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One drug for two targets: Biological evaluation of antiretroviral agents endowed with antiproliferative activity

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ABSTRACT

AIDS-related cancer diseases are malignancies with low incidence on healthy people that affect mostly subjects already immunocompromised. The connection between HIV/AIDS and these cancers has not been established yet, but a weakened immune system is certainly the main cause. We envisaged the possibility to screen a small library of compounds synthesized in our laboratory against opportunistic tumors mainly due to HIV infection like Burkitt's Lymphoma. From cellular assays and gene expression analysis we identified two promising compounds. These derivatives have the dual action required inhibiting HIV replication in human TZM-bl cells infected with HIV-1 NL4.3 and showing cytotoxic activity on human colon HT-29 and breast adenocarcinoma MCF-7 cells. In addition, preclinical *in vitro* adsorption, distribution, metabolism, and excretion studies highlighted a satisfactory pharmacokinetic profile.

Kaposi's Sarcoma (KS), Hodgkin's Lymphoma (HL) and Burkitt's Lymphoma (BL), are orphan AIDS-related cancers diseases.[1] These cancers are rare among the healthy population but have high incidence in immune-compromised patients (e.g. persons infected by HIV).[2] The current therapy for KS, BL and HL still require the use of surgery, debilitating radiotherapy, and anticancer compounds such as Taxol® and Doxorubicin for which heavy side effects are well known.[3] Moreover, the treatment of these cancers is complicated by the concomitant intake of antiretroviral drugs.

The development of a preclinical drug candidates active both against rare AIDS-related cancers and against HIV replication (one drug for two targets) will reduce the total number of drugs that a patient should assume. Moreover the use of a drug with a dual mode of action has also a clear benefit in terms of possible side effects and multi-drug interactions.[4]

Recently, we reported the discovery and synthesis of a series of compounds endowed with anti-retroviral activity which proved to be strongly active against HIV virus.[5] The general structure of these compounds is shown in Fig. 1 (general structure A). The antiretroviral mode of action of these molecules resides in the inhibition of the gp120-CD4 protein-protein interaction, detrimental for the entry process of the viral infection. A block at this stage of the infection results in the inability for the virus to infect healthy cells. Two compounds of that series (compounds **3** and **4**, Fig. 2), combined with another small library synthesized in our laboratory (general structures **B** and **C**, Fig. 1), were screened against human MCF-7 breast cancer cells, HT-29 colon cancer cells and Ramos Human Caucasian Burkitt's Lymphoma cells.

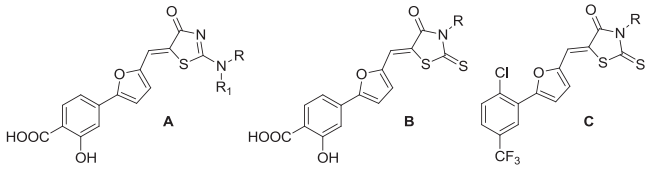

Fig. 1. General structures of the compounds of the library.

To obtain compounds with general structure **A** we used a one pot microwave assisted multicomponent reaction protocol (Scheme 1).[6,7] This microwave-assisted procedure allowed us to obtain, in short reaction time and easy workup, different final

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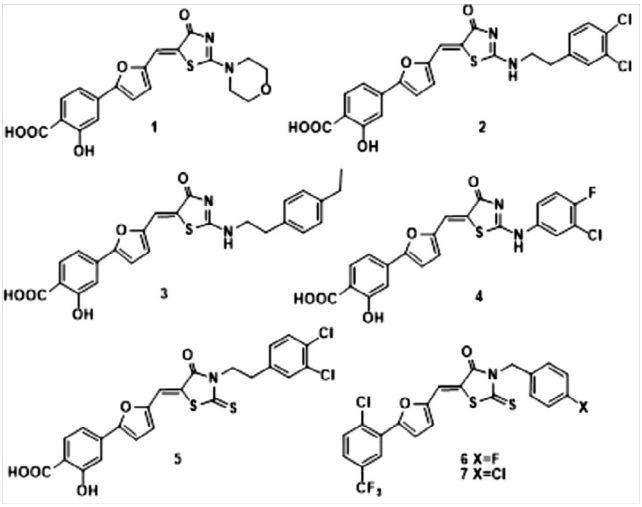
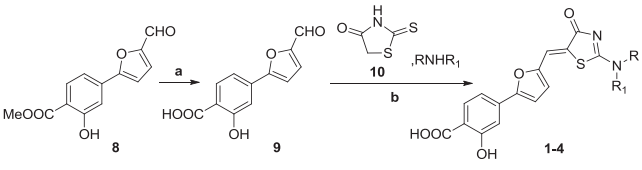


Fig. 2. Structure of compounds synthesized and tested.

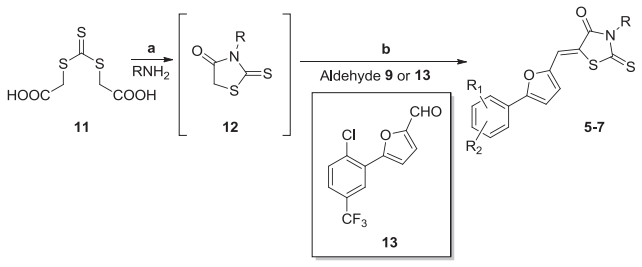
products by changing only the amine employed. To generate compound **1** morpholine was used as secondary amine whilst 2-(3,4-dichlorophenyl)ethanamine, 2-(4-ethylphenyl)ethanamine and 3-chloro-4-fluoroaniline were used as primary amines to generate compounds **2**–**4**, respectively.

The other series of substituted rhodanine derivatives with general structures **B** (compound **5**) and **C** (compounds **6** and **7**) were obtained by exploiting a practical and rapid procedure developed by us and showed in Scheme 2.[8] This methodology consists of a sequential, one-pot, two-steps microwave-assisted process with the formation of the desired final compounds in few minutes and high purity (Scheme 2).

All the synthesized compounds were tested *in vitro* to evaluate their ability to inhibit HIV replication in human TZM-bl cells infected with HIV-1 NL4.3 (a CXCR4-tropic strain) and their biological results are listed in Table 1. All these derivatives showed antiviral activity in the low micromolar range, with compounds **3** and **4** being the more potent of the series (see Table 1).[5] Furthermore, we carried out a measurement of the cytotoxic activity of our synthetic compounds **1**–**7** using three tumoral cells, the human



Scheme 1. Synthesis of derivatives **1**–**4** (a) 1 M NaOH aq., THF/MeOH, reflux, 2 h (99%). (b) 2-thioxothiazolidin-4-one **10**, amine $RNHR_1$, EtOH, MW (300 W), 150 °C, 20 min. Yields: **1** (49%); **2** (39%); **3** (80%); **4** (54%).



Scheme 2. Synthesis of derivatives **5**–**7** (a) DME, Et_3N, MW (300 W), 90 °C, 10 min. (b) aldehyde **9** or **13**, MW (300 W), 110 °C, 5 min. Yields: **5** (29%); **6** (18%); **7** (54%).

colon HT-29, the breast adenocarcinoma MCF-7 and the Human Caucasian Burkitt's Lymphoma Ramos, as well as two non-tumoral cell lines, the human embryonic kidney cell line HEK-293 and the bovine aortic endothelial cells BAE.[9] Table 1 shows the cytotoxicity values for compounds **1**–**7**, expressed as the compound concentration (μM) that causes 50% inhibition of cell growth (IC_{50}). Table 1further shows the selectivity indexes, named here as α, β, γ, δ, ε and ζ obtained by dividing the IC_{50} values of the non-tumoral cell lines HEK-293 or BAE by those of HT-29, MCF-7 or Ramos cell lines respectively (see footnote in Table 1). The higher the value of either coefficients, the higher the therapeutic safety margin of the compound in the corresponding cell line.

The cytotoxicity of compounds **1**, **3**, **4**, **6** and **7** is in the low micromolar range. Regarding the HT-29 line, compounds **1** and **4** showed promising inhibitory activity and as a consequence high α and γ values. For MCF-7 cells, compounds **6** and **7** showed the lowest IC_{50} values, having β and δ values reasonably high. Moreover, compounds **1**, **4**, **6**, **7** showed good IC_{50} values on Ramos cell line. Among all these compounds, derivatives **1** and **4** are the ones that combine high cytotoxicity towards HT-29 cell line, acceptable inhibitory activity against MCF-7 and Ramos and low cytotoxicity towards non-tumoral cell lines HEK-293 and BAE.

Moreover, all these compounds were further investigated for the inhibition of gene expressions. Most of the viruses responsible for the AIDS-related cancers aforementioned, in fact, promote the up-regulation of proto-oncogenes like c-*Myc* and genes like *hTERT*.[10] These two genes are overexpressed as a result of the infection from oncoviruses like the Epstein Barr virus (EBV) and the human virus 8 (HHV-8), responsible for BL, HL and KS.[11] To study the ability of our compounds to inhibit *hTERT* and c-*Myc* gene expression, HT-29 cells were incubated with a non-cytotoxic concentration of each compound (concentration lower than their IC_{50} value), then the RNA was extracted and retrotranscripted to cDNA to quantify the amount of gene expressed.

In order to determine whether the synthesized compounds were able to downregulate the expression of *hTERT* and c-Myc genes, we have performed a reverse transcription quantitative PCR (RT-qPCR) analysis using HT-29 tumoral cells (see Experimental Section).[12]

For these measurements compounds **2** and **5** were not selected because they were not cytotoxic towards HT-29 cells (IC_{50} values higher than 100 μM). In these assays, concentrations lower than the IC_{50} values towards the HT-29 cell line were used. Accordingly, concentrations were always 1 μM except for compound **6**, which was used at a concentration of 5 μM, and compound **3**, which was used at a concentration of 20 μM, because of their lower cytotoxicity on HT-29 cells.

Results for the selected compounds are depicted in Fig. 3 which shows the percentage of *hTERT* gene expression after 48 h of incubation in the presence of DMSO (control experiment) and in the presence of each of the compounds investigated at a concentration of 1 μM (lower than their IC_{50} values). All values were standardized (100%) to control (DMSO) and to β-actin.

It is worth noting that all selected compounds are able to significantly downregulate *hTERT* gene expression around **50%**.

The most remarkable compound is **7**, which has the strongest inhibitory activity on the *hTERT* gene expression, downregulating *hTERT* gene expression to 37%.

In order to determine whether the studied compounds were able to regulate the expression of the *c-Myc* gene, we have performed a RT-qPCR analysis using again HT-29 tumoral cells. The cells were incubated for 48 h in the presence of DMSO (control) and, as above, 1 μM of each of the studied compounds (for **6**, 5 μM and for **3**, 20 μM) were used. Results, standardized (100%) to the control (DMSO) and to β-actin, are depicted in Fig. 4.

Table 1
IC_{50} values (μM), LD_{50} values (μM) and selectivity indexes for compounds **1**–**7**.[a]

Comp.	NL4.3	TZM-bl[b]	HT-29	MCF-7	Ramos	HEK-293	BAE	α^c	β^d	γ^e	δ^f	ε^g	ζ^h
1	13.4 ± 6.4	>50	2,3 ± 1,0	21 ± 3	3,14 ± 1	53 ± 3	76 ± 9	23,04	2,52	33,04	3,62	16,9	24,2
2	1.8 ± 0.4	>50	>100	>100	>100	>100	>100	–	–	–	–	–	–
3	1 ± 0.3	>50	26 ± 3	4,5 ± 1,6	37 ± 2	3,4 ± 0,4	20 ± 4	0,13	0,76	0,77	4,44	0,09	0,54
4	1 ± 0.8	>50	1,1 ± 0,6	13 ± 7	3,1 ± 0,7	12,0 ± 0,8	25 ± 4	10,91	0,92	22,72	1,92	3,9	8,0
5	4.8 ± 0.7	>50	>100	>100	>100	29 ± 7	86 ± 10	<0,29	<0,29	<0,86	<0,86	<0,29	<0,86
6	6.9 ± 1.2	>50	8,3 ± 1,9	1,13 ± 0,21	3,8 ± 1,1	4,2 ± 2,3	2,4 ± 0,5	0,51	3,72	0,29	2,12	1,1	0,63
7	17.3 ± 3.8	>50	4,6 ± 1,8	1,2 ± 0,4	4,1 ± 0,5	2,4 ± 0,9	2,2 ± 0,5	0,52	2,00	0,48	1,83	0,59	0,53
RAL	0.021	>50	–	–	–	–	–	–	–	–	–	–	–
RTV	–	–	137 ± 8	34 ± 6	37 ± 5	17.0 ± 2.6	57 ± 5	–	–	–	–	–	–

[a]Values are the average (±s.d.) of three different measurements performed as described in the Experimental Section. [b]50% lethal dose LD_{50}. α^c = IC_{50} (HEK-293)/IC_{50} (HT-29). β^d = IC_{50} (HEK-293)/IC_{50} (MCF-7). γ^e = IC_{50} (BAE)/IC_{50} (HT-29). δ^f = IC_{50} (BAE)/IC_{50} (MCF-7). ε^g = IC_{50} (HEK-293)/IC_{50} (Ramos). ζ^h IC_{50} (BAE)/IC_{50} (Ramos). Values of α, β, γ and δ have been rounded off to a decimal figure. With RAL and RSV are indicated the positive controls Raltegravir and Resveratrol respectively.

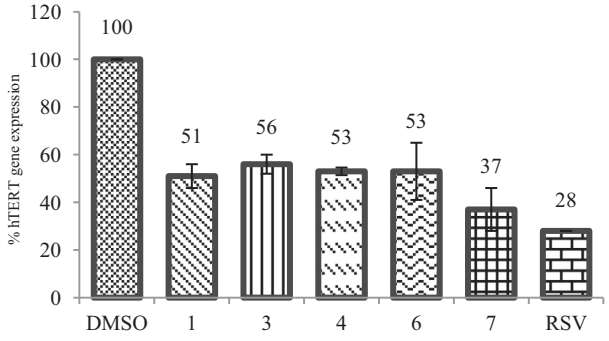


Fig. 3. Expression percentage of the *hTERT* gene after 48 h of incubation of HT-29 cells determined by means of the RT-qPCR methodology. At least three measurements were performed in each case. Concentration of all compounds was 1 μM (for 3, 20 μM and for 6, 5 μM). With RSV is indicated the positive control Resveratrol. Error bars indicate standard errors of the mean. The statistical significance was evaluated using one-sample *t*-tests (P < 0.001).

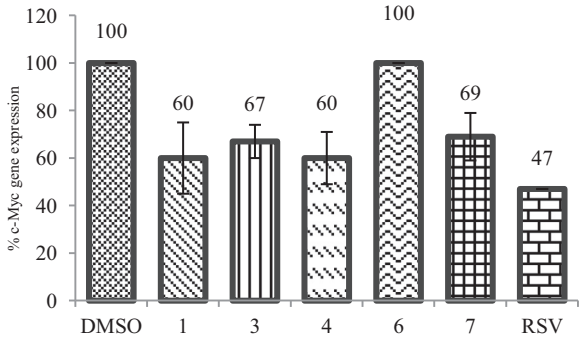


Fig. 4. Expression percentage of the c-Myc gene after 48 h of incubation of HT-29 cells determined by means of the RT-qPCR methodology. At least three measurements were performed in each case. Concentration of all compounds was 1 μM (for 6, 5 μM and for 3, 20 μM). With RSV is indicated the positive control Resveratrol. Error bars indicate standard errors of the mean. The statistical significance was evaluated using one-sample *t*-tests (P < 0.001).

Compounds **1** and **4** showed the highest inhibitory effect on the expression of the *c-Myc* gene, downregulating *c-Myc* gene expression to 60%. In addition, compounds **3** and **7** displayed a similar ability to inhibit the expression of the *c-Myc* gene (reduction to 67% and 69% of the control value, respectively). In contrast, compound **6** showed no significant decrease in *c-Myc* gene expression.

In Fig. 5, values of the percentage of *hTERT* gene expression are graphically represented versus the percentage of c-*Myc* gene expression. Compounds with approximately the same inhibition levels on both biological targets (*hTERT* and c-*Myc* gene expressions) are located in the diagonal. This correlation between inhibition levels of *hTERT* and c-*Myc* gene expressions suggests that these compounds are able to downregulate *hTERT* gene expression through downregulation of c-*Myc* transcription factor gene expression. Compound **1** and **4**, at a concentration of 1 μM, and compound **3,** at 20 μM, are able to decrease both gene expressions approximately at the same level. Among them, it is important to note the activities showed by compounds **1** and **4**, which are capable to downregulate simultaneously *hTERT* and *c-Myc* gene expression around 50–60% with a concentration of 1 μM.

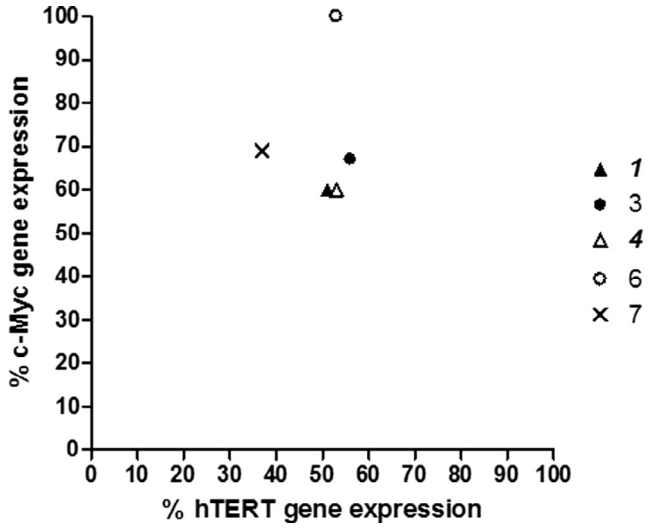


Fig. 5. Percentage of hTERT gene expression vs. percentage of c-Myc gene expression.

The administration of a sub-toxic concentration of compounds sensibly reduced the expression of the protooncogene *c-Myc* and of the gene *hTERT* (Fig. 5).[13] These results clearly showed the antiproliferative effects of this small library of compounds especially of the two derivatives **1** and **4**.

We initiated early preclinical *in vitro* adsorption, distribution, metabolism, and excretion (ADME) studies with hit compounds **1** and **4** to determine their aqueous solubility, parallel artificial membrane permeability (PAMPA), and human liver microsomes (HLM) stability in order to early assess the absorption/stability profiles of these drug candidates. Passive membrane permeability was evaluated with the PAMPA assay, while compound solubility was evaluated according to the method developed by Avdeef.[14]

Metabolic stability was finally evaluated by incubating the above mentioned compounds with 5 μL of human pooled HLM for 1 h at 37 °C in order to simulate phase I metabolism.

The ADME properties of the two hit compounds **1** and **4** analyzed by means of PAMPA and HLM are shown in Table 2.[15] These

Table 2
ADME profile of compounds **1** and **4**.

Comp.	Apparent permeability (cm/s)	Solubility in H_2O (μM)	Metabolic stability (%)
1	$1.28 \cdot 10^{-6}$	9.7	98.7
4	$1.58 \cdot 10^{-6}$	11.8	99.1
Caffeine[a]	$<0.1 \cdot 10^{-6}$	–	–
Propanolol[b]	$9.68 \cdot 10^{-6}$	–	–
Testosterone[c]	–	–	76.2

[a] Tested as reference compound for low permeability.
[b] Tested as reference compound for high permeability.
[c] Tested as reference compound for CYP-dependent metabolism in human liver microsomes.

two molecules showed good metabolic stability in HLM assay, low water solubility and scarce permeability in PAMPA test. However, it has been reported that salicylic acid derivatives might pass through cellular membranes by active transport; thus, further experiments in this direction are necessary to better characterize the permeability profiles of these compounds.[16] According to the obtained results, compound **4** is the most interesting because it shows higher permeability, solubility and metabolic stability than compound **1**.

In conclusion, a small library of compounds synthesized in our laboratory exhibited a dual activity against cancer and HIV infected cell lines inhibiting TZM-bl cells infected with HIV-1 NL4.3 *in vitro* and HT-29, MCF-7 cancer cell lines. These derivatives were also active against opportunistic tumor, due to an immunocompromised state and closely associated with virus infections like Burkitt's Lymphoma as shown by the data on activity against Ramos cells in Table 1.

The origin of this antiviral and antiproliferative activity is not well understood but from *hTERT* and *c-Myc* gene expression evaluations, downregulation of the expression of these two genes emerged. The most active compounds were derivatives **1** and **4**, because they were able to simultaneously reduce the *hTERT* and *c-Myc* gene expression at a concentration of 1 μM. Derivative **7** could be also considered an active compound, as it was able to reduce the expression of the *hTERT*, and partially of *c-Myc* gene, at 1 μM concentration. The ability of reducing the *c-Myc* gene expression could also explain the high cytotoxic activity of these compounds on Ramos cells, which showed an overexpression of this gene.[17] Moreover, compounds **1** and **4** showed high metabolic stability as revealed by human liver microsomes (HLM) assay.

In summary, compound **4** is the most promising of the series showing dual activity against cancer and HIV-1 infected cell lines, and being able to downregulate *hTERT* and *c-Myc* gene expressions. It represents a good starting point for future studies aimed at the identification of the mode of action of this series of compounds and also at an optimization campaign in order to increase the inhibition activity and the ADME properties, especially the membrane permeability.

Acknowledgments

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.03.097.

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