	-	5.05	-4.59
33	NHAc	4-Cl-Ph	28.4 (6.8)	-	-	20.3 (2.9)	-	5.23	-5.19
34	NHAc	3,4-diCl-Ph	33.9 (11.8)	-	-	63.2 (11.6)	-	6.39	-5.48

Only compounds with ≥70% activity against NTS at 10 µM shown. Numbers in parentheses are the standard deviations of the data. For full results, see Supporting Information. †49.1% dead at 10 µM.

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358 Table 1. *In vitro* activities of synthesized analogs.

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	IC_{50}, adult *S. mansoni* (µM)	IC_{50}, L6 cells (µM)	SI	WBR (%)
27	0.31	5.7	18.3	9.3
29	0.28	12.5	44.6	46.4
30	0.19	1.7	8.9	12.5

praziquantel	0.1[a]	>96[a]	>960[a]	94.1[b]

SI = selectivity index ($IC_{50,L6}/IC_{50,S.\ mansoni}$). WBR = worm burden reduction. a) Ref. 30. b) Ref. 34.

Table 2. IC_{50} and worm burden reduction values of synthesized analogs.

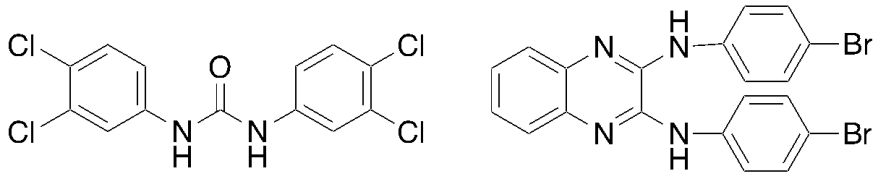


Figure 1. Hit compounds against S. mansoni from the MMV Malaria Box (7).

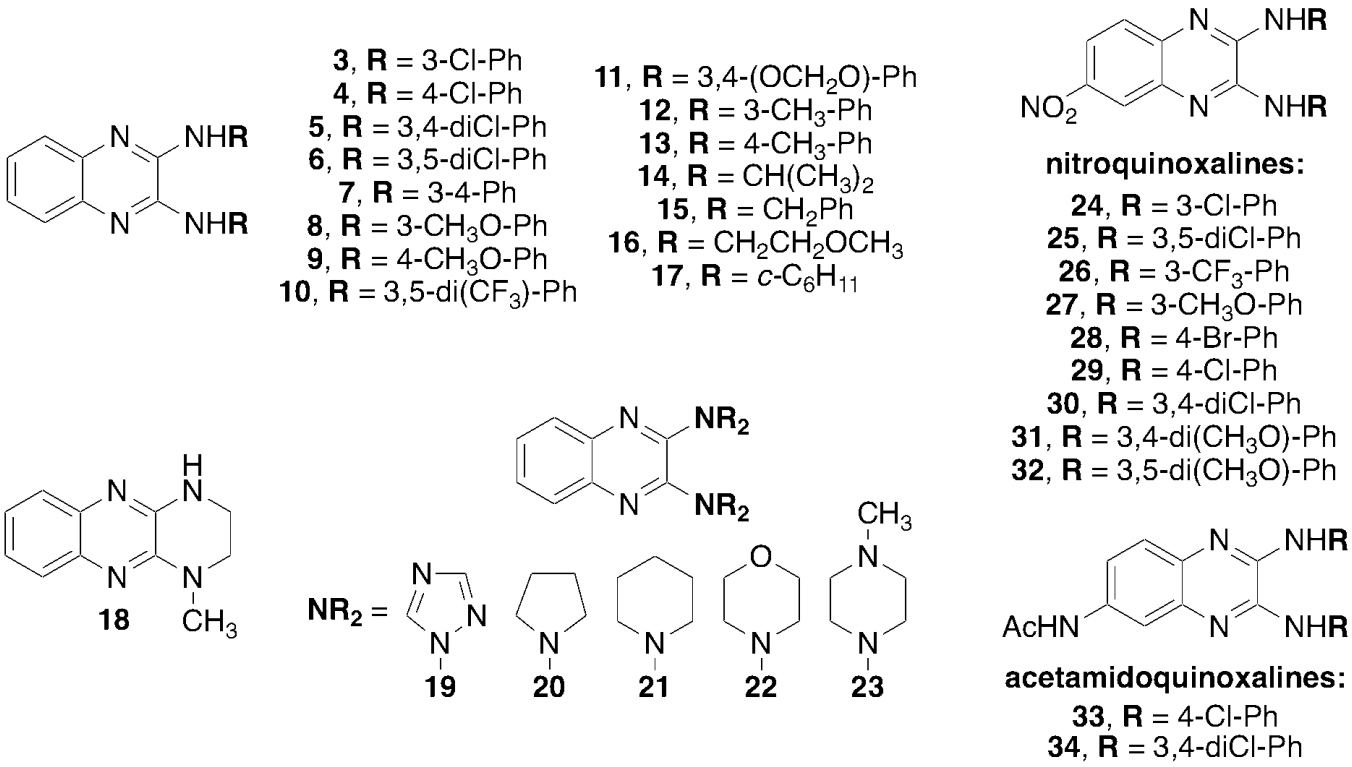
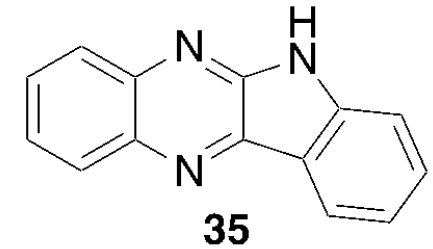
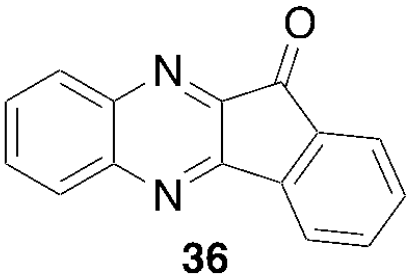


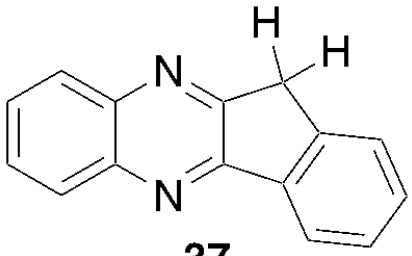
Figure 2. Quinoxaline analogs synthesized.



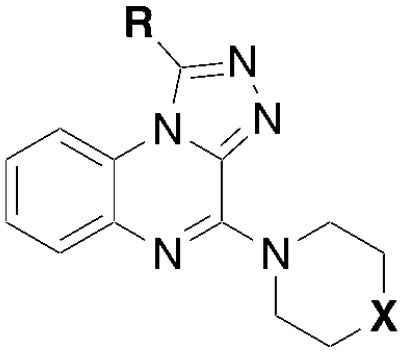
35



36



37



triazoloquinoxalines:

38, **R** = H, **X** = CH_2
39, **R** = H, **X** = O
40, **R** = H, **X** = NCH_3
41, **R** = CH_3, **X** = CH_2
42, **R** = CH_3, **X** = O
43, **R** = CH_3, **X** = NCH_3
44, **R** = Ph, **X** = CH_2
45, **R** = Ph, **X** = O
46, **R** = Ph, **X** = NCH_3
47, **R** = 4-F-Ph, **X** = CH_2
48, **R** = 4-F-Ph, **X** = O
49, **R** = 4-F-Ph, **X** = NCH_3

Figure 3. Tetracyclic compounds 35-37 and triazoloquinoxalines 38-49.