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Synthesis and biological activity of glycosyl conjugates of *N*-(4-hydroxyphenyl)retinamide

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Abstract

Three glycosyl (glucosyl, galactosyl, mannosyl) conjugates of 4-hydroxyphenylretinamide have been synthesized and tested on a broad variety of tumor cells. All three compounds are active on promyelocytic leukemia cell lines HL60 but less than the parent compound 4-HPR. Among them, the mannosyl analog stands out by its very low toxicity. © 2001 Elsevier Science S.A. All rights reserved.

Keywords: Antiproliferative compounds; Glycolipids; Glycosylation; Retinoid

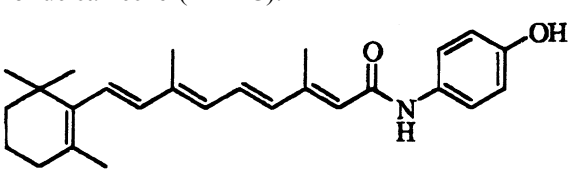
1. Introduction

Retinoic acid (RA) and its synthetic analogs are involved in numerous biological processes such as fetal development, differentiation, morphogenesis, metabolism and homeostasis [1]. These compounds are known to be effective against various skin diseases and are now being considered as potential drugs for treatment and prevention of several cancers [2]. However, systemic administration has been limited by their high toxicity.

All-*trans*-*N*-(4-hydroxyphenyl)retinamide or 4-HPR (Fenretinide®) (Chart 1), a synthetic derivative of RA [3,4], has shown mild toxicity and seems promising in terms of its effectiveness relative to toxicity. 4-HPR displays antiproliferative effects in vitro against human breast carcinoma cells [5] and induces apoptosis in hemopoietic cell lines [6]. Its mode of action is unknown [7], but a recent study has assumed that 4-HPR is a highly selective activator of the retinoic acid receptor γ (RARγ) [8]. Recent studies have shown that *N*-(4-hydroxyphenyl)retinamide-*O*-glucuronide shows greater potency and reduced toxicity relative to 4-HPR when tested as an antiproliferative agent in the culture of breast carcinoma cells [5] and also as a chemopreventive agent against the development and growth of 7,12-dimethylbenzanthracene (DMBA) induced rat mammary tumor [7].

These results and our recent efforts [9] to synthesize new retinoid derivatives with potential antiproliferative properties, have led us to investigate the influence of a glycosyl moiety on the activity and the toxicity of 4-HPR. The carbohydrate moiety can be expected to play the role of drug carrier and influence the selectivity of compounds for cancerous cell lines. Results in this way have been already obtained by Iglesias-Gerra et al. [10] with alkylating agents.

In the present study, we describe the synthesis and report on the activity of glucosyl, galactosyl and mannosyl derivatives of 4-HPR in a screen of: (1) their antiproliferative potential in vitro using four human cancer lines and (2) their toxicity on normal cells such as human fetal fibroblast and peripheral blood mononuclear cells (PBMC).



4-Hydroxyphenylretinamide

Chart 1.

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1.1. Chemistry

The different glycosyl conjugates of 4-HPR have been prepared in five steps as described in Scheme 1.

The peracetylated monosaccharides were brominated selectively on the anomeric position using bismuth bromide as catalyst [11]. The coupling with *p*-nitrophenol was accomplished by a procedure described by Kato et al. [12]. The β-anomer was obtained in the case of glucose and galactose, and the α-anomer for mannose as shown by the physical data [13–15]. Reduction of the nitro group, using hydrogen with Pearlman's catalyst afforded the corresponding aniline derivatives.

RA was then coupled by a procedure recently described [16], which involve the use of the couple EDC/DMAP in stoichiometric amount. In the three cases, the compounds were obtained with yields superior to 80%. The cleavage of the acetate protecting groups by a saturated solution of ammonia in methanol led to the three expected glycosyl conjugates of 4-HPR **5a**, **5b** and **5c** (Table 1) (Scheme 1).

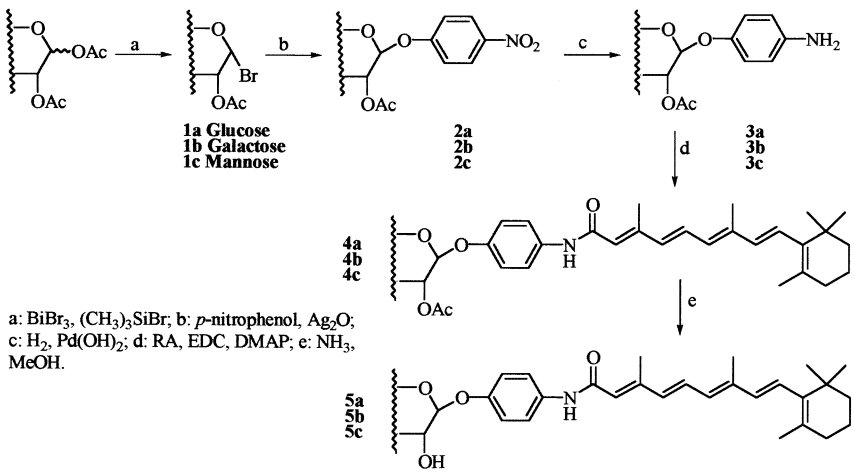
1.2. Biological assays

Antiproliferative activity of the compounds was determined after 7 days using four cancer cell lines in the exponential growth phase: NCI-H460, HT29, MCF-7 and HL60. The normal MRC5 fibroblast cell line was used as control. With MRC5 cells, cytotoxic (MRC5-Tox) and antiproliferative (MRC5-Antip) effects of compounds were determined in the stationary and exponential growth phase, respectively. Compounds were added to cultures at concentrations ranging from 0.01 to 100 μM for the 7 days. All compounds were dissolved in dimethylsulfoxide (DMSO). The DMSO amount in all treatments and in controls never exceeded 1%. After 7 days of culture, cell growth was determined using the MTT assay as previously described by Park et al. [17].

The cytotoxic concentration (IC_{50}) of each compound was determined on the human T lymphoblastoid cell line CEM.

The cells were treated with a concentration of compound ranging from 0.01 to 100 μM and cultivated 4 days at exponential rate. At the end of this period, cells were incubated with propidium iodide (50 μg/ml). Total cell number and viability were measured in duplicates by flow cytometry on a Facscalibur (Becton Dickinson) cell analyzer. Fluorescence was measured at 575 nm (excitation 480 nm).

PBMC from healthy donors were also used to determined the cytotoxicity of compounds. The cells were



Scheme 1.

Table 1
Antiproliferative activity [a]

	MRC5 Tox	MRC5 Antip.	NCIH460		HT29		MCF7		HL60	
	IC_{50}	IC_{50}	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI
4-HPR	14.7 ± 1.3	4.7 ± 1.3	2.8 ± 0.5	1.7	1.6 ± 0.6	2.9	2.3 ± 0.05	2.0	0.21 ± 0.01	22.4
5a	20.0 ± 1.6	15.5 ± 0.5	29.3 ± 6.3	0.53	7.5 ± 2.9	2.1	29.3 ± 1.1	0.53	7.5 ± 1.8	2.1
5b	25.1 ± 1.9	14.0 ± 1.0	28.6 ± 0.6	0.49	20.5 ± 0.5	0.68	26.3 ± 3.3	0.53	5.3 ± 0.3	2.6
5c	79.0 ± 4.4	72.4 ± 5.1	30.8 ± 2.0	2.3	23.2 ± 4.8	3.1	25.0 ± 4.0	2.9	4.9 ± 2.4	14.7

[a] IC_{50}: concentration (μM) that inhibits 50% of cellular proliferation or cell viability (MRC5-Tox); SI: selectivity index (ratio between IC_{50} for the non-tumoral MRC5 cell line and IC_{50} for the tumoral cell line considered).

Table 2
Cytotoxicity comparison between 4-HPR and compound **5c**

	MRC5 Tox	MRC5 Antip.	CEM	PBMC
4-HPR IC_{50} (μM)	14.7 ± 1.3	4.7 ± 1.3	0.75 ± 0.16	3.6 ± 0.2
5c IC_{50} (μM)	79.0 ± 4.4	72.4 ± 5.1	29 ± 0.3	30 ± 1.0
Ratio IC_{50} **5c**/IC_{50} 4-HPR	5.3	15.4	38.6	8.3

cultured with 0.01–100 μM of each compound, or with DMSO for control. After 7 days, the cell number and viability were measured by flow cytometry as described previously.

IC_{50} values in Table 1 are from three independent experiments ± standard error.

2. Results and discussion

The antiproliferative activity of each compound and their selectivity were evaluated by determining the ratio between the IC_{50} for non-tumoral and tumoral cells (SI). In the case of the reference molecule (4-HPR), the antiproliferative activity (IC_{50}) was in the micromolar range in agreement with previous results reported by Curley et al. [18] but the selectivity index was low. 4-HPR showed growth inhibitory effect at lower concentration on the HL60 cell line: 0.21 ± 0.01 μM.

Among the different derivatives tested, only the mannosyl conjugate **5c** exhibits an interesting antiproliferative activity on the HL60 leukemia cell line with a selectivity index close to 15. In spite of a 23 times lower activity on HL60 cells, this compound shows reduced effects on MRC5 as compared with the reference molecule (4-HPR). Indeed the cytotoxic and the antiproliferative activities of **5c** on MRC5 are, respectively, 5 and 15 times lower than what observed for 4-HPR (Table 2). Cytotoxic concentrations of 4-HPR and **5c** were also determined on the human T lymphoblastoid cell line (CEM) and on PBMC activated by phytohemaglutinin P and interleukin-2.

These complementary determinations are consistent with the characteristics obtained on MRC5 cells. Compound **5c** is 38.6 and 8.3 times less cytotoxic than 4-HPR on CEM and PBMC, respectively.

These preliminary results are quite encouraging in view of loss of toxicity obtained with the mannosyl derivative **5c**. Although the loss of activity was significant, the IC_{50} value remained below 5 μM.

Further investigations of the structure–toxicity and structure–activity relationship of these kind of compounds are currently underway in our group. Results will be published elsewhere.

3. Experimental

3.1. Chemistry

Elemental analysis were performed in the microanalysis laboratory of ENSCM (Montpellier, France). ^{1}H NMR spectra were recorded with an AC 250 Brücker spectrometer using $CHCl_3$-*d* as solvent. Chemical shifts are expressed in parts per million, with TMS as reference. The multiplicity is indicated as: s (singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet), l (large) and combination of these signals. Fast atom bombardment mass spectra (FABMS) were recorded in positive mode on a JEOL DX 300 spectrometer using NOBA (nitro-benzylic alcohol) as matrix.

3.1.1. 2,3,4,6-Tetra-O-acetyl-α-D-glycopyranosyl bromide

3.1.1.1. General procedure. To a stirred solution of a pentaacetylated hexopyranose (1 equiv., 0.5 g) and $BiBr_3$ (0.05 equiv.) in 5 ml of methylene chloride, was added under nitrogen bromotrimethylsilane (4 equiv.). The reaction was stirred at room temperature (r.t.) and monitored by TLC, then poured into cold saturated aqueous solution of $NaHCO_3$ and extracted twice with methylene chloride. The combined organic layers were dried over anhydrous Na_2SO_4, and concentrated in vacuo to give the desired pure compound.

*3.1.1.2. 2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide (**1a**).* Yield: 98%; m.p.: 88–89°C, ^{1}H NMR ($CHCl_3$-*d*): δ 6.55 (d, 1H, $J = 4$ Hz), 5.5 (t, 1H, $J = 10$ Hz), 5.1 (t, 1H, $J = 10$ Hz), 4.75 (d, 1H, $J = 4$ Hz), 4.1–4.2 (m, 3H), 2 (m, 12H). FAB MS; *m*/*z*: 413 $[M + H]^+$, 435 $[M + Na]^+$, 827 $[2M + H]^+$.

*3.1.1.3. 2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl bromide (**1b**).* Yield: 99%; m.p.: 79–81°C, ^{1}H NMR ($CHCl_3$-*d*): δ 6.65 (d, 1H, $J = 4$ Hz), 5.45 (d, 1H, $J = 3$ Hz), 5.35 (d, 1H, $J = 4$ Hz), 4.9 (d, 1H, $J = 4$ Hz), 4.45 (t, 1H, $J = 6$ Hz), 4.15 (m, 2H), 2 (m, 12H). FAB MS; *m*/*z*: 413 $[M + H]^+$, 435 $[M + Na]^+$, 827 $[2M + H]^+$.

*3.1.1.4. 2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl bromide (**1c**).* Yield: 99%; m.p.: 48–50°C, ^{1}H NMR ($CHCl_3$-*d*): δ 6.25 (d, 1H, $J = 1$ Hz), 5.65 (d, 1H, $J = 3$ Hz), 5.35 (d, 1H, $J = 2$ Hz), 5.25 (m, 1H), 4.25 (d, 1H, $J = 5$ Hz), 4.15 (m, 1H), 4.05 (d, 1H, $J = 2$ Hz), 2 (m, 12H). FAB MS; *m*/*z*: 413 $[M + H]^+$, 435 $[M + Na]^+$, 827 $[2M + H]^+$.

3.1.2. p-Nitrophenyl-2,3,4,6-tetra-O-acetyl-D-glycopyranoside

3.1.2.1. General procedure. A mixture of 1 equiv. of tetra-*O*-acetyl-α-D-glycopyranosyl bromide, 2 equiv. of

p-nitrophenol and 1.3 equiv. of silver oxide(I) in acetonitrile was refluxed for 1 h. After filtration, the mixture was concentrated in vacuo. The residue was diluted in ethanol, and was refluxed for 1 h in the presence of animal charcoal. After filtration, the solution was concentrated in vacuo until precipitation of the product. The mixture was allowed to stand at 4°C for one night, filtered and washed several times with cold ethanol. The expected compound was obtained as a white powder.

*3.1.2.2. p-Nitrophenyl-2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (**2a**).* Yield: 70%; m.p.: 174–176°C, ^{1}H NMR ($CHCl_3$-*d*): δ 8.3 (d, 2H, $J = 9$ Hz), 7.2 (d, 2H, $J = 9$ Hz), 5.2–5.4 (m, 4H), 4.25 (m, 2H), 3.95 (m, 1H), 2.1 (m, 12H). FAB MS; *m/z*: 470 $[M + H]^+$, 492 $[M + Na]^+$, 331 $[M - OC_6H_5NO_2]^+$.

*3.1.2.3. p-Nitrophenyl-2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (**2b**).* Yield: 50%; m.p.: 143–145°C, ^{1}H NMR ($CHCl_3$-*d*): δ 8.15 (d, 2H, $J = 9$ Hz), 7 (d, 2H, $J = 9$ Hz), 5.5 (m, 2H), 5.1 (m, 2H), 4.2 (m, 3H), 2.1 (m, 12H). FAB MS; *m/z*: 470 $[M + H]^+$, 492 $[M + Na]^+$, 331 $[M - OC_6H_5NO_2]^+$.

*3.1.2.4. p-Nitrophenyl-2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside (**2c**).* Yield: 35%; m.p.: 154–156°C, ^{1}H NMR ($CHCl_3$-*d*): δ 8.2 (d, 2H, $J = 9$ Hz), 7.2 (d, 2H, $J = 9$ Hz), 5.6 (s, 1H), 5.3–5.5 (m, 3H), 4.2 (m, 1H), 4.1 (m, 2H), 2.1 (m, 12H). FAB MS; *m/z*: 470 $[M + H]^+$, 492 $[M + Na]^+$, 331 $[M - OC_6H_5NO_2]^+$.

3.1.3. p-Aminophenyl-2,3,4,6-tetra-O-acetyl-D-glycopyranoside

3.1.3.1. General procedure. *p*-Nitrophenyl-2,3,4,6-tetra-*O*-acetyl-D-glycopyranoside (0.5 g) was dissolved in a mixture of methanol–methylene chloride 5:1 in the presence of 0.5 g of palladium hydroxide. The mixture was stirred for 2 h under hydrogen atmosphere. After filtration on celite the solution was concentrated in vacuo to give the pure expected compound.

*3.1.3.2. p-Aminophenyl-2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (**3a**).* Yield: 98%; m.p.: 100–102°C, ^{1}H NMR ($CHCl_3$-*d*): δ 6.9 (d, 2H, $J = 8$ Hz), 6.6 (d, 2H, $J = 8$ Hz), 4.9–5.3 (m, 4H), 4.2 (m, 2H), 3.8 (m, 1H), 3.2 (sl, 2H), 2.1 (m, 12H). FAB MS; *m/z*: 439 $[M]^+$, 440 $[M + H]^+$, 462 $[M + Na]^+$.

*3.1.3.3. p-Aminophenyl-2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (**3b**).* Yield: 98%; m.p.: 55–58°C, ^{1}H NMR ($CHCl_3$-*d*): δ 6.9 (d, 2H, $J = 7$ Hz), 6.7 (d, 2H, $J = 7$ Hz), 5.4 (m, 2H), 5.1–4.8 (m, 2H), 4 (m, 3H), 3.3 (sl, 2H), 2.1 (m, 12H). FAB MS; *m/z*: 439 $[M]^+$, 440 $[M + H]^+$, 462 $[M + Na]^+$.

*3.1.3.4. p-Aminophenyl-2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside (**3c**).* Yield: 96%; m.p.: 58–60°C, ^{1}H NMR ($CHCl_3$-*d*): δ 6.9 (d, 2H, $J = 10$ Hz), 6.7 (d, 2H, $J = 10$ Hz), 5.6–5.2 (m, 3H), 4–4.3 (m, 3H), 3.2 (sl, 2H), 2.1 (m, 12H). FAB MS; *m/z*: 439 $[M]^+$, 440 $[M + H]^+$, 462 $[M + Na]^+$.

3.1.4. 4-Retinamidophenyl-2,3,4,6-tetra-O-acetyl-D-glycopyranoside

3.1.4.1. General procedure. One equiv. of RA, 1.5 equiv. of *p*-aminophenyl-2,3,4,6-tetra-*O*-acetyl-D-glycopyranoside, and 1.5 equiv. of DMAP were dissolved in methylene chloride. Then 1.5 equiv. of EDC was added, and the reaction was stirred at r.t. for 20 min. The mixture was diluted with methylene chloride, washed three times with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified on silica gel.

*3.1.4.2. 4-Retinamidophenyl-2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (**4a**).* Yield: 65%; m.p.: 92–94°C, ^{1}H NMR ($CHCl_3$-*d*): δ 7.5 (d, 1H, $J = 9$ Hz), 7–7.2 (m, 4H), 6.1–6.4 (m, 5H), 5.8 (s, 1H), 5–5.3 (m, 4H), 4.2–4.4 (m, 2H), 3.85 (m, 1H), 2.4 (s, 3H), 2 (m, 17H), 1.8 (s, 3H), 1.65 (m, 2H), 1.5 (m, 2H), 1 (s, 6H). FAB MS; *m/z*: 721 $[M]^+$, 722 $[M + H]^+$, 744 $[M + Na]^+$, 760 $[M + K]^+$.

*3.1.4.3. 4-Retinamidophenyl-2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (**4b**).* Yield: 70%; m.p.: 88–90°C, ^{1}H NMR ($CHCl_3$-*d*): δ 7.6 (d, 1H, $J = 9$ Hz), 7–7.3 (m, 4H), 6.15–6.4 (m, 5H), 5.85 (s, 1H), 5–5.5 (m, 4H), 4–4,3 (m, 3H), 2.4 (s, 3H), 2–2.1 (m, 17H) 1.7 (s, 3H), 1.6 (m, 2H), 1.5 (m, 2H), 1.05 (s, 6H). FAB MS; *m/z*: 721 $[M]^+$, 722 $[M + H]^+$, 744 $[M + Na]^+$, 760 $[M + K]^+$.

*3.1.4.4. 4-Retinamidophenyl-2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside (**4c**).* Yield: 68%; m.p.: 68–70°C, ^{1}H NMR ($CHCl_3$-*d*): δ 7.5 (d, 1H, $J = 9$ Hz), 6.9–7.2 (m, 4H), 6.1–6.3 (m, 5H), 5.85 (s, 1H), 5–5.5 (m, 4H), 4–4,3 (m, 2H), 3.5–3.7 (m, 1H), 2.4 (s, 3H), 2–2.2 (m, 17H), 1.75 (s, 3H), 1.6 (m, 2H), 1.5 (m, 2H), 1.05 (s, 6H). FAB MS; *m/z*: 721 $[M]^+$, 722 $[M + H]^+$, 744 $[M + Na]^+$, 760 $[M + K]^+$.

3.1.5. 4-Retinamidophenyl-D-glycopyranoside

3.1.5.1. General procedure. 4-Retinamidophenyl-2,3,4,6-tetra-*O*-acetyl-D-glycopyranoside (0.1 g) was dissolved in 10 ml of a saturated solution of ammonia in methanol. The mixture was allowed to stand at 4°C for 48 h, and concentrated in vacuo. The residue was purified on silica gel (eluent: methylene chloride–methanol 8:2).

*3.1.5.2. 4-Retinamidophenyl-β-D-glucopyranoside (**5a**).* Yield: 95%; ^{1}H NMR ($CHCl_3$-*d*) 7.5 (d, 1H, $J = 9$ Hz), 7–7.2 (m, 4H), 6.1–6.4 (m, 5H), 5.8 (s, 1H), 5–5.3 (m, 4H), 4.2–4.4 (m, 2H), 3.85 (m, 1H), 2.4 (s, 3H), 1.8 (s, 3H), 1.65 (m, 2H), 1.5 (m, 2H), 1 (s, 6H). FAB MS; *m*/*z*: 553 $[M]^+$, 554 $[M + H]^+$, 576 $[M + Na]^+$, 592 $[M + K]^+$. *Anal.* Calc. for $C_{32}H_{43}NO_7$ (553.69): C, 69.42; H, 7.83. Found: C, 69.71; H, 7.90%.

*3.1.5.3. 4-Retinamidophenyl-β-D-galactopyranoside (**5b**).* Yield: 97%; ^{1}H NMR ($CHCl_3$-*d*) 7.6 (d, 1H, $J = 9$ Hz), 7–7.3 (m, 4H), 6.15–6.4 (m, 5H), 5.85 (s, 1H), 5–5.5 (m, 4H), 4–4.3 (m, 3H), 2.4 (s, 3H), 1.7 (s, 3H), 1.6 (m, 2H), 1.5 (m, 2H), 1.05 (s, 6H). FAB MS; *m*/*z*: 553 $[M]^+$, 554 $[M + H]^+$, 576 $[M + Na]^+$, 592 $[M + K]^+$. *Anal.* Calc. for $C_{32}H_{43}NO_7$ (553.69): C, 69.42; H, 7.83. Found: C, 69.62; H, 7.87%.

*3.1.5.4. 4-Retinamidophenyl-α-D-mannopyranoside (**5c**).* Yield: 96%; ^{1}H NMR ($CHCl_3$-*d*) 7.5 (d, 1H, $J = 9$ Hz), 6.9–7.2 (m, 4H), 6.1–6.3 (m, 5H), 5.85 (s, 1H), 5–5.5 (m, 4H), 4–4.3 (m, 2H), 3.5–3.7 (m, 1H), 2.4 (s, 3H), 1.75 (s, 3H), 1.6 (m, 2H), 1.5 (m, 2H), 1.05 (s, 6H). FAB MS; *m*/*z*: 553 $[M]^+$, 554 $[M + H]^+$, 576 $[M + Na]^+$, 592 $[M + K]^+$. *Anal.* Calc. for $C_{32}H_{43}NO_7$ (553.69): C, 69.42; H 7.83. Found: C, 69.69; H 7.88%.

3.2. Biological assays

3.2.1. Cell lines

The different cell lines were obtained from the American Type Culture Collection (Rockville, MD). NCI-H460, lung large cell carcinoma, ATCC # HTB-177; HT29, colon adenocarcinoma, ATCC # HTB-38; MCF-7, breast carcinoma, ATCC # HTB-22; HL60, promyelocytic leukemia, ATCC # CCL-240; MRC5, fetal lung fibroblast, ATCC # CCL-171, CEM, human T lymphoblastoid cells ATCC # CL119).

3.2.2. Cell culture

NCI-H460, HT29, MCF-7 and HL60 cell lines were seeded at a density of 100, 150, 6000 and 750 cells per well, respectively, in 96 wells Falcon dishes. The culture medium was RPMI 1640 supplemented with 25 mM Hepes, 2 mM glutamin, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate and 5% fetal calf serum. Cytotoxic (MRC5-Tox) and antiproliferative (MRC5-Antip) activities were determined when MRC5 cells were seeded at density of 30 000 and 6000 cells per well, respectively. The cells were allowed to attach for 24 h before treatment.

CEM cells were seeded at 7500 cells per well. PBMC were activated by PHA (1 μg/ml) and IL_2 (20 UI/ml) and seeded at 2.10^5 cells per well. The culture medium was RPMI supplemented with 20 mM Hepes, 2mM glutamin, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, 10% fetal calf serum and 100 μg/ml Penicillin–Streptomycin.

3.2.3. MTT test [17]

The MTT test is based on the enzymatic reduction of the tetrazolium salt MTT [3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] in living, metabolically active cells but not in dead cells. The reaction product, a purple-colored formazan soluble in dimethylsulfoxide, is measured colorimetrically at 550 nm, using multiwell plate reader. Measurements are currently duplicated. These results are from two distinct manipulations.

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