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Anti-HIV activity of stilbene-related heterocyclic compounds

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Abstract—Viral transcription has not been routinely targeted in the development of new antiviral drugs. This crucial step of the viral cycle depends on the concerted action of cellular and viral proteins such as NF-κB and Tat. In the present study, stilbene-related heterocyclic compounds including benzalphthalide, phthalazinone, imidazoindole and pyrimidoisoindole derivatives are tested for their anti-HIV activity. Original assays based on recombinant viruses were used to evaluate HIV replication inhibition and stably transfected cell lines were used to evaluate inhibition of Tat and NF-κB proteins. Some of the stilbene-related heterocyclic compounds analysed displayed anti-HIV activity through interference with NF-κB and Tat function. Moreover, compounds inhibiting both targets displayed a stronger activity on viral replication.

Human immunodeficiency virus (HIV) is the etiological agent of acquired immunodeficiency syndrome (AIDS). This immunological disease remains the number one cause of mortality produced by infectious agents.[1] Drug discovery efforts yield new compounds with anti-HIV activity every year. However, these agents only interfere with three viral proteins, reverse transcriptase, protease and gp41 or fusion peptide. Moreover, emergence of resistance makes currently available drugs insufficient to maintain a safe therapeutic arsenal against HIV. Effective synergy has been found when combinations of drugs directed against different targets are used. Thus, development of new compounds against diverse steps of HIV life cycle is envisaged.

HIV viral cycle is divided into early and late events. Early events begin with the viral entry into the host cell and conclude with the integration of the HIV provirus into the cell genome. As an integrated provirus HIV can remain in a latent state from weeks to several years. Transition from latency to HIV replication occurs mainly when cells are activated and requires the concerted action of cellular transcription factors and regulatory HIV proteins.[2]

Reactivation from latent state is mediated by viral and cellular proteins such as Tat and NF-κB. Both could be possible targets for anti-HIV chemotherapy. The NF-κB family of transcription factors is involved in different processes such as embryonic development, apoptosis control, inflammatory response regulation and immune system activation.[3] In addition, NF-κB is the major inducible regulatory element involved in Long terminal repeats (LTR) transactivation and HIV-1 replication in CD4 lymphocytes.[4,5]

The virally encoded Tat protein is a potent activator of HIV expression[6,7] by, at least, two pathways.[8] First through binding the transactivation response element (TAR), a stable RNA stem loop located downstream of the transcriptional initiation site, leading to full RNA elongation.[9,6] Second through the interaction with

Keywords: NF-κB; Tat; HIV replication; Benzalphthalides; Phthalazinones; Imidazoisoindoles; Pyrimidoisoindoles.
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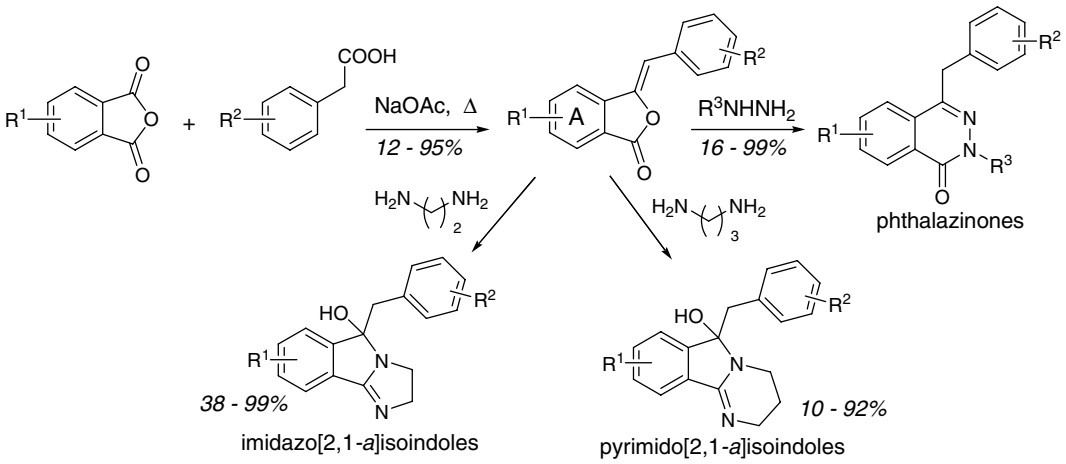
cellular transcription factors that bind to the enhancer region of the HIV-LTR, thus increasing HIV transcription. In fact, functional relations between Tat and NF-κB have been described.[5,10] Moreover, Tat is also able to modify the expression of genes involved in apoptosis.[11]

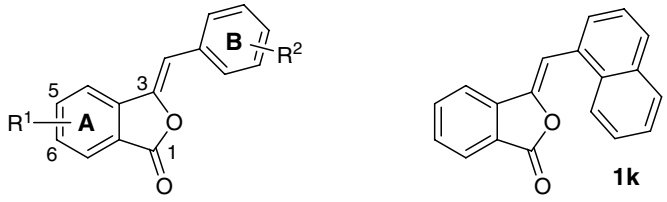
Therefore, simultaneous targeting of both, Tat and NF-κB,[12,13] could provide better expectancy against drug resistance than that attained in current AIDS therapy.[14,15]

Stilbenoids have previously shown anti-HIV activity,[16,17] in particular anti-Tat activity.[18] Compounds tested here contain the stilbene or dihydrostilbene skeletal basis supporting a fused heterocyclic system and can be divided in four main groups, benzalphthalides, phthalazinones, imidazoisoindoles and pyrimidoisoindoles. Some compounds belonging to these families displayed some activity against protozoa[19–21] and also promoted aorta ring vasorelaxation[22] and in vivo anxiolytic effects on mice.[23] The compounds evaluated in this research were selected as part of a preliminary systematic screening, and were prepared through chemical procedures already reported by us[21] that are summarized in Scheme 1. Aiming to recognise the respective influence on the activity, a number of natural-like and unnatural substituents, with different electronic, lipophilic, polarity and size attributes,



Scheme 1. The synthesis of fused heterocyclic compounds related to stilbene-2-carboxylic acid.

Table 1. Results of anti-HIV and cytotoxicity evaluations for some selected benzalphthalides[a]



Compound	Yield (%)	Structure		Antiviral activity				Cytotoxicity	Index
		R^1	R^2	5.1+TNF 100 μM	HeLa Tat-luc 100 μM	HeLa Tet-ON 100 μM	RVA IC_{50} μM	Propidium iodide CC_{50} μM	CC_{50}/IC_{50}
1b	35	H	2-Cl	<0	**76.8**	**S**	**24.4**	>195	>8.0
1c	70		4-Cl	84.8	84.6	U	**23.8**	>195	>8.2
1e	69		4-SCH_3	86.7	89.7	U	**23.4**	>187	>8.0
1h	25		3,4-OCH_2O–	91.8	87.2	U	**12.2**	138	11.3
1k	15		1-Naphthyl	<0	6.5	ND	>50	ND	—
2a	65	5(6)-CH_3	H	93.7	77.7	U	**22.3**	ND	—
2b	95		2-Cl	**71.0**	<0	**S**	133	>185	>1.4
2c	90		4-Cl	96.8	91.8	U	**15.8**	ND	—
2d	59		4-OCH_3	87.6	74.0	U	**14.3**	ND	—
2f	54		2,4-Cl_2	31.2	29.7	ND	**20.5**	>164	8.0
3c	12	4-NO_2	4-Cl	<0	63.7	U	119	<20.7	<0.2
4c	85	5,6-Cl_2	4-Cl	3.2	<0	ND	45.6	>154	>3.4

IC_{50}, concentration that reduces luciferase activity, thus RV replication, by 50%. CC_{50}, cytotoxic concentration that reduces viability of uninfected cells by 50%. ND, not determined; S, specific; U, nonspecific.

Results for HeLa Tat-Luc and 5.1 assays expressed as percentage of inhibition relative to control. <0 values indicate enhancement of the corresponding factor. Compounds **2a**–**g** were evaluated as regioisomeric 1:1 mixtures.

[a] Complete data related to Table 1 can be found in Supplementary data.

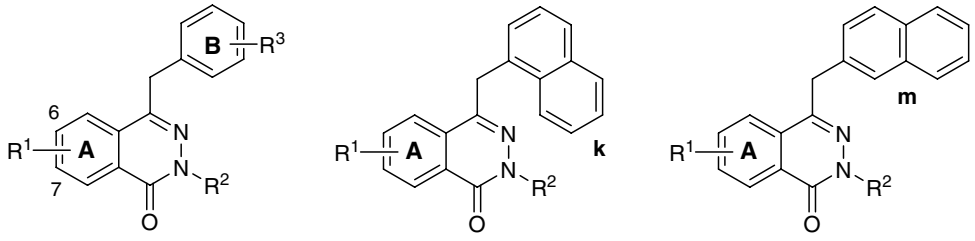
were located at certain positions on the structure of each group of compounds. Compounds submitted to evaluation were obtained in pure form, either through recrystallisation or preparative CC or TLC. All of them were characterised through their IR, ^{1}H and ^{13}C NMR spectral data. Experimental CHN analytical data were in agreement with the molecular formulae.

To evaluate the antiviral activity of these compounds, an original viral replication assay was performed using HIV clones, in which luciferase reporter genes have been cloned.[24] In this model, inhibition of viral replication is directly evaluated through measurement of luciferase activity in cell lysates in the presence or the absence of the different compounds. Inhibition of NF-κB was evaluated using 5.1 LTR Luc stably transfected cells, which contain a plasmid in which the reporter luciferase gene is driven by HIV-1 LTR promoter and it is responsive to the NF-κB activator cytokine TNFα. To perform the screening of anti-Tat activity, HeLa Tat-Luc stably transfected cell line was used. This cell line contains the same reporter plasmid as 5.1 cells together with the *tat* gene regulated by CMV promoter. Thus, LTR is highly activated in this cell line secondarily to expression of intracellular Tat protein.

Compounds were considered active when percentage of inhibition of NF-κB and/or Tat was higher than 50% and 30%, respectively, as compared to an untreated control. In order to rule out nonspecific mechanism of action, active compounds were evaluated through a HeLa Tet-On-Luc assay, in which the luciferase gene is under the control of an artificial promoter regulated by doxycycline.[25] Compounds lacking NF-κB and Tat activities were not submitted to specificity evaluation.

Twenty-seven benzalphthalides were evaluated and the results are partially shown in Table 1. Among them, nine compounds were NF-κB inhibitors at 100 μM and just compound **2b** displayed a specific mode of action. Tat inhibition was demonstrated for ten compounds, but only **1b** was specific.

Table 2. Results of anti-HIV and cytotoxicity evaluations for some selected phthalazinones[a]



Compound	Yield (%)	Structure			Antiviral activity				Cytotoxicity	Index
		R^1	R^2	R^3	5.1+TNF 100 μM	HeLa Tat-luc 100 μM	HeLa Tet-ON 100 μM	RVA IC_{50} (μM)	Propidium iodide CC_{50} (μM)	CC_{50}/IC_{50}
5b	93	H	H	2-Cl	67.3	29.8	**S**	41.6	>185	>4.5
5j	97		H	3,4,5-$(OCH_3)_3$	**96.5**	<0	**S**	51.7	135	2.6
6b	64		CH_3	2-Cl	73.0	18.3	S	**21.6**	>176	>8.2
6h	61		CH_3	3,4-OCH_2O–	**97.2**	25.5	**S**	27.4	131	4.8
6k	92		CH_3	1-Naphthyl	**100**	43.2	U	**11.0**	62.9	5.7
6m	99		CH_3	2-Naphthyl	**99.9**	11.8	U	**16.6**	57.2	3.5
7d	99		Et	4-OCH_3	**93.5**	14.2	**S**	**22.3**	89.5	4.0
8d	90		Allyl	4-OCH_3	50.0	2.9	**S**	30.5	68.6	2.3
9i	79		*n*-Bu	3,4-$(OCH_3)_2$	83.0	6.7	U	**16.0**	70.3	4.4
10c	39		*t*-Bu	4-Cl	82.1	11.5	**S**	**12.8**	72.1	5.6
11c	23		Ph	4-Cl	**86.4**	<0	**S**	58.7	67.7	1.2
12c	16		4-BrPh	4-Cl	<0	<0	ND	88.1	91.8	1.0
13c	62		4-NO_2Ph	4-Cl	54.8	29.6	**S**	89.5	96.3	1.1
14c	74	6(7)-CH_3	H	4-Cl	76.6	**99.0**	S	**14.5**	129	8.9
14g	84		H	3,4-Cl_2	14.9	28.4	ND	**19.5**	64.7	3.3
15f	81		CH_3	2,4-Cl_2	<0	14.0	ND	**18.8**	112	6.0
16b	64	6,7-Cl_2	H	2-Cl	65.9	11.2	**S**	**22.0**	>147	>6.7
16d	88		H	4-OCH_3	12.6	<0	ND	**15.7**	>149	>9.5
16f	55		H	2,4-Cl_2	**99.0**	53.9	**S**	96.2	58.0	0.6
17a	60		CH_3	H	19.4	11.4	ND	47.5	>156	3.3

IC_{50}, concentration that reduces luciferase activity by 50%. CC_{50}, concentration that reduces viability of uninfected cells by 50%. ND, not determined; S, specific; U, nonspecific.

Results of HeLa Tat-Luc and 5.1 assays expressed as percentage of inhibition relative to control. <0 values indicate enhancement of the corresponding factor. Compounds **5a–g** and **14b–g** were assayed as regioisomeric 1:1 mixtures.

[a] Complete data related to Table 2 can be found in Supplementary data.

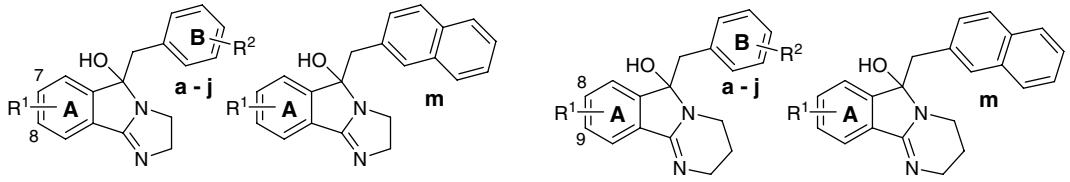
Concerning antiviral activity, the recombinant virus assay (RVA) revealed low potency values except for compounds **1b**, **2f** and **4c**. Compound **2b** showed a specific mode of action, although percentage of NF-κB inhibition was in the medium range. The most interesting compounds of this family were: **1b**, which interferes HIV replication by inhibiting Tat activity through an NF-κB-independent mechanism, and **2f** and **4c** which should act by a mechanism not related to those analysed here. Interestingly, those benzalphthalides displaying anti-HIV activity resulted less cytotoxic and it should be noticed that they contain chlorine atoms at positions 2 or 4 of the phenyl substituent.

The results of antiviral screening for phthalazinones are partially shown in Table 2. Thirty-four compounds (68%) inhibited HIV replication in the RV assay and its mode of action was specific. The activity of these compounds could be mainly due to NF-κB inhibition, since twenty-two specific compounds (44% of the total) inhibit TNFα activation of 5.1 LTR Luc cells. However, some compounds that do not target neither NF-κB nor Tat, remain active in the RVA, suggesting that some compounds of this family could act on other targets.

Apart from the general observations about phthalazinones (good NF-κB inhibitors, with medium potency in the RVA and not too cytotoxic compounds), other SAR considerations on the results shown in Table 2 could not be easily deduced. Nevertheless, it can be observed that within the subfamily of phthalazinones without substituent on ring A (compounds **5**–**13**), the alkylation of the nitrogen atom at position 2 and the presence of a chlorine atom at positions 2 or 4 on ring B (compounds **5b** and **5c**, respectively) seem to be important features for NF-κB inhibition and for the specificity of antiviral activity. However, this appreciation clearly fails in the case of ^{2}N-allyl (compounds **8**) or ^{2}N-phenyl (compounds **11**–**13**) derivatives. On the other hand, the existence of methyl- or dichloro-substitutions on ring A makes the absence of a substituent on ^{2}N preferred (compounds **14** vs **15**), not only for the above-mentioned aspects of antiviral activity, but also for enhancing substantially the Tat inhibitory activity (99% at 100 μM, for compound **14c**). Additionally, and considering all of the evaluated phthalazinones, three compounds merit to be mentioned especially. Compound **14c** was the only compound with a specific and potent antiviral activity, inhibiting both targets (NF-κB and Tat) and displaying a good RVA activity/cytotoxicity ratio (index: 8.9; Table 2). Compound **10c** resulted more potent than **14c** in the RVA, with an IC_{50} value of 12.8 μM, being also specific, and displaying a fair NF-κB inhibitory activity. Compound **6b** behaved similarly, it was less potent as antiviral than **10c**, but also less cytotoxic. Finally, like benzalphthalides, some phthalazinones (e.g., **8e**, **14g**, **15f** and **16d**) showed relatively high potency in the RVA, without notable inhibitory activities on NF-κB nor Tat, suggesting that they would act through another mechanism.

The results for the two related families of imidazoisoindoles and pyrimidoisoindoles are partially shown in Table 3. Inhibition of HIV replication was observed

Table 3. Results of anti-HIV and cytotoxicity evaluations for some selected imidazoisoindoles and pyrimidoisoindoles[a]



imidazo[2,1-*a*]isoindoles (18, 19, 20) **pyrimido[2,1-*a*]isoindoles (21)**

Compound	Yield (%)	Structure		Antiviral activity				Cytotoxicity	Index
		R^1	R^2	5.1+TNF 100 μM	HeLa Tat-luc 100 μM	HeLa Tet-ON 100 μM	RVA IC_{50} (μM)	Propidium iodide CC_{50} (μM)	CC_{50}/IC_{50}
18c	88	H	4-Cl	<0	38.4	**S**	71.0	>168	2.4
18f	99	H	2,4-Cl_2	<0	**86.6**	**S**	**13.8**	87.5	6.3
19c	96	7(8)-CH_3	4-Cl	**98.9**	78.9	**S**	25.9	34.4	1.3
19g	38	7(8)-CH_3	3,4-Cl_2	**100**	**84.0**	**S**	40.1	37.8	0.9
20b	82	7,8-Cl_2	2-Cl	**100**	**99.6**	U	**22.8**	30.1	1.3
20d	65	7,8-Cl_2	4-OCH_3	**100**	75.3	**S**	27.6	44.1	1.6
21b	26	H	2-Cl	**99.7**	<0	**S**	66.7	78.6	1.2
21d	25	H	4-OCH_3	<0	3.8	ND	74.2	>162	>2.2
21e	39	H	4-SCH_3	**99.8**	13.9	**S**	61.5	142	2.3
21g	76	H	3,4-Cl_2	**67.7**	**60.0**	**S**	26.1	85.2	3.3
21m	71	H	2-Naphthyl	**100**	**51.2**	**S**	60.8	81.0	1.3

IC_{50}, concentration that reduces luciferase activity by 50%. CC_{50}, concentration that reduces viability of uninfected cells by 50%. ND, not determined; S, specific; U, nonspecific.

Results of HeLa Tat-Luc and 5.1 assays expressed as percentage of inhibition relative to control. <0 values indicate enhancement of the corresponding factor. Compounds **19a**–**c** were evaluated as regioisomeric 1:1 mixtures.

[a] Complete data related to Table 3 can be found in Supplementary data. No attempts were made to optimise reactions and yields.

for almost all the compounds tested when RVA was used. After discarding for further studies nonspecific and toxic compounds, imidazoisoindoles **18f**, **19b**, **19c** and **20d**, and pyrimidoisoindoles **21d**, **21e**, **21g** and **21m** displayed HIV replication interference. Nevertheless, CC_{50} cytotoxicity values were close to the IC_{50} values observed in the RV assay, and the cytotoxicity/activity indexes were too low, as seen in Table 3. This toxicity could also justify the erratic changes of activity observed for the series of compounds **18a**–**m**. Among these compounds, **18f** showed the highest therapeutic index (6.3), but still modest, and its mode of action seems to be related to Tat inhibition.

In summary, it can be concluded that the four families of stilbenoid-related compounds inhibit HIV replication and act through inhibition of NF-κB and/or Tat. Results among benzalphthalides showed activity in just four of them, but only **1b** was interesting. This Tat inhibitor strongly inhibits HIV replication. Imidazoisoindoles and pyrimidoisoindoles were also effective against HIV replication in the RV assay, acting through Tat, NF-κB inhibition or both, but most probably their activity correlates with their cytotoxicity. On the other hand, benzylphthalazinones are less cytotoxic than the isoindole derivatives and constitute a new family of compounds that inhibit NF-κB, since 50% of those evaluated interfere with this activity and block HIV replication in the RV assay.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2006.04.087.

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