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Full Paper

Modified *N,O*-Nucleosides: Design, Synthesis, and Anti-tumour Activity

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A preliminary library of modified *N,O*-nucleosides was prepared and tested on a selected number of human cancer lines that include SKOV3, SW480, and K562. Thymine, *N*-benzyl substituents, and aromatic rings contribute to an increase of the biological activity, up to 10–25 μM, that appeared also reliant on the calculated lipophilicity of the nucleosides, expressed as cLogP, where P represents the partition coefficient of a solute between n-octanol and water.

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Introduction

In recent years, a large number of nucleoside analogues with antiviral and/or antitumour properties have been designed and synthesized.[1,2] Nucleosides, in fact, comprise the largest class of clinically useful antiviral agents and they continue to be excellent candidates as anticancer drugs.[3]

In the search for effective, selective, and non-toxic agents, a variety of modifications of the naturally occurring structure have been devised on both the sugar and the nucleobase. Of particular interest are nucleoside analogues in which the furanose ring has been replaced by *N,O*-heterocyclic systems, as in isoxazolidine **1** and isoxazoline **2** derivatives, in which B represents pyrimidine or purine nucleobases (Fig. 1).[4–6]

Most of the *N,O*-containing nucleoside analogues described in the literature possess remarkable antiviral activity but a modest potential activity towards human cancer cell lines,[7,8] contrary to what is observed with substituted nucleosides and thionucleosides, in particular 4′-thio-β-D-arabinofuranosylcytosine (4′-Thio-Ara-C).[3,9–11]

In a previous study, we reported the efficient synthesis of several *N,O*-nucleosides by direct 1,3-dipolar cyclization methodology.[12,13] The cyclization was carried out between selected nitrones **3** and a set of unprotected vinyl nucleobases **4** under microwave (MW) irradiation, in the absence of solvent or catalyst (Scheme 1). The cycloadducts are formed in good yield, with complete regioselectivity and notable *cis* diastereoselectivity, up to 98 %, resulting from a dominant (*Z*)-*exo* nitrone–alkene approach.[13]

The *N,O*-nucleosides of the previous study were evaluated by in vitro assays for their antiproliferative activity on LCL_s, JiJoye, and Jurkat cell lines, and were found particularly promising.[13]

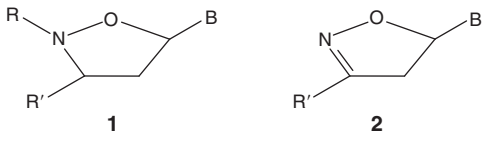
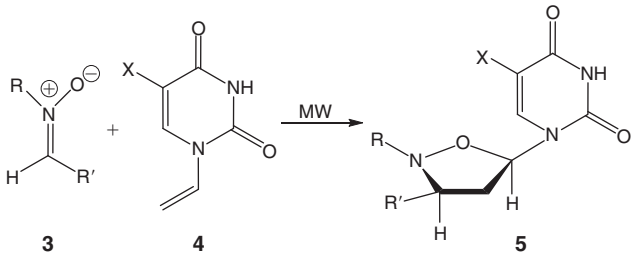


Fig. 1. General structure of *N,O*-nucleosides.



Scheme 1. Synthesis of *N,O*-nucleosides via 1,3-dipolar cycloaddition of nitrones **3** with vinyl nucleobases **4** by microwave (MW) irradiation.

Results and Discussion

In analogy with specific absorption rate (SAR) tests designed to prove the minimal structural requirements for antiproliferative activity in the NCI 60 panel of human cancers,[14] we investigated variations in the four canonical quadrants of our compounds **5** (Fig. 2), and some of these were submitted to growth inhibition assays on SKOV3 (cisplatin-resistant) and SW480 cell lines established for ovarian and colon cancers respectively (Table 1). It was found that compound **5d** exhibited greater potency (25–50 μM) than either compounds **5a** or **5e** on these

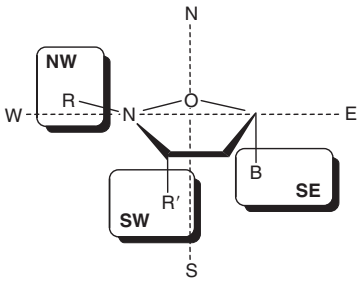
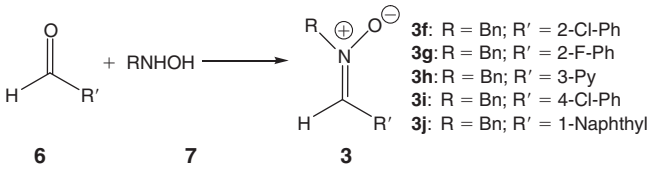


Fig. 2. Representation of isoxazolidines in quadrants for changes of B, R and R′.

Table 1. Estimated IC_{50} values in cisplatin-resistant SKOV3 and SW480 cell lines, as determined by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide solution (MTT) assay

Data are the mean ± s.d. of three independent experiments performed in triplicate

R	R′	Nucleobase (B)	Compound	IC_{50} (μM)	
				SKOV3	SW480
Bu^t	Ph	Thy	**5a**	>100	>100
Me	Ph	Thy	**5b**	>100	>100
Bu^t	Ph	F-Ura	**5c**	>100	>100
Bn	Ph	Thy	**5d**	43 ± 5	50 ± 6
Me	2-Cl-Ph	Thy	**5e**	>100	>100
			Cisplatin	>50	
Bn	2-Cl-Ph	Thy	**5f**	24 ± 6	36 ± 1
Bn	2-F-Ph	Thy	**5g**	39 ± 1	39 ± 1
Bn	3-Py	Thy	**5h**	>100	>100



Scheme 2. Synthesis of aryl-substituted nitrones **3f**–**j**.

tumoural lines, comparable with that of cisplatin *cis*-$[PtCl_2(NH_3)_2]$ used as model reference.

Motivated by the above finding, a novel series of *N*,*O*-isoxazolidines was designed, modifying the nucleobases and/or the substituents on the isoxazolidine ring. Taking into account these findings and as pyrimidine nucleoside analogues are essential components of haematological malignancy therapy and are also used in the treatment of solid tumours,[15] we prepared a new set of *N*,*O*-nucleosides varying the SE quadrant with thymine or fluorouracil as nucleobases, and the R and R′ substituents in the NW and SW quadrants respectively.

Initially, in analogy to compound **5d**, we chose to maintain unchanged the R and B groups (benzyl and thymine respectively), varying only the SW quadrant.

The procedure for the preparation of the new nucleosides is based on the direct 1,3-dipolar cycloaddition of the nitrone **3** with the vinyl nucleobase **4** in the absence of solvent, under microwave irradiation.

In particular, nitrones **3f**–**j** were obtained in excellent yields and high purity by condensation of the precursor aldehydes **6** with suitable hydroxylamines **7** in acetate-buffered water/ethanol solution (Scheme 2).[16]

With the set of nitrones **3f**–**j** in hand, we initially synthesized compounds **5f**–**h** by 1,3-dipolar cycloaddition with vinylthymine as nucleobase **4**, according to the general procedure described in the *Experimental* section. Then, the *N*,*O*-nucleoside derivatives **5f**–**h** were evaluated by in vitro assays for their antiproliferative activity against SKOV3 and SW480 cell lines. Among the new derivatives, the *ortho*-chloro **5f** and *ortho*-fluoro **5g** compounds displayed a biological activity similar to or even enhanced relative to that shown by **5d**, thus confirming that thymine, *N*-benzyl substituents, and aromatic rings are the optimal combination for biological activity.

The small library of *N*,*O*-nucleosides **5a**–**h** was analysed taking into consideration physicochemical data related to solubility and permeability.[17] These properties are easily obtained from research databases such as SciFinder® or may be calculated.[18] Table 2 lists the calculated lipophilicity expressed as cLogP, where P represents the partition coefficient of a solute between n-octanol and water, the molecular weight (MW), and the number of hydrogen donor (H_{don}) and acceptor (H_{acc}) sites of our *N*,*O*-nucleosides, which in all cases is in

Table 2. Estimated IC_{50} values in cisplatin-resistant SKOV3, SKOV480, K562, MDA, HCT116, and A2780 cancer cell lines, as determined by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide solution (MTT) assay

Data are the mean ± s.d. of three independent experiments performed in triplicate. cLogP, calculated lipophilicity of the nucleosides, where P represents the partition coefficient of a solute between n-octanol and water; MW, molecular weight; H_{acc}, number of hydrogen acceptor sites of the *N*,*O*-nucleosides; H_{don}, number of hydrogen donor sites of the *N*,*O*-nucleosides

R	Ar	B	Compound	IC_{50} (μM)						cLogP	MW	H_{acc}	H_{don}
				SKOV3	SW480	K562	MDA	HCT116	A2780				
Bu^t	Ph	Thy	**5a**	>100	>100	>100	>100	>100	>100	2.906	329	6	1
Me	Ph	Thy	**5b**	>100	>100	>100	>100	>100	>100	1.656	287	6	1
Bu^t	Ph	F-Ura	**5c**	>100	>100	>100	>100	>100	>100	2.586	333	6	1
Bn	Ph	Thy	**5d**	43 ± 5	56 ± 6	30 ± 3	60 ± 3	45 ± 1	60 ± 3	3.579	363	6	1
Me	2-Cl-Ph	Thy	**5e**	>100	>100	>100	>100	>100	>100	2.381	321	6	1
Bn	2-Cl-Ph	Thy	**5f**	24 ± 6	36 ± 1	30 ± 3	31 ± 3	41 ± 4	47 ± 3	4.066	397	6	1
Bn	2-F-Ph	Thy	**5g**	39 ± 1	39 ± 1	45 ± 4	33 ± 5	55 ± 8	49 ± 9	3.555	381	6	1
Bn	3-Py	Thy	**5h**	>100	>100	>100	>100	>100	>100	2.165	364	7	1
Bn	2-Cl-Ph	F-Ura	**5i**	>100	>100	50 ± 5	>100	>100	>100	3.850	401	6	1
Bn	4-Cl-Ph	Thy	**5j**	25 ± 3	15 ± 1	31 ± 3	34 ± 1	24 ± 3	21 ± 1	4.070	397	6	1
Bn	1-Naphthyl	Thy	**5k**	19 ± 2	28 ± 1	11 ± 3	31 ± 1	17 ± 3	29 ± 1	4.397	413	6	1

accordance with the 'rule of five', several requirements used for the development of orally bioavailable drug candidates.[17,19] These rules have been enhanced by other authors with additional parameters such as the number of rotatable bonds; however, the original proposal still maintains its validity.[20] In the present case, all the compounds display similar parameters with respect to MW and H donor and acceptor numbers. The only exception is seen in calculated lipophilicity, which is higher for the most biologically active compounds **5d**, **5f**, and **5g**. Following this line of reasoning, *N*,*O*-nucleosides substituted with *N*-benzyl groups and aromatic rings, preferably with thymine as nucleobase and displaying calculated lipophilicity values in the range 3.5–4.5 should represent the optimal combination for increased biological activity. To support this hypothesis, we planned the synthesis of three new *N*,*O*-nucleosides (**5i**–**k**) with cLogP values higher than 3.5 and we extended the number of human cancer cell lines for biological evaluation with A2780 (cisplatin-sensitive human ovarian cancer), HCT116 (colon cancer), MDA (breast cancer), and K562 (human Caucasian chronic myelogenous leukaemia). The related results are collected in the lower part of Table 2, which includes the biological activities of **5d**, **5f**, and **5g** with the new cancer lines as well.

The presence of nucleobases other than thymine is detrimental for cytotoxicity, as demonstrated by the results found with compound **5f** (B = Thy), the half-maximal inhibitory concentration (IC_{50}) in the range 25–50 μM, and **5i** (B = F-Ura) $IC_{50} > 100$ μM. However, within the set of nucleosides bearing *N*-benzyl, thymine, and aromatic substituents, increasing biological activity was observed for compounds with increased values of cLogP. The best results in fact, with cytotoxicity ~10–25 μM, were displayed by compounds **5f**, **5j**, and **5k**, all possessing a cLogP value higher than 4. The position of chlorine substituents on the aromatic ring, *ortho* for **5f** and *para* for **5j**, cannot be underestimated because a quite significant decrease of IC_{50} was observed with the latter compound in four of the six cancer lines investigated.

Conclusion

In conclusion, we prepared and tested a preliminary library of *N*,*O*-nucleosides that demonstrate the impact of substitution in the three assessable canonical quadrants of lead antiproliferative agent compounds **5j** and **5k**, with a cytotoxic activity in the range 10–25 μM. To our knowledge, this is the first example of modified *N*,*O*-nucleosides showing such promising inhibitory activity against different lines of ovarian (SKOV3, A2780) and colon (SW480, HCT116) carcinoma.

Experimental

General

Commercial starting materials were used without further purification. Solvents were distilled before use. ^1H and ^{13}C NMR spectra were recorded at 500 and 125 MHz respectively in $CDCl_3$ using tetramethylsilane (TMS) as internal standard (Bruker ACP 500 MHz). Chemical shifts are given in parts per million and coupling constants in Hertz. High-resolution mass spectra (HRMS) were acquired on a Q-star pulsar-i (MDS Sciex Applied Biosystems, Toronto, Canada) equipped with an ion-spray source at 10000 atomic mass unit (amu) resolution. GC-MS spectra were carried out on a Shimadzu QP 5000. HPLC analyses were performed on a Hewlett-Packard 1100 Series, monitored by a UV detector at 254 nm, using a Jupiter 10 μ C-18 (25 cm) column, H_2O/MeOH 9 : 1, 4.0 mL min^{-1}. The value of log*P* of substrates **5a**–**5k** was calculated with the *ACDlogP* program (SciFinder®).

Nitrones **3f**–**j** and vinyl nucleobases **4** were synthesized according to published procedures.[12,16,21–27] The full characterization of compounds **3f**–**j** can be found in references [16] and [24–26], and for **5a**–**e** in reference [13].

Synthesis

General Procedure of Synthesis of Compounds 5f–k

The selected nitrone **3** (0.2 mmol) and vinyl nucleobase **4** (0.1 mmol) were ground together in a mortar and further mixed in a vortexer. The mixture of the two solids was transferred into a 50-mL Pyrex container, which was placed within an unmodified household microwave oven, at 600-W irradiation power. After the appropriate time, from 10 to 15 min, the reaction mixture was dissolved in a minimum quantity of $CHCl_3$ and submitted to flash chromatographic separation, using variable mixtures of chloroform and methanol. The excess nitrone was recovered and can be re-used. The cycloadducts were analysed by HPLC and ^1H NMR to establish the diastereoisomeric *cis* : *trans* ratio, which ranges from 80 : 20 to 85 : 15. Yields were calculated based on isolated compounds.

Further purification and separation of the *cis* cycloadducts were carried out by semi-preparative HPLC chromatography.

cis-4′-Aza-4′-(N-benzyl)-3′-(2-chlorophenyl)-2′,3′-dideoxythymidine 5f

White solid, yield 77 %, mp 148–149°C. Diastereoisomer *cis* : *trans* ratio 80 : 20. δ_H (500 MHz, $CDCl_3$) 1.80 (d, *J* 1.1, 3H, Thy CH_3), 2.2 (ddd, *J* 3.8, 9.4, 13.9, 1H, H_{2C}), 3.51 (ddd, *J* 7.6, 7.9, 13.9, 1H, H'_{2C}), 3.82 (d, *J* 14.3, 1H, H_{Bn}), 4.08 (d, *J* 14.3, 1H, H_{Bn}), 4.48 (dd, *J* 7.9, 9.4, 1H, H_{3C}), 6.06 (dd, *J* 3.8, 7.6, 1H, H_{1C}), 7.24–7.45 (m, 9H, Ar), 7.55–7.63 (m, 1H, Thy H_6), 8.75 (bs, 1H, NH). δ_C (500 MHz, $CDCl_3$) 12.54, 46.37, 60.03, 66.08, 83.56, 109.86, 127.41, 127.68, 127.87, 128.54, 128.84, 129.25, 130.10, 133.93, 135.09, 135.76, 136.81, 150.35, 163.96. *m*/*z* (electrospray ionisation (ESI)-HRMS) calc. for $[C_{21}H_{20}ClN_3O_3 + H]^+$ 398.1271; found 398.1264.

cis-4′-Aza-4′-(N-benzyl)-3′-(2-fluorophenyl)-2′,3′-dideoxythymidine 5g

White solid, yield 75 %, mp 176–177°C. Diastereoisomer *cis* : *trans* ratio 85 : 15. δ_H (500 MHz, $CDCl_3$) 1.81 (d, *J* 0.76, 3H, Thy CH_3), 2.40 (ddd, *J* 3.8, 9.8, 13.9, 1H, H_{2C}), 3.34 (dt, *J* 7.5, 13.9, 1H, H'_{2C}), 3.78 (d, *J* 14.4, 1H, H_{Bn}), 4.05 (d, *J* 14.4, 1H, H_{Bn}), 4.26 (dd, *J* 7.6, 9.8,1H, H_{3C}), 6.09 (dd, *J* 3.8, 7.5, 1H, H_{1C}), 7.04–7.52 (m,10H, Ar + Thy H_6), 8.94 (bs, 1H, NH). δ_C (500 MHz, $CDCl_3$) 12.51, 46.25, 59.96, 63.46, 83.45, 109.93, 116.16, 123.97, 124.86, 127.81, 128.29, 128.49, 129.93, 135.99, 136.81, 150.47, 160.12, 162.09, 164.11. *m*/*z* (ESI-HRMS) calc. for $[C_{21}H_{20}FN_3O_3 + Na]^+$ 404.1386; found 404.1382.

cis-4′-Aza-4′-(N-benzyl)-3′-(3-pyridyl)-2′,3′-dideoxythymidine 5h

White solid, yield 74 %, mp 160–161°C. Diastereoisomer *cis : trans* ratio 87 : 13. δ_H (500 MHz, $CDCl_3$) 1.80 (d, *J* 0.76, 3H, Thy CH_3), 2.37 (ddd, *J* 3.1, 9.8, 14.0, 1H, H_{2C}), 3.40 (ddd, *J* 7.6, 14.0, 1H, H'_{2C}), 3.77 (d, *J* 13.9, 1H, H_{Bn}), 3.97 (dd, *J* 7.9, 9.8, 1H, H_{3C}), 4.20 (d, *J* 13.9, 1H, H_{Bn}), 6.03 (dd, *J* 3.1, 7.6, 1H, H_{1C}), 7.20–7.42 (m, 7H, Ar), 7.68–7.74 (m,1H, Thy H_6), 8.58–8.64 (m, 2H, Ar), 9.25 (bs, 1H, NH). δ_C (500 MHz, $CDCl_3$) 12.56, 48.36, 59.66, 67.95, 83.44, 110.09, 124.04, 127.98, 128.54,

128.93, 132.68, 135.09, 135.41, 136.31, 149.43, 150.15, 150.51, 164.08. *m/z* (ESI-HRMS) calc. for $[C_{20}H_{20}N_4O_3 + H]^+$ 365.16140; found 365.16091; *m/z* calc. for $[C_{20}H_{20}N_4O_3 + Na]^+$ 387.1433; found 387.1427.

cis-*4′-Aza-4′-(*N*-benzyl)-3′-(2-chlorophenyl)-2′,3′-dideoxy-5-fluorouridine **5i**

White solid, yield 65 %, mp 155–156°C. Diastereoisomer *cis* : *trans* ratio 80 : 20. δ_H (500 MHz, $CDCl_3$) 2.32 (ddd, *J* 3.4, 8.7, 14.0, 1H, H_{2C}), 3.37 (ddd, *J* 7.5, 8.8, 14.0, 1H, H'_{2C}), 3.90 (d, *J* 14.1, 1H, H_{Bn}), 4.13 (d, *J* 14.1, 1H, H_{Bn}), 4.48 (dd, *J* 7.5, 8.7, 1H, H_{3C}), 6.03 (dd, *J* 3.4, 8.8, 1H, H_{1C}), 7.15–7.65 (m, 9H, Ar), F-Ura H_6 not detected owing to large *J*(HF), 9.05 (bs, 1H, NH). δ_C (500 MHz, $CDCl_3$) 47.57, 61.10, 68.08, 85.43, 110.23, 127.54, 127.70, 127.95, 128.65, 128.90, 129.53, 131.43, 134.85, 135.09, 135.76, 142.81, 152.45, 164.56. *m/z* (ESI-HRMS) calc. for $[C_{20}H_{17}ClFN_3O_3 + H]^+$ 402.1021; found 402.1032.

cis-*4′-Aza-4′-(*N*-benzyl)-3′-(4-chlorophenyl)-2′,3′-dideoxythymidine **5j**

White solid, yield 75 %, mp 163–164°C. Diastereoisomer *cis* : *trans* ratio 91 : 9. δ_H (500 MHz, $CDCl_3$) 1.79 (d, *J* 1.3, 3H, Thy CH_3), 2.31 (ddd, *J* 3.8, 9.8, 14.2, 1H, H_{2C}), 3.34 (dt, *J* 7.7, 14.2,1H, H'_{2C}), 3.70 (d, *J* 14.2, 1H, H_{Bn}), 3.91 (dd, *J* 7.7, 9.8, 1H, H_{3C}), 4.01 (d, *J* 14.2, 1H, H_{Bn}), 6.02 (dd, *J* 3.8, 7.7, 1H, H_{1C}), 7.29–7.45 (m, 10H, Ar + Thy H_6), 9.23 (bs,1H, NH). δ_C (500 MHz, $CDCl_3$) 12.46, 48.27, 59.51, 69.72, 83.38, 109.91, 127.80, 128.44, 128.84, 128.92, 129.32, 134.41, 135.25, 135.55, 136.61, 150.40, 164.02. *m/z* (ESI-HRMS) calc. for $[C_{21}H_{20}ClN_3O_3 + H]^+$ 398.1272; found 398.1269.

cis-*4′-Aza-4′-(*N*-benzyl)-3′-(1-naphthyl)-2′,3′-dideoxythymidine **5k**

White solid, yield 76 %, mp 179–180°C. *Diastereoisomer cis* : *trans* ratio 77 : 23. δ_H (500 MHz, $CDCl_3$) 1.83 (d, *J* 1.1, 3H, Thy CH_3), 2.41 (ddd, *J* 3.9, 9.6, 14.2, 1H, H_{2C}), 3.56 (dt, *J* 7.9, 14.2, 1H, H'_{2C}), 3.80 (d, *J* 14.2, 1H, H_{Bn}), 4.16 (d, *J* 14.2, 1H, H_{Bn}), 4.70 (dd, *J* 7.9, 9.6,1H, H_{3C}), 6.15 (dd, *J* 3.9, 7.9, 1H, H_{1C}), 7.18–8.28 (m,13H, Ar + Thy H_6), 8.85 (bs, 1H, NH). δ_C (500 MHz, $CDCl_3$) 12.39, 43.84, 47.16, 60.11, 66.51, 83.54, 109.83, 112.61, 123.73, 125.66, 125.95, 126.45, 127.71, 128.43, 128.45, 128.67, 128.84, 129.15, 133.10, 135.80, 136.92, 150.32, 163.82. *m/z* (ESI-HRMS) calc. for $[C_{25}H_{23}N_3O_3 + H]^+$ 414.1818; found 414.1821.

Methodology for In Vitro Biological Evaluation

MDA, A2780, SKOV3, SW480, HCT116, and K562 cell lines were maintained in RPMI-1640 supplemented with 10 % newborn bovine serum (HyClone; Thermo Fisher Scientific Inc., Waltham, MA), penicillin (100 units mL^{-1}), and streptomycin (100 U mL^{-1}), and glutamine (2 mM); the pH of the medium was 7.2 and incubation was at 37°C in a 5 % CO_2 atmosphere. Adherent cells were routinely used at 70 % confluence and passaged every 3 days by treatment with 0.05 % trypsin-EDTA. K562 cells were routinely fed every 3 days. The antiproliferative activity of compounds was tested with the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide solution (MTT) assay, which is based on the conversion of the yellow tetrazolium salt MTT into purple formazan crystals by metabolically active cells and provides a quantitative determination of viable cells. Cells were seeded in triplicate in 96-well plates at a density of 25×10^3 cells in 50 µL fully defined serum-free medium (AIM V medium; Life Technologies) and treated with the test compounds at concentrations ranging from 10 to 200 µM. Stock solutions (10 mM) of each compound were made up in dimethylsulfoxide (DMSO) and diluted in AIM V medium to give final concentrations. The final concentrations of DMSO in the solutions ranged from 0.1 % to 1 %. At 1 %, DMSO alone had no effect on cell viability.

Untreated cells were placed in every plate as a negative control. Cisplatin was employed as a control for cisplatin-resistant SKOV3. After 72 h, 25 µL of a 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT) (12 mM) was added to each well and plates were incubated at 37°C and in 5 % CO_2. After 2 h, the MTT crystals were solubilized with 100 µL of lysing buffer (50 % DMF + 20 % sodium dodecyl sulphate (SDS), pH 4.7); after 24 h, the spectrophotometric absorbance of each sample was measured at 570 nm.

MDA, SKOV3, SW480, HCT116, and K562 cell lines were purchased from American Tissue Culture Collection (ATCC; Manassas, VA). The A2780 cell line was kindly donated by Dr Paola Bergamini, Department of Chemical and Pharmaceutical Sciences, University of Ferrara (Italy).

Supplementary Material

HPLC chromatograms, and ^1H and ^{13}C NMR spectra of compounds **5f**–**k** are available from the Journal's website. Graphs of biological activity for compounds **5d** and **5f**–**k** are provided as supplementary material.

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