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Synthesis and evaluation of the anticoccidial activity of trifluoropyrido[1,2-*a*]pyrimidin-2-one derivatives

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ABSTRACT

Screening of our chemical library to discover new molecules exhibiting in vitro activity against the invasion of host cells by *Eimeria tenella* revealed a lead compound with an IC_{50} of 15 μM. Structure–activity relationship studies were conducted with 34 newly synthesized compounds to identify more active molecules and enhance in vitro activity against the parasite. Four compounds were more effective in inhibiting MDBK cell invasion in vitro than the lead compound.

Belonging to the apicomplexan phylum, Coccidian parasites (*Toxoplasma*, *Eimeria*) are a significant threat for both animals and humans. Although coccidia can infect a wide variety of animals, they are usually species-specific.[1] However, one well-known exception is toxoplasmosis caused by *Toxoplasma gondii*. Spreading from one animal to another, coccidiosis can be acquired by the fecal contamination of food and water by oocysts, the resistant stage that develops in the environment. Moreover, the success of these parasites is due to their rapid and efficient invasion of targeted host cells, using actin-myosin-dependent glideosome motility and specialized proteins from their microneme and rhoptry secretory organelles.[2–4] Of the seven most common *Eimeria* spp. species in chickens, *Eimeria tenella* causes the cecal or bloody type of coccidiosis, *Eimeria necatrix* causes bloody intestinal coccidiosis, and *Eimeria acervulina* and *Eimeria maxima* cause chronic intestinal coccidiosis. These species are extremely important as they mostly affect broilers, that represent a huge part of the poultry industry. In recent years, our laboratory has focused on avian coccidiosis and the interactions between sporozoites, the first invasive stage, and their host cells,[5] especially the highly host-specific *E. tenella*, that infects chicken ceca epithelial cells. The symptoms related to this disease are morbidity, weight loss, decreased food consumption and hemorrhagic diarrheas, leading to mortality in the most severe cases. The prevention and control of coccidiosis in commercial poultry is mainly based on the use of vaccines and administration of anticoccidial drugs in feed.[6] Due to their cost, most vaccines are limited to certain production lines and the rapid emergence of drug-resistant parasites has led scientists to search for new approaches to controlling coccidiosis through the synthesis of new compounds. Screening of 87 synthetic compounds representing the different methodological approaches developed by our laboratory,[7–11] enabled us to identify 7-bromo-9-iodo-pyrido[1,2-*a*]pyrimidin-2-one **3g** as biologically active, with an IC_{50} of 15 μM. We describe here the identification and structure–activity relationship (SAR) of the pyrido[1,2-*a*]pyrimidin-2-one core and the non-cytotoxic inhibitory effect of the newly synthesized compounds (improved to 0.8 μM for **3k**) against the invasion of host cells by *E. tenella* sporozoites (even at IC_{100} dosage).

Eighty-seven compounds of varying heterocyclic structure (phthalides, imidazopyridine, coumarines, triazoles, butenolides) selected from our chemical library were screened to evaluate their ability to impede invasion of host cells by *Eimeria tenella*. In the absence of avian cell lines, the assays were conducted in vitro on Madin-Darby Bovine Kidney (MDBK) cells. Those epithelial cells are routinely used for in vitro invasion studies of *Eimeria*.[12] All compounds were first tested at concentrations as high as 100 μM

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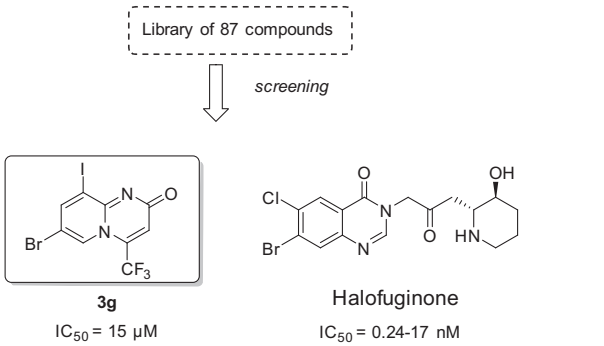
0960-894X/

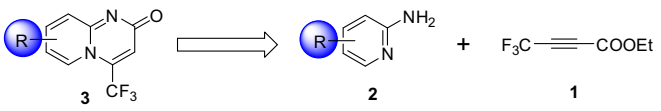
to facilitate screening. To perform this assay, an *Eimeria tenella* strain expressing the yellow fluorescent protein (YFP+) was used, previously produced in our laboratory. The effectiveness of the compounds was assessed on the basis of invasion of epithelial MDBK cells by the sporozoites. Calculating the number of infected cells in relation to the number of total cells determined the percentage of invasion. The percentage was greatly reduced in the presence of an effective compound. Compounds that reduced invasion levels to 20% compared to control wells were considered to be potential leads. Five of the compounds tested possessing different cores were identified as having modest activity, with IC_{50} ranging from 10 to 40 μM (Scheme 1). The dose-dependent efficacy of the biologically active **3g** was good (IC_{50} = 15 μM), with an absence of cytotoxicity toward the MDBK cells. Moreover, **3g** was synthesized in a very straightforward manner and was found to be a good candidate for SAR analysis. It is of note that our lead compound possessed structural similarities (halogen atoms, heteroaromatic ring) to Halofuginone (Stenorol®), an antiprotozoal drug originally synthesized in the late 1960s. Halofuginone was first used as an antimalarial and then as an anticoccidial feed additive for broilers. The antiprotozoal activity of Halofuginone in vitro is highly efficient, with IC_{50} values ranging from 17 nM to 0.24 nM, according to parasite/host cell models.[13–16] Following identification of this hit, we synthesized several analogs with the aim of establishing preliminary structure–activity relationships allowing optimization of the hit.

As described above, compound **3g** was shown to impede the invasion of MDBK cells by *E. tenella* sporozoites and was subsequently chosen as our lead. Preparation of this compound was inspired by the work of Harriman et al.[17], and consisted of the condensation of the corresponding 2-aminopyridine **2g** on ethyl 4,4,4-trifluorobut-2-ynoate **1** (Scheme 2). It is of note that our group has previously studied the reactivity of this alkyne with other α-aminoazaheterocycles, such as substituted 3-aminopyridazines[7] and 2-amino-1,3,4-thiadiazoles.[18] All the corresponding heterocycles were included in the screening, but they did not afford significant biological activity.

In order to set up a SAR starting from our lead **3g**, we modified the substituent of the pyridine core, but left the trifluoromethylpyrimidin-2-one core unmodified. The condensation step of such 2-aminopyridine is only possible with activated alkyne as



Scheme 1. Hit identification and validation.



Scheme 2. Strategy for the pharmacomodulation of **3**.

1. Moreover, as the CF_3 moiety is known to influence metabolic stability, binding activity and lipophilicity,[19,20] we assumed that the introduction of such a group would be likely to have a valuable effect and improve biological activity. We report here the synthesis of several pyrido[1,2-*a*]pyrimidin-2-ones **3** using various synthesized (**2a**–**n**)[21] or commercially available (**2o**–**z**) 2-aminopyridines as starting material (Table 1).

Through these cyclization reactions, we investigated the reactivity of 2-aminopyridines substituted with different R^1, R^2, R^3 and R^4 groups when exposed to ethyl 4,4,4-trifluorobutynoate **1**. Mono- and polyhalogenated products were easily synthesized (entries 1–14), and the corresponding products **3a**–**3n** were obtained in good to excellent yields, ranging from 41% to 95%. However, in the case of 2-aminopyridines substituted at R^4 with a halogen atom such as **2o**–**2q** (entries 15–17), the reaction did not proceed and only the starting substrate was recovered. The strongly deactivating nitro substituent, led to modest yields (entries 18 and 19). Similarly, reactions in the presence of 2-aminopyridines bearing ester (entries 20–22), aldehyde (entry 23) and nitrile functions (entry 24) or a trifluoromethyl group (entry 25) afforded products **3t**–**y** isolated at acceptable yields (45–75%). Finally, no cyclization product was obtained in the case of 2-aminonicotinic acid **2z**, (entry 26).

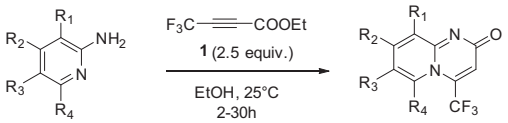
Other structural modifications of the substituents on the pyridine ring were then envisaged, starting from halogenated trifluoromethylpyrido[1,2-*a*]pyrimidin-2-ones **3a**–**h**. We planned to introduce various aromatic rings by exposing these cores to Suzuki–Miyaura cross-coupling conditions. Unfortunately only the starting material was recovered when testing several coupling conditions, despite the use of high temperatures and long reaction times. The synthesis of analogs such as **5a**–**g** was finally possible starting from the corresponding arylated 2-aminopyridines, and the methodology described by Liu et al.[22] allowed us to prepare **4a**–**g**. This efficient method was performed with ethylene glycol using $Pd(PPh_3)_4$ as catalyst in the absence of ligand in short reaction times under air. The reactions of **2a**, **2c** and **2h** in the presence of aryl boronic acids all provided **4a**–**g** in excellent yields. These coupling reactions were followed by the same cyclization step with a twofold excess of **1**. They afforded good overall yields of arylated substituted pyrido[1,2-*a*]pyrimidin-2-ones **5a**–**g** (Scheme 3).

The series of synthesized molecules with varying substituents was subjected to in vitro assays to evaluate the activity of the compounds. Each well seeded with MDBK cells was treated with a defined concentration of each compound ranging from 0 to 50 μM. In order to study the influence of substituents on the ability of the compounds to inhibit the invasion of the host cell by sporozoites, the steric hindrance and electronic factors of the chosen substituents were taken into account. Preliminary SAR was implemented by observing the influence of these parameters according to their position on the pyridine ring. All results are reported in Table 2.

First, the C(7)-substituted derivatives were explored. C(7)-substituents with minor steric hindrance, electron-withdrawing properties or bulky phenyl, *p*-methoxyphenyl or *p*-fluorophenyl (entries 1–6) substituents had IC_{50} values higher than 50 μM. However, in the presence of the cyano substituent (entry 3) the IC_{50} was 37.7 μM.

Having revealed restricted tolerance for variations when using C (7)-substituents, we then focused on compounds affording C(9)-substituents. Unfortunately, regiochemistry was not found to enhance activity. Indeed, former C(7)-substituents were used at C (9) and all had IC_{50} values greater than 50 μM (entries 7–9). Similarly, electron-deficient cores bearing small C(9)-bromine, C(9)-nitro or C(9)-methyl ester (entries 10–12) substituents were rendered ineffective (IC_{50} > 50 μM). However, when testing a carboxaldehyde substituent derivative (entry 13) activity was slightly

Table 1
Synthesis of trifluoromethylpyrido[1,2-*a*]pyrimidin-2-ones **3a**–**3aa**[a]



2a-aa **3a-aa**

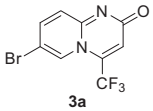
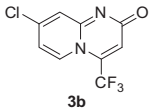
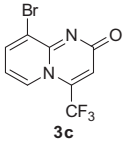
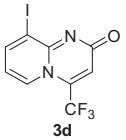
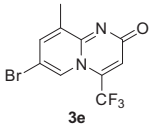
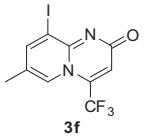
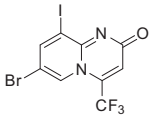
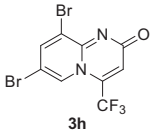
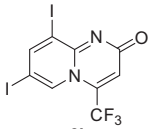
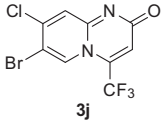
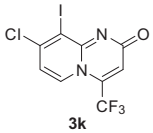
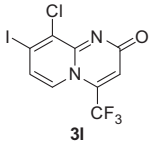
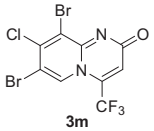
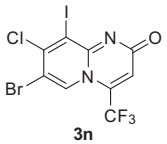
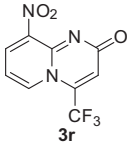
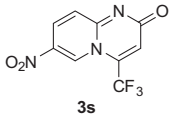
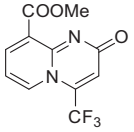
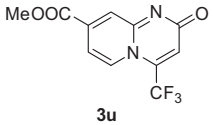
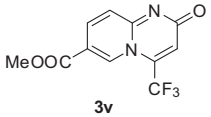
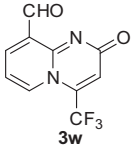
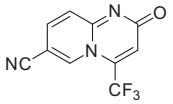
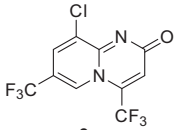
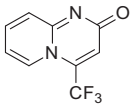
Entry	Substrate	Product (N°)	Yield[b] (%)
1	**2a**	 **3a**	76
2	**2b**	 **3b**	82
3	**2c**	 **3c**	95
4	**2d**	 **3d**	80
5	**2e**	 **3e**	85
6	**2f**	 **3f**	74
7	**2g**	 **3g**	80
8	**2h**	 **3h**	72
9	**2i**	 **3i**	77
10	**2j**	 **3j**	78
11	**2k**	 **3k**	78

Table 1 (*continued*)

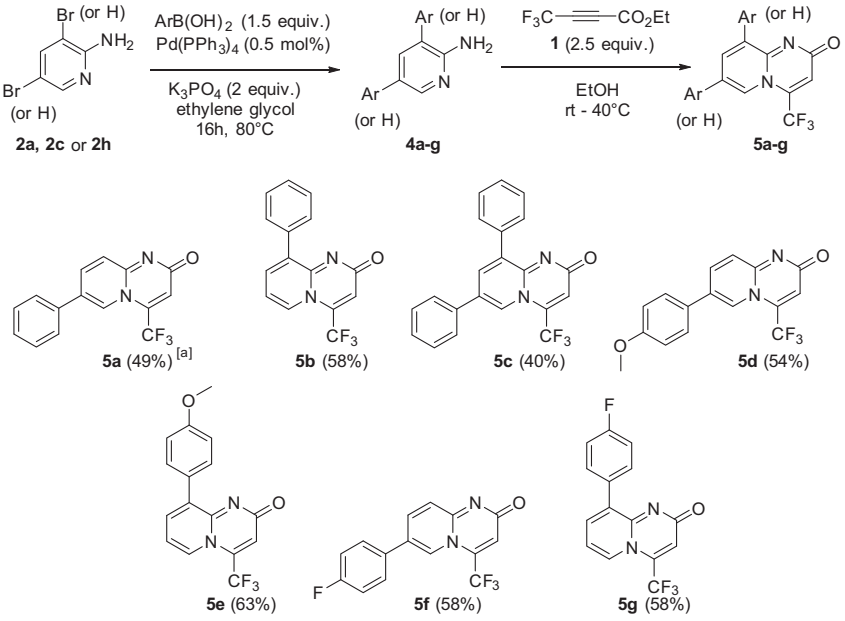
Entry	Substrate	Product (N°)	Yield[b] (%)
12	**2l**	 **3l**	87
13[c]	**2m**	 **3m**	41
14[c]	**2n**	 **3n**	48
15[d]	**2o**	/	0
16[d]	**2p**	/	0
17[d]	**2q**	/	0
18	**2r**	 **3r**	22
19	**2s**	 **3s**	32
20	**2t**	 **3t**	45
21	**2u**	 **3u**	55
22	**2v**	 **3v**	50
23	**2w**	 **3w**	52
24	**2x**	 **3x**	50
25	**2y**	 **3y**	75

(*continued on next page*)

Table 1 (*continued*)

Entry	Substrate	Product (N°)	Yield[b] (%)
26[d]	**2z**	/	0
27	**2aa**	 **3aa**	97

[a] Progress of the reaction was followed by TLC.
[b] Isolated yield.
[c] Reaction mixture was heated at 40 °C to solubilize the substrate.
[d] Starting material was recovered when the reaction was at room temperature, only degradation compounds were obtained after 2 h of reflux.



Scheme 3. Preparation of 4,4,4-trifluoromethylpyrido[1,2-*a*] pyrimidin-2-ones **5a**–**g**. [a] Overall yields over two steps.

recovered, with an IC_{50} value of 42.2 μM. Finally, the 7,9-diphenyl compound **5c** (entry 14) was not effective to inhibit MDBK cells invasion against invasion of MDBK cells by *Eimeria* sporozoites (IC_{50} > 50 μM).

Following the loss of activity of these C(7) and C(9)-substituted compounds, study then focused on the influence of C(9)-iodine present in the lead compound. The presence of small substituents such as C(9)-methyl and C(9)-bromine, and even the absence of substitution (entries 15–20) demonstrated no improvement in activity, IC_{50} values of the latter all being above 50 μM. This SAR suggests that the presence of C(9)-iodine has a prominent role in the activity of this family of molecules.

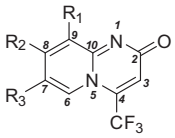
Consequently, as the active lead compound **3g** was a polyhalogenated core (entry 21), we studied the activity of several variously halogenated pyrido[1,2-*a*]pyrimidin-2-ones. The dihalogenated (7-bromo, 8-chloro) compound **3j** (entry 22) was nine times less effective than its dihalogenated analogs **3k** and **3l** (entries 23 and 24). Indeed, a significant regain in activity was observed when testing these compounds with IC_{50} values as low as 0.8 μM. This suggests that the presence of halides at C(9) is of greater value than at C(7). Furthermore, comparison of the IC_{50} values of compounds **3k** (IC_{50} = 0.8 μM), **3l** (IC_{50} = 1.1 μM) and **3b** (IC_{50} >50 μM) confirmed the preference for iodine at C(9).

To extend the SAR, trihalogenated compounds **3m** and **3n** (entries 26 and 27) were synthesized to combine substituents of our lead with those of the most active compound **3k**. These new compounds were active, with an average IC_{50} value of 3.1 μM. However, this last approach did not provide more effective inhibitors; IC_{50} values did not exceed those of the most active inhibitor **3k**.

In conclusion, the use of bulky substituents for this SAR study significantly reduced compound effectiveness to impede the invasion of host cells by *Eimeria* sporozoites. No correlation was observed among electron withdrawing groups that incorporated a hydrogen bond acceptor such as carboxaldehyde, nitro, ester or even cyano substituents. This absence of relationship may have been due to the steric hindrance of these substituents. However, the incorporation of nucleofuge halides showed the best levels of activity. The formation of covalent bonds between these compounds and the biological material could be envisaged at this stage. For instance, a glutathione metabolic pathway of 2-chloropyridines activated by GSH has already been reported in the literature.[23]

To determine whether the inhibitory effects observed were specific to the host cells or the sporozoites, these biological materials were preincubated independently with each active compound (**3k**–**n** including **3g**), and then washed by centrifugation, before proceeding with the invasion assay. Two new combinations were assayed for invasion: inhibitor-treated sporozoites were added to untreated host cells (dark gray) whereas untreated sporozoites

Table 2
Structure–activity relationship



Entry	Product	R^1 (**C9**)	R^2(**C8**)	R^3(**C7**)	IC_{50} (μM)
1	**3s**	H	H	NO_2	>50
2	**3u**	H	H	COOMe	>50
3	**3x**	H	H	CN	37.7
4	**5a**	H	H	Ph	>50
5	**5d**	H	H	*p*-$MeOC_6H_4$	>50
6	**5f**	H	H	*p*-FC_6H_4	>50
7	**5b**	Ph	H	H	>50
8	**5e**	*p*-$MeOC_6H_4$	H	H	>50
9	**5g**	*p*-FC_6H_4	H	H	>50
10	**3c**	Br	H	H	>50
11	**3r**	NO_2	H	H	>50
12	**3v**	COOMe	H	H	>50
13	**3w**	CHO	H	H	42.2
14	**5c**	Ph	H	Ph	>50
15	**3e**	CH_3	H	Br	>50
16	**3h**	Br	H	Br	>50
17	**3a**	H	H	Br	>50
18	**3d**	I	H	H	>50
19	**3f**	I	H	CH_3	>50
20	**3i**	I	H	I	>50
21	**3g**	I	H	Br	15
22	**3j**	H	Cl	Br	9.3
23	**3k**	I	Cl	H	0.8
24	**3l**	Cl	I	H	1.1
25	**3b**	H	Cl	H	>50
26	**3m**	Br	Cl	Br	3.8
27	**3n**	I	Cl	Br	3.1
28	**3aa**	H	H	H	>50

were added to inhibitor-treated host cells (light gray), and the results were compared to the initial screening (Fig. 1).

In the presence of compounds **3l** and **3k**, all assays led to high levels of invasion after washing out the compounds; these inhibitors were considered to act specifically on sporozoites but in a reversible manner. However, in the presence of compounds **3m** and **3n**, the inhibitory effects were conserved only when treated and washed sporozoites were added to untreated host cells, since the level of invasion control was restored when untreated sporozoites were added to treated and washed host cells. Compounds **3m** and **3n** were classified as irreversible inhibitors specifically targeting the sporozoites. The screening thus successfully identified four new reversible and irreversible inhibitors acting specifically towards sporozoites. To determine whether the active compounds that impeded the invasion process were cytotoxic, two assays were developed: one using a tetrazolium salt and measuring effects towards cells, and the other using Evans blue dye to measure effects on sporozoites.

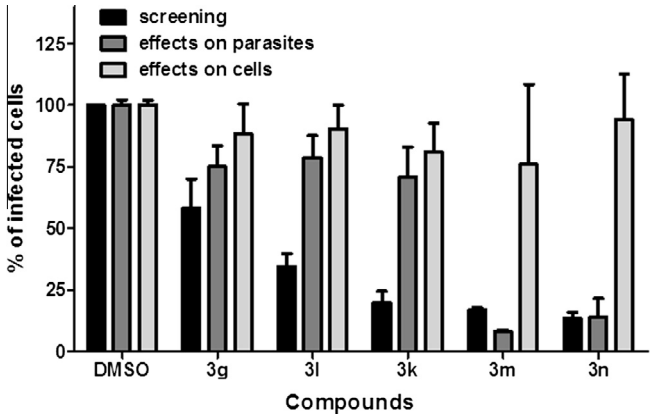


Figure 1. Anticoccidial activity of compounds in in vitro assay. All compounds were tested at 15 μM.

To determine toxicity towards host cells, these five active compounds were subjected to the cytotoxicity assay using MTT as reagent. This fast method for quantifying viable cells makes possible the reduction of yellow tetrazolium salt to purple formazan only in the presence of metabolically active cells. Reading of absorbance and presenting these values on a diagram enabled us to reveal cytotoxicity effects. Compounds that maintained the metabolic activity of cells to levels $\geqslant$80% compared to control wells were considered to be non-cytotoxic. Following analysis of these results at the compounds' IC_{100} value, no cytotoxicity was observed according to our criterion (Fig. 2). The toxicity assay was also performed at IC_{50} (data not shown) to determine the IC_{50} value against MDBK cells. The therapeutic index was estimated for each compound (Table 3).

The absence of cytotoxicity effects observed at the compounds' IC_{100} as well as their specific activity towards sporozoites led us to perform a viability assay on parasites. This complementary assay was performed to establish whether the inhibitory effect observed was caused by blocking of the invasion process or by sporozoite death. To further investigate this property the assay was conducted in the presence of Evans blue and based on penetration of the dye into non-viable cells. Sporozoites previously treated with the active compound were treated with the dye; any non-living parasite was displayed in red when visualized with a fluorescence microscope. During the test, sporozoites killed by heat were used as negative controls to judge the reliability of the test. Furthermore, unlike a MTT cytotoxicity assay, this test was carried out without sporozoite lysis (Fig. 3).

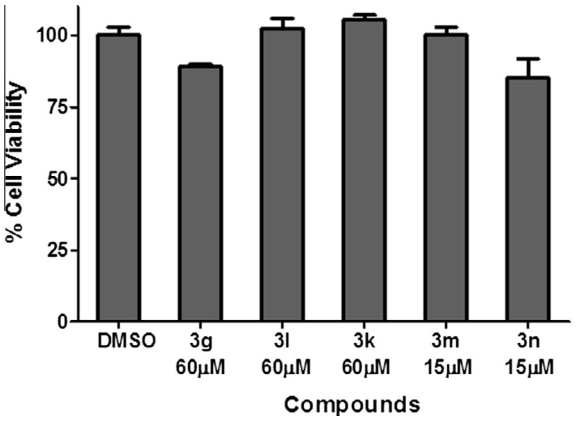


Figure 2. Cytotoxicity of compounds towards MDBK cells was evaluated by MTT assay. Each compound was added at the IC_{100} concentration.

Table 3
Therapeutic index (MDBK IC_{50}/invasion IC_{50})

Product	Invasion IC_{50} (μM)	MDBK IC_{50} (μM)	Therapeutic Index
3g	15	196	13.1
3l	1.1	102	92.7
3k	0.8	16	2
3m	3.8	23	6.1
3n	3.1	21	6.8

MDBK IC_{50} was determined by cytotoxicity assay against MDBK, performed at doses that inhibited 50% and 100% of cell invasion by sporozoites.

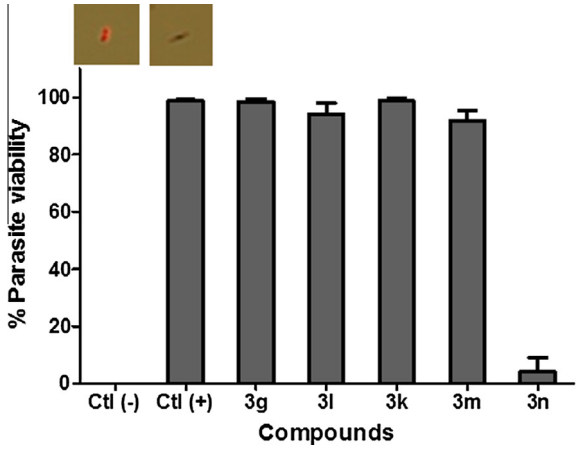


Figure 3. Anticoccidial activity of compounds evaluated on extracellular sporozoites after 1 h incubation with compounds at IC_{50} concentration. Heat killed sporozoites were used as a negative control. In the positive control, freshly excysted sporozoites were incubated without compound in Evans blue dye.

This criterion was chosen so that any compound allowing 90% of viable sporozoites would be considered non-active against extracellular sporozoites. Lead compound and compounds **3l**, **3k** and **3m** showed no activity against extracellular sporozoites, their percentages of viability being 98%, 95%, 96% and 92%, respectively. Surprisingly, compound **3n** showed the highest anticoccidial activity against extracellular sporozoites valued (100% compared to controls). The inhibitory activity of this compound was thus due to sporozoite death preventing any invasion process. As the molecular target of compounds is unknown, speculation about the interaction of compounds with their possible target is not possible. Compounds **3m** and **3n** were irreversible compounds, and it is accepted that irreversible compounds may have off-target interactions which can reduce non-mechanism-based toxicity.[24] This may be the only explanation possible of why only **3n** was highly active against extracellular *Eimeria* sporozoites.

Novel trifluoropyrido[1,2-*a*]pyrimidin-2-ones were synthesized as potential new anticoccidial drugs. The first lead compound **3g** showed in vitro anticoccidial activity (IC_{50} = 15 μM) with no cytotoxicity towards host cells. After SAR analysis, four new reversible (**3l** and **3k**) and irreversible (**3m** and **3n**) compounds were identified. They showed better levels of activity (IC_{50} = 0.8–3.8 μM) and no cytotoxicity towards host cells. Three of these compounds inhibited cell invasion by *E. tenella* parasites (**3l, 3k** and **3m**) whereas **3n** showed high anticoccidial activity towards *E. tenella* parasites, killing extracellular sporozoites.

Further studies will be necessary to identify the drug target in the parasite. If the target is conserved throughout the Apicomplexan parasite phylum, it will be interesting to evaluate antiparasitic activity against *T. gondii* and *Plasmodium falciparum*.

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Supplementary data

Supplementary data (general biological methods and chemical experimental procedures for the synthesis of 2-aminopyridines and trifluoromethylpyrido[1,2-*a*]pyrimidin-2-ones) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.11.018.

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