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Discovery of novel antileishmanial agents in an attempt to synthesize aplysinopsin pentamidine hybrid molecule

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In an attempt to synthesize pentamidine aplysinopsin hybrid molecule (**A**), a lead molecule **8** (containing *Z*-configured aplysinopsin moiety) was identified for antileishmanial activity. Optimization of lead **8** provided **24** (containing *E*- configured aplysinopsin) possessing 10 times more activity and 450 fold less toxicity than drug pentamidine. Synthesis of **24** was possible, surprisingly, due to two innate reactivity of indole-3-carbaldehyde which provided it in diastereo and regioselectively pure form without recourse to protection deprotection and long reaction pathway.

Leishmaniasis is a vector born parasitic disease of tropics and subtropics which is manifested in four major clinical forms (cutaneous leishmaniasis, mucocutaneous leishmaniasis, visceral leishmaniasis and post kala-azar dermal leishmaniasis or PKDL) depending on causative species of protozoan *Leishmania*. In all above forms of leishmaniasis, visceral leishmanaiasis (VL) is lethal, if left untreated. There are ≈ 70 000 deaths and 1.5 million new cases emerges per year due to VL. Majority of VL cases (≥ 90%) occurs in just six countries India, Nepal, Bangladesh, Sudan, Ethiopia and Brazil. Situation has become complicated by emergence of PKDL which appears in 0-6 months after successful cure of VL.[1] The WHO has declared VL a neglected and emerging disease.[2]

Antimonials are first line treatment options for VL, which was discovered almost 70 years ago. These suffer from major side effects including cardiac arrhythmia, pancreatitis. Despite their toxicity treatment failure to antimonials treatment has increased; some times as high as 62% in some of the regions.[1] Second line treatment options for VL are pentamidine, miltefosin and amphotericin B. However, all of these drugs suffer from several moderate to sever side effects. Pentamidine, an aromatic diamidine, is not active orally and can lead to renal, pancreatic, and hepatic toxicity along with hypotension and dysglycemia.[3] Miltefosine, an alkylphosphocholine, has long half life (100-200 h) in humans and low therapeutic ratio, characteristics that could encourage development of resistance. It is not suitable for pregnancy due to tetratogenecity and also cause mild to severe gastrointestinal side effect.[4] Liposomal amphotericin B is highly effective option; however this drug formulation is very expensive (US$ 2800 per treatment), limiting its use in most endemic regions. Although recent clinical trial with injectable paromomycin have shown encouraging results, an expanded catalogue of new drugs for VL causing parasite *L. donovani* is required to tackle the problem of resistance.

In spite of some side effects of pentamidine, the broad range of its (dicationic class of molecules in general) biological activities,[5] relatively less propensity towards development of resistance (resistance to pentamidine itself has never been a significant problem in the field, despite its widespread use as a prophylactic) [6] and our previous work on this class on molecules [7] prompted us to develop some novel dicationic class of molecules as potential antileishmanial agents with improved efficacy and selectivity than pentamidine. From literature search we found that aplysinopsins (a class of natural products possessing cyclic guanidine function) [8] acts on similar biological targets (plasmepsin II and serotonin receptors) as dicationic class of molecules.[9] Taking inspiration from fragment based drug discovery (FBDD) approach, [10, 11] we designed a hybrid molecule **A** (Fig. 1), where one amidinophenoxy function of pentamidine has been replaced with aplysinopsin. Due to their synthesis in biological settings small molecule natural products have in built selectivity and phamacokinetic profile, necessary for a drug molecule.[12] Hence, incorporating a drug fragment with natural product may provide molecules with better activity and less toxicity.[13, 14]

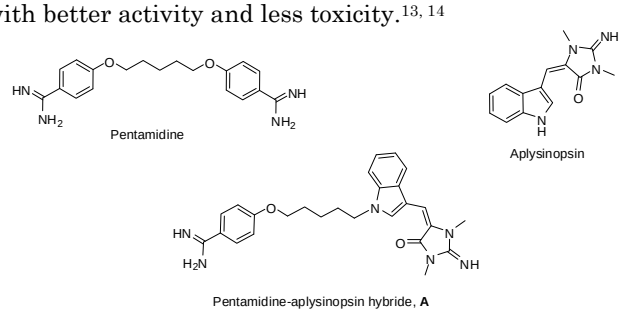


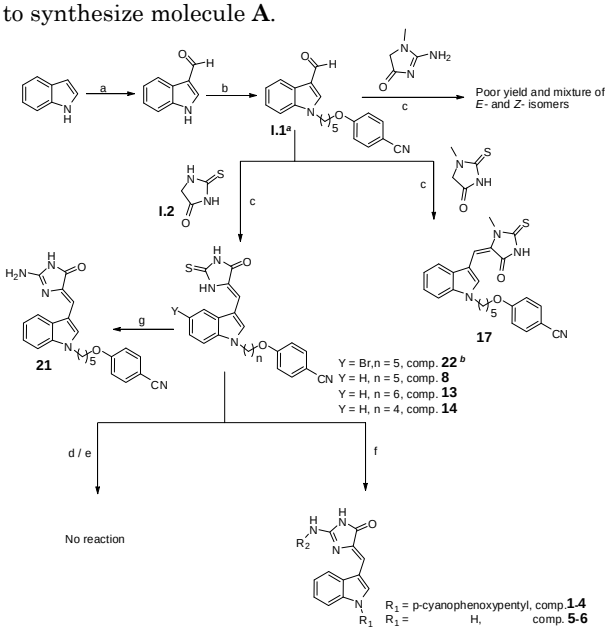
Figure 1: Hybrid of pentamidine and aplysinopsin

Herein, we describe our efforts for synthesis of hybrid molecule **A** and its analogues and discovery of new class of antileishmanial in this endeavor.

To synthesize compound **A** and its analogues we adopted route depicted in scheme 1. Direct synthesis of aplysinposin and its subsequent coupling with pentamidine fragment wasn't feasible due to poor yield in synthesis of aplysinopsin and lack of chemo selectivity in its subsequent coupling with petamidine fragment [mainly p-(hydroxypentyl)benzamidine]. As we proceeded according to Scheme 1, we encountered a problem in direct condensation of 1-methyl-2-imino-4-imidazolidinon (creatinine) which occurred in poor yield and provided a mixture of diastereomers (*E*- & *Z*-). Therefore, we first condensed 2-thio-4-imidazolidinon (2-thiohydantoin) with intermediate **I.1** to provide compounds **8**, **13**, **14** in its *Z*- geometry. Conversion of cyano function of comp. **8** to amidine (Pinner synthesis) again proved difficult due to its high insolubility and many NH centers. We tried a novel approach for the conversion of cyano function to amidine; by firstcomplexing it with Zn^{2+} (using

anhydrous $ZnCl_2$) and then using neat amine to form desired amidine.

Scheme 1: Compounds formed (**1**-**9**) in initially attempt to synthesize molecule **A**.



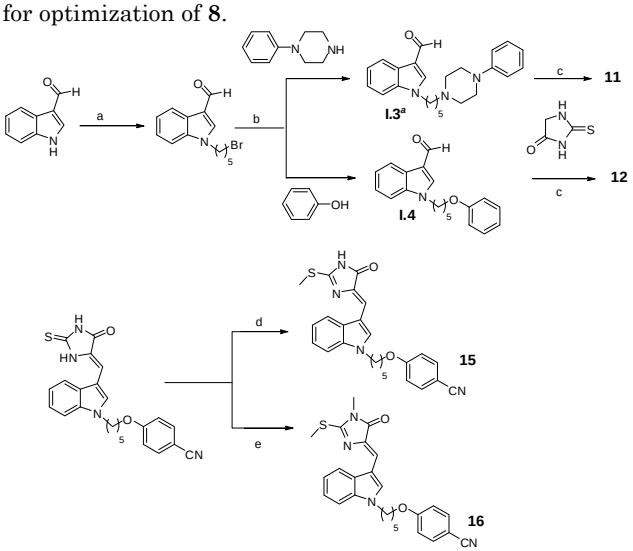
Reagents and conditions: (a) DMF, $POCl_3$, 0°C-rt. (b) p-cyanophenoxypentylbromide, tolune, NaOH (aq.), TBAB, rt. (c) ethanolamine (1 equiv.), ethanol (absolute), 60°C . (d) MeOH (dry), HCl (g). (e) MeOH, MeONa. (f) $ZnCl_2$ (anhy.), RNH_2, neat, 80-90°C. (g) TBHP/ NH_3 (aq.), MeOH, steel vessel. [a] **I.1** denote intermediate 1 and so on. [b] See Supp. Info

But, instead we observed guanylation of amine with the substrate **8** to form comp. **1**-**6** (Scheme 1). Guanylation of amine was known using $HgCl_2$, Bi(III) salts and Mukaiyama's reagent.[15] This is the first example of use of anhydrous $ZnCl_2$ in guanylation of amine. In absence of anhydrous $ZnCl_2$, guanylation occur in very poor yield with many side products.

To synthesize intermediate **I.3** and **I.4** (Scheme 2) containing other than p-cyanophenoxy moiety, we have to change the sequence of coupling by first replacing one bromo group of dibromopentane with indole-3-carbaldehyde and then replacing the other with piperazine and phenol, respectively. Similarly, intermediate **I.5** (Scheme 3) was synthesized to finally obtain comp. **18**. S-methylated derivative **15** was synthesized under controlled methylation condition (Scheme 2).

Amide analogue **20** was synthesized (Scheme 3) by first converting cyano function of p-cyanophenoxypentyl bromide to amide and then proceeding as for comp. **8**. Comp. **21** was obtained from comp. **8** by oxidative amination of thiocarbonyl function. Synthesis of comp. **23** was started from alkylation of NH- of azaindole followed by Vilsmeir formylation. Direct synthesis of comp. **19** from **8** by reaction of hydroxylamine hydrochloride provided inseparable mixture of unknown compounds, which was characterized neither *Z*- nor *E*-isomers. Therefore, we prepared intermediate **I.6** for synthesis of **19** and **24**; by coupling of p-amidoximophenol with 1-bromopentylindole-3-carbaldehyde. Two regioisomers were formed in the reaction, but desired isomer **I.6** (phenolic OH- substituted isomer) precipitated out in pure form after initial purification

Scheme 2: Synthesis of compounds (**11, 12, 15,** and **16**)[b] for optimization of **8**.



Reagents and conditions: (a) dibromopentan (5 equiv.), K_2CO_3, acetone, 60°C. (b) DIPEA, AcCN, 80°C. (c) ethanolamine (1 equiv.), ethanol (absolute), 60°C. (d) MeI, K_2CO_3, acetone, 0°C. (e) MeI, K_2CO_3, DMF, 70°C. [a] **I.3** denote intermediate 3 and so on. [b] See Scheme 1 for **10**, **13**, **14**.

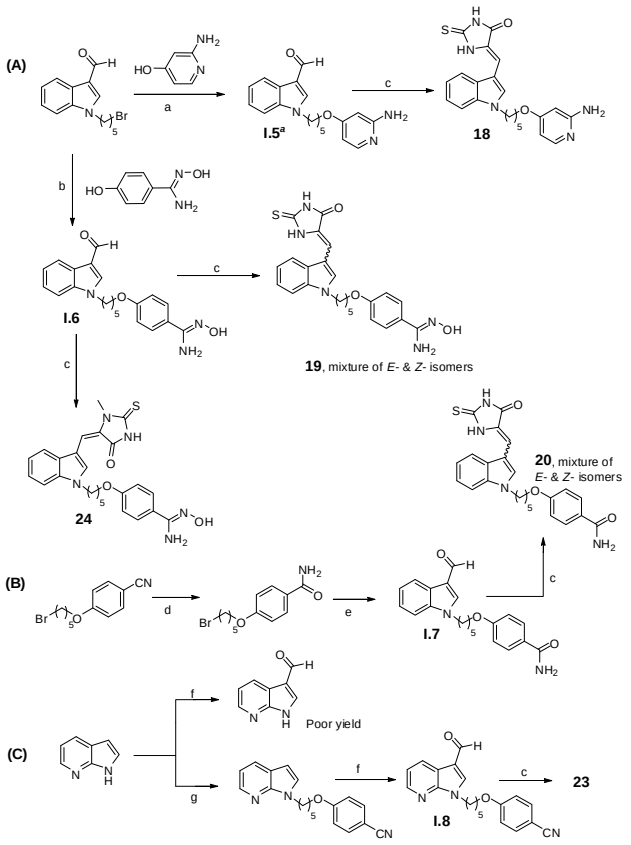
by column chromatography (Scheme 3). In contrast to above, simple n-alkylation of p-amidoximophenol provided inseparable mixture of two regioisomers. Comp. **17** and **24** were formed in exclusive *E*-configuration due to innate reactivity of indole-3-carbaldehyde (Scheme 1 & 3 respectively).[16]

Indole-3-carbaldehyde derivatives I.6 and I.7 (Scheme 3) behaves exceptionally in Knoevenagel condensation with 2-thiohydantoin. These derivatives instead of providing usual Z- configured molecules provided almost 1:1 mixtures of *E*- and *Z*- isomers. The behavior of these indole-3-carbaldehydes derivatives where indole NH- was substituted with p-amidino- and p-amidoximophenoxypentyl, respectively was surprising in comparison to p-cyanophenoxypentyl substituted derivative **I.1**, which provided only *Z*-isomer. Change of cyano function of p-cyanophenoxypentyl indole-3-aldehyde **I.1** to amidoxim or amide function may have disturbed the geometry of Knoevenagel condensation of CH acid (2-thiohydantoin) by interference in the transition state (TS) of the reaction.

To find new antileishmanial agents we screened initially formed guanylated derivatives **1**-**4** against promastigote stage of *L. donovani*. The structures were first screened at 10μg/ml. If greater than 90% inhibition were observed, the compounds were further screened against amastigote stage of parasite. Compounds **2**-**4** have shown >90% inhibition of promastigote stage of parasites, but none have shown activity against amastigote stage of *L. donovani*. Later compounds **5** and **6**, in which p-cyanophenoxypentyl chain has been removed, were screened. This change even diminished compounds' anti promastigote activity. Finally the screening of

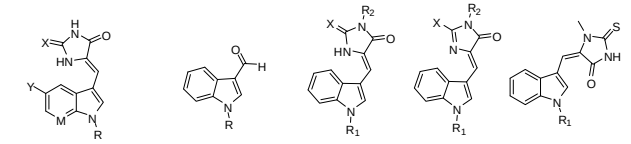
precursor compounds **7**-**9** provided a hit molecule, **8** which showed 62% growth inhibition of amastigotes in infected macrophages at 10μg/ml without showing any toxicity (Table 1).

Scheme 3: Synthesis of comp. **18**-**24** in a further attempt of optimization of **8**.



Reagents and conditions: (a) K_2CO_3, acetone, 60°C. (b) AcCN, K_2CO_3, 60°C. (c) 2-thiohydantoin or 1-Me-2-thiohydantoin ethanolamine (1 equiv.), ethanol (absolute), 60°C. (d) NaOH (aq.), H_2O_2, MeOH. (e) indole-3-carbaldehyde, K_2CO_3, acetone, 60°C.(f) DMF, $POCl_3$, 0°C-rt. (g)NaH, DMF,rt, under N_2. [a] **I.5** denote intermediate 5 and so on.

Surprisingly, the same compound **8** was also found active in vivo with almost complete bioavailability and no toxicity (Table 2).



1-8 (M= C, Y=H, Table 1) **18-24** (Table 4) | **9 (= I.1)** (Table 1) | **10-14** (Table 3) | **15-16** (Table 3) | **17** (Table 3) **24** (Table 4)

Figure 2: Structure of compounds listed in Table 1 to 4

From above promising data about comp. **8**, we next proceeded to optimize this lead molecule. A series of compounds **10**-**17** were synthesized (with variation in chain length, thiocarbonyl, cyano and phenolic moieties) and screened for antileishmanial activity. As is evident from Table 3 none of these compounds was found as promising as comp. **8**. Hence, it became evident that p-cyanophenoxypentyl and 2-thiohydantoin moieties are essential for activity of comp. **8**.

To enhance the activity of comp. **8** we next focused on to modify −CN functionality by converting it to amide, amidoxim etc. We also envisaged synthesizing 2-imino analogue of comp. **8** to see the effect on activity. A series of comp. **18**-**24** were synthesized

Table 1: Preliminary antileishmanial screening of initially formed compounds **1**-**9** (See Fig 2)

Comp.	R	X	% Inhibition [a]	
			Prom.[b]	Amat.[c]
1			88.00	NI[d]
2			93.76	NI
3			92.83	NI
4			93.90	NI
5	H		NI	NI
6	H		NI	NI
7	H	S	NI	NI
8		S	95.10	62.00 [e]
9		NIL	NI	NI

[a] percent inhibition measured at 10μg/ml (for Prom.) and at 12.5μg/ml (for Amat.), [b] Prom. : promastigote, [c] Amat. : intracellular amastigote, [d] NI: no inhibition, [e] Toxicity IC_{50} against J774 cell line >100 μg/ml.

Table 2: In vivo antileishmanial activity of comp. **8**

Comp.	Dosage route [a]	Dosage	% Parasitemia reduction [b]	Toxicity
8	ip	4x50mg/kg	62%	NT [c]

[a] ip: intra peritonial, [b] average over four hamsters, [c] all hamsters survived.

Table 3: Antileishmanial activity of compounds **10**-**17** (See Fig 2)

Comp.	R_1	R_2	X	Toxicity [e] IC_{50} (μg/ml)	% Inhibition [a]	
					Prom.[b]	Amat.[c]
10		H	O	33.04	NI [d]	NI
11		H	S	NT [f]	88.30	NI
12		H	S	NT	40.74	ND [g]
13		H	S	2.27	71.28	ND
14		H	S	NT	58.85	ND
15		H	SMe	NT	75.37	ND
16		Me	SMe	NT	63.19	ND
17		-	-	14.77	46.44	ND

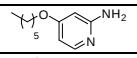
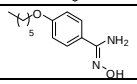
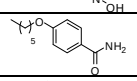
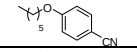
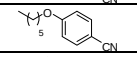
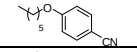
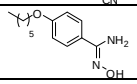
[a, b, c, d] See corresponding entries of Table 1, [e] cytotoxicity for J774 cell line, [f] NT: not toxic i.e. IC_{50} > 100 μg/ml, [g] ND: not determined (due to poor activity than **8** in Prom. assay).

In the third phase and directly screened against amastigote internalized in macrophages (which is more relevant for drug discovery) due to their high probability to be active.

In this endeavor we found that introduction of 2-aminopyridine in place of p-cyanophenoxy moiety in comp. **8**, which mimic amidine function, proved to be almost ineffective (comp. **18**, Table 3). This may be due to one carbon shortage compared p-

amidinophenoxypentyl fragment of pentamidine. Conversion of cyano function of comp. **8** to amidoxim proved to be totally ineffective (comp. **19**) and conversion to amide also didn't provide any significant activity (comp. **20**). Reduction in active

Table: 4 Antileishmanial activity of comp. **18**-**24** (See Fig 2)

Comp.	R	X	M	Y	Inhibition Amastigote [a]		Tox. [c] IC_{50} (µg/ml)	SI
					% [b]	IC_{50} (µg/ml)		
18		S	C	H	32.25	ND [d]	ND	N/A [e]
19		S	C	H	0	ND	ND	N/A
20		S	C	H	48.92	ND	ND	N/A
21		NH	C	H	0	ND	ND	N/A
22		S	C	Br	12.58	ND	ND	N/A
23		S	N	H	97.05	2.82	80.66	28.6
24		S	C	H	99.32	0.97 (2.0µM)	51.75	52.73
	Control [f]	-	-	-	--	12.11 (20.4µM)	1.61	<1

[a] intracellular amastigote, [b] % inhibition at 10µg/ml, [c] cytotoxicity for KB cell line, [d] ND: not determined, [e] N/A: not applicable, [f] Control: pentamidine isethionate salt.

diastereomer may be the reason for that, since both compounds were formed as ≈1:1 mixture of *E*- & *Z*-isomers. Surprisingly, comp. **24** with pure *E*-configuration proved to be most promising compounds of the series with IC_{50} = 2µM and SI = 52.73 for inhibition of parasitemia of infected macrophages. The compound was 10 times more active and 405 times less toxic than drug pentamidine. Replacing indole ring of comp. **8** with azaindole (comp. **23**) has only improved the activity of comp. **8**, in all synthesized compounds with *Z*- configuration. There were two other interesting findings. First, the conversion of 2-thio function of comp. **8** to 2-imino or substituted imino, which is equivalent to cyclic guanidine (**21**, **1**-**4**) totally diminished activity. Lipophilicity of cationic center of diamidines has already been reported as enhancer of biological activity against similar species of protozoa, [17] this may be the reason for activity enhancement of comp. **8** compared to **21**. Second, amidoxim containing comp. **24** (possessing *E*-configuration) has shown better activity profile than its cyano counterpart **17**. p-Amidinophenoxy functionality is already known as recognition motif for various transporter involved in pemtamidine uptake.[18] The selective enhancement of parasite cell permeability may be the reason for better activity of **24** over **17**.

In conclusion, in an effort to optimize activity of lead molecule **8** − based on aplysinopsin pentamidine hybrid scaffold − we found active molecules in both *E*-(comp. **24**) and *Z*- (comp. **23**) subseries of compounds. Incorporation of pentamidine substructure to 2-thio analogue of natural product aplysinopsin (comp. **24**) increased both selectivity and activity of parent drug pentamidine. The concept may be useful in improving bioactivity profile of privileged natural product structure for other therapeutic areas also. Future studies will be directed to more detailed study of SAR (by finding suitable chemistry to modify cyano function of comp. **8** in *Z*-subseries and by variation of cationic and linker part of comp. **24** in *E*-subseries) to optimize in vivo efficacy of this class of molecules.

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Supporting Information available: Assay methods, synthesis procedures and analytical data of compounds. This material is available free of charge via internet at http://pubs.acs.org.

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