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4-Phenylcoumarins as HIV transcription inhibitors

Luis M. Bedoya,[a] Manuela Beltrán,[a] Rocío Sancho,[b] Dionisio A. Olmedo,[c,d] Sonsoles Sánchez-Palomino,[a] Esther del Olmo,[d] José L. López-Pérez,[d,*] Eduardo Muñoz,[b] Arturo San Feliciano[d] and José Alcamí[a,*]

[a]*Unidad de Inmunopatalogía, Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220-Majadahonda, Madrid, Spain*
[b]*Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba, Avda. Menéndez Pidal s/n. 14004-Córdoba, Spain*
[c]*CIFLORPAN, Facultad de Farmacia, Universidad de Panamá, R. de Panamá, Panamá*
[d]*Departamento de Química Farmacéutica, Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, 37007-Salamanca, Spain*

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Abstract—We have evaluated the anti-HIV activity of eleven natural 4-phenylcoumarins isolated from *Marila pluricostata* and three of their derivatives. Antiviral activity was assessed on MT-2 cells infected with viral clones carrying the luciferase gene as reporter. Inhibitions of HIV transcription and Tat function were tested on cells stably transfected with the HIV-LTR and Tat protein. Most of the coumarins tested displayed NF-κB inhibition. Two coumarins were also Tat antagonists and the presence of both activities correlated with a stronger inhibition of HIV replication. Our results show that antiviral effect of 4-phenylcoumarins can be related to the inhibition of NF-κB and Tat, and suggest that these types of compounds can be useful in the treatment of HIV infection as viral transcription inhibitors.

The viral cycle of the HIV can be divided into early and late events. Early events comprise several steps, from viral attachment on the cell surface to integration in the host genome. Late events include the processes of HIV mRNA synthesis, protein expression and morphogenesis. Once integrated, HIV can remain in a latent state in resting lymphocytes or undergo active replication. Transition from latency to HIV expression occurs mainly when cells are activated and requires the concerted action of cellular transcription factors and regulatory HIV proteins.[1,2] Among the transcription factors involved in LTR transactivation, the HIV proximal enhancer contains three binding sites for SP1 transcription factor and two binding sites for NF-κB. The NF-κB/Rel family of transcription factors represents a major inducible regulatory element involved in HIV transcription.[3] Located downstream of the basal promoter TAR sequence is the RNA target for the viral protein Tat, which acts in concert with other cellular factors[4] to generate full-length RNA transcripts.[5] Furthermore, NF-κB and Tat cooperate in driving HIV replication from the state of latency. Therefore, inhibition of the activity of these critical proteins should result in an effective blocking of viral replication.[6–8]

It has been reported that natural coumarins and derivatives can display anti-HIV activity through different mechanisms, including blockade of viral entry, inhibition of reverse transcriptase and interference with viral integration.[9,10] Some phenylcoumarins and chalcones, as well as tannins and lignins, have been proposed as suppressors of LTR-dependent transcription, but the mechanism of action has not been fully characterised.[11] More interestingly, (+)-calanolide A, a natural dipyranocoumarin currently undergoing anti-AIDS clinical trials,[12] has also proven to be an effective antimycobacterial against drug-sensible and drug-resistant *Mycobacterium tuberculosis* strains. This relevant fact should promote further studies to take advantage of this therapeutic ambivalence and to evaluate the possibility of using coumarins for treating patients suffering from

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* Corresponding authors. Tel.: +34 918223943; fax: +34 8223943; e-mail: ppalcami@isciii.es

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both AIDS and tuberculosis diseases.[13] This could also contribute to ameliorate patient adhesion to the treatment.

Here, we report on the results of anti-HIV evaluation of 4-phenylcoumarins through inhibition of NF-κB and Tat activity. The structures of the assayed 4-phenylcoumarins are shown in Figure 1. They were mainly isolated from the dichloromethane extract of leaves of *Marila pluricostata* (Fam. *Clusiaceae*) collected in Panama and belong to the prenylated mammea and mesuol types of coumarins, commonly found in other *Clusiaceae* species. Their structures were established by spectroscopic and chemical means, and the details of their characterisation have been reported recently.[14]

As it can be seen, apart from the 4-phenyl group, all the coumarins tested contain oxygenated functions at positions C-5 and C-7. Additionally, two coumarins have a prenyl (**1**) or acyl (**2**) monosubstitution at C-8; eight compounds (**3**–**7**) are disubstituted at positions C-6 and C-8 with prenyl, acyl, or hydroxyalkenyl groups in different combinations and in the last four compounds (**8**–**11**) the oxygen at position C-7 is involved in the formation of fused dihydrofuran (**8**, **9**) or pyran (**10**, **11**) rings. Thus, in spite of the small number of compounds tested, a wide variety of structural arrangements have been covered, which would facilitate comparisons of the influence of the type and position of the substituents on the antiviral activities tested.

As part of the characterisation of anti-HIV activity, the effect of these coumarins on Tat and NF-κB functions has been analysed. To this aim, we have used two stably transfected cell lines. Previously described 5.1 cell line[15] is a Jurkat-derived clone stably transfected with a plasmid containing the luciferase gene under the control of HIV-LTR. In this cell clone, activation with TNFα induces NF-κB activation and subsequent HIV-1 expression. We have also analysed the anti-HIV activity in HeLa-Tat-Luc cells,[16] in which the HIV-1 LTR is directly activated by the HIV-1 Tat protein. A compound was considered active in one assay if it inhibited the target function by more than 50% (NF-κB) or 30% (Tat) at either 25 or 50 μM concentration. The active compounds were submitted for further evaluation through a HeLa-Tet-ON assay, as previously described.[17] Compounds with a non-specific mode of action were not examined further, and those showing specific activity were analysed using a recombinant virus (RV) assay. In addition, those compounds that were specific and did not reach the threshold of NF-κB and anti-Tat activity were also tested in RV assay to rule out anti-HIV activity through other pathways. In this system, a luciferase reporter gene has been cloned in full-length infectious DNA. The measured luciferase activity is therefore directly proportional to the replication of the virus. This assay is more sensitive and reliable than classic infectious assays (Bedoya et al. manuscript in preparation). The complete results of anti-HIV evaluation of coumarins **1**–**11** are shown in Table 1.

The RV assay displayed anti-HIV activity in the case of coumarins **1**, **2**, **3**, **6a**, **7**, **10**, and **11**, and their mode of action was specific. AZT was used as a reference compound. Coumarins **4**, **5**, **6**, **6b**, **6c**, **8**, and **9** were not

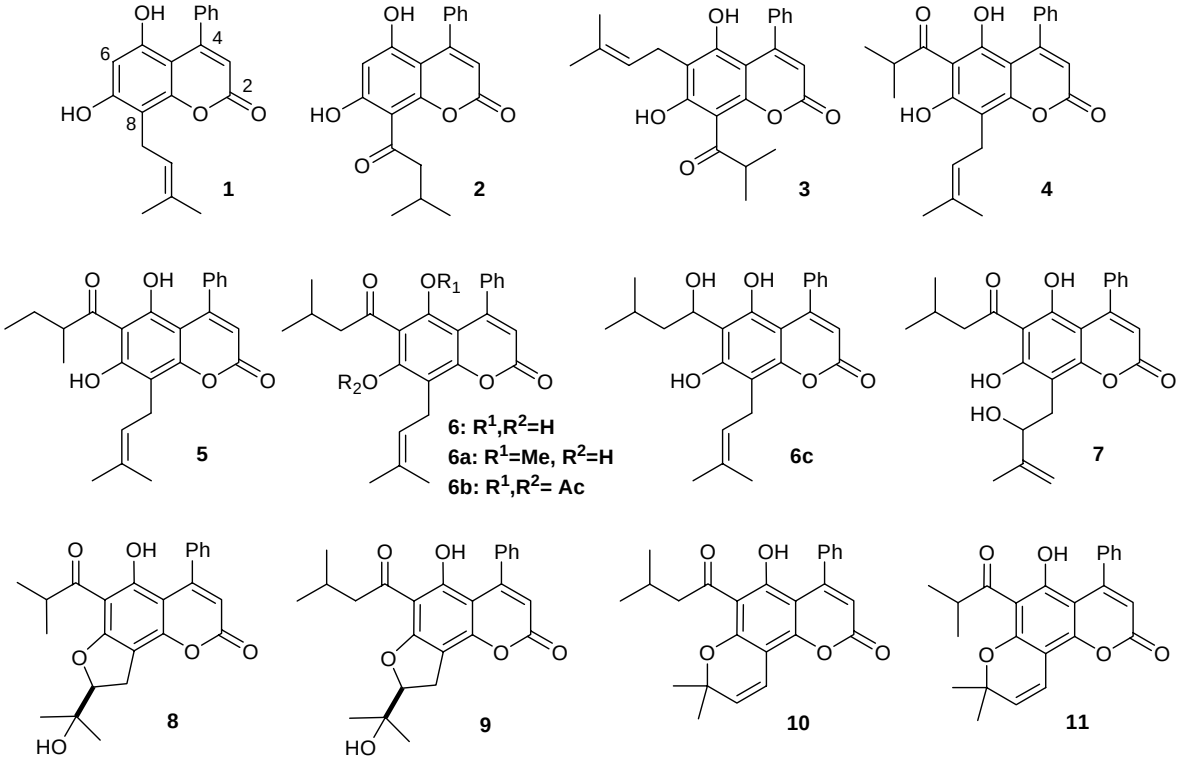

Figure 1. Natural 4-phenylcoumarins evaluated as anti-HIV agents.

Table 1. Results of anti-HIV evaluation of 4-phenylcoumarins

Compound	Antiviral activity						Toxicity
	RV (VIH) IC50 (μM)	5,1 + TNFα		HeLa-Tat-Luc		HeLa-Tet-ON	Cell death (%) at 50 μM
		25 μM	50 μM	25 μM	50 μM	50 μM	
1	11.2	33.4	84.5	NT	15.4	NT	34.4
2	**6.9**	**81.0**	**85.6**	**42.0**	**69.4**	**S**	**17.2**
3	**31.9**	**67.0**	**67.1**	**36.7**	**34.3**	**S**	**11.5**
4	NT	67.1	77.9	11.5	71.3	U	22.5
5	NT	NT	85.2	NT	86.7	U	28.8
6	NT	NT	58.8	NT	53.1	U	16.6
6a	22.2	NT	43.6	NT	13.5	NT	4.8
6b	NT	NT	81.1	NT	50.4	U	15.7
6c	NT	NT	60.0	NT	47.6	U	8.0
7	**0.5**	**55.4**	**53.1**	**43.6**	**70.4**	**S**	**18.5**
8	NT	NT	59.4	NT	59.5	U	79.5
9	NT	66.9	86.3	NT	22.0	U	34.1
10	123.8	5.9	15.7	2.0	3.0	NT	4.7
11	32.1	10.8	20.2	0.0	0.0	NT	4.5
AZT	0.01	NT	NT	NT	NT	NT	>100
Mesuol	2.5	71.0	77.9	NT	71.3	S	22.5

IC_{50}, concentration that inhibits infection at 50%.
Rest of the values are percentages of inhibition. HeLa-Tet-ON assay: NT, not tested; S, specific; U, unspecific.

tested in the RV assay because of their non-specific mode of action. All the coumarins tested were non-toxic at 50 μM excepting compound 8 that displayed high cytotoxicity. Other coumarins, as imperatorin, induce cell cycle arrest in the G1 phase,[18] but the analysis of compound 8 suggests a clear apoptotic effect at concentrations as low as 25 μM (data not shown).[19]

Coumarins **1**, **2**, **3**, and **7** showed inhibitory activity in the NF-κB test at 50 μM, and coumarins **2**, **3**, and **7** also at 25 μM. Regarding the anti-Tat activity, coumarins **2**, **3**, and **7** showed anti-Tat activity in the HeLa-Tat-Luc assay and their mode of action was specific, although coumarin **3** inhibition percentage at 50 μM was lower than 40%. Mesuol was used as a reference inhibitor of NF-κB and Tat activities.[20] All these compounds showed activity in the RV assay. Interestingly, coumarins **2** and **7**, displaying both anti-NF-κB and anti-Tat activities, also became active in the RV assay, with IC_{50} values of 6.9 and 0.5 μM, respectively. Thus, coumarins inhibiting both targets, NF-κB and Tat, are more potent inhibitors of the HIV replication, suggesting a possible synergy between NF-κB and Tat, as it has previously been shown in peripheral blood lymphocytes (PBLs).[5] Despite of coumarin 2 was more potent as NF-κB inhibitor, coumarin 7 displayed better activity in the RV assay, although anti-Tat activity was similar in both. These results suggest that additional target in viral replication could not be ruled out for coumarin 7, as previously stated for other coumarins as imperatorin.[18]

Coumarin **3** was less active as Tat inhibitor and accordingly its IC_{50} value was higher, 31.9 μM. Coumarins **6a** and **11** were active in the RV assay, with IC_{50} values of 22.2 and 32.1 μM, but did not show either anti NF-κB or anti-Tat activity. Overall, our data suggest that inhibition of viral transcription would be the main target of these natural coumarins. However, other mechanisms could be involved,[10] as it can be deduced for compounds **6a** and **11**, although their antiviral activity is lower than that of the anti-NF-κB or anti-Tat natural coumarins. Therefore, the potential role of this type of coumarin as possible therapeutic agents for HIV infection points to their capacity for inhibiting HIV expression from latency, mainly through interference of NF-κB and Tat functions.

Although all the compounds tested contain the same 4-phenylcoumarin skeleton and the double hydroxyl substitution at C-5 and/or C-7, no other structural features can be identified to be determinant factors of the activity and specificity observed in the most interesting compounds. Compounds **2** and **3**, which contain an acyl group attached to C-8, differ in the respective absence or presence of the prenyl chain at C-6. Neither the presence of the mentioned acyl chain at C-8 could be considered necessary for the activity, because it is absent in compound **7**, which in turn, contains a β-hydroxyalkenyl substituent at C-8. Compounds **2** and **7** have shown more potency in the RV assay and target both NF-κB and Tat proteins. It has been described that Tat can activate NF-κB by yet unidentified pathways[21] and both proteins act synergistically in driving HIV transcription.

These considerations along with the results shown here suggest that 4-phenylcoumarins, and specially compounds **2** and **7**, are HIV transcription inhibitors. Further studies including the synthesis and the evaluation of a number of related coumarins are in progress and a deeper insight to determine the mechanism of action is required for assessing the actual potential and interest of these 4-phenylcoumarins as anti-HIV drugs.

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