Bioorganic & Medicinal Chemistry 17 (2009) 523–529

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and antitumor activity of novel amsacrine analogs: The critical role of the acridine moiety in determining their biological activity

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ARTICLE INFO

Article history:
Received 27 August 2008
Revised 26 November 2008
Accepted 29 November 2008
Available online 6 December 2008

Keywords:
Amsacrine analogs
Pyranoquinoline
Anticancer drugs
DNA

ABSTRACT

A new series of *N*-[4-(2′-oxo-2*H*-pyrano[2,3-*b*]quinolin-5′-ylamino)-phenyl]-methanesulfonamides was prepared and analyzed as novel amsacrine-like derivatives. Our preliminary biological evaluation has shown that the replacement of the acridine moiety with the analogous 2-oxo-2*H*-pyrano[2,3-*b*]quinoline system drastically reduced both their anticancer activity and their propency to intercalate into double stranded DNA.

1. Introduction

Chemotherapy is often the treatment of choice for many types of cancer and the search for new chemotherapeutic agents still plays a major role in the fight against cancer.

A worthwhile approach in this area deals with the use of compounds interacting with DNA and/or inhibiting enzymes critical for cell life and replication. A good example of such a compound is *m*-amsacrine (*m*-AMSA, Fig. 1), a well known antiproliferative agent used to treat some types of cancers including acute adult leukemia.[1,2] *m*-AMSA and, in general, 9-aminoacridine derivatives work inhibiting DNA topoisomerase II (topoII).[3] Their strong activity was due to the ability of acridine nucleus to intercalate into DNA base pair, stabilizing the DNA–topoII cleavable complex, and forming the so-called 'ternary complex' which involve DNA, intercalated compound and topoII. The poisoning of topoII activity inhibits the religation process and causes lethal double-strand breaks in DNA, leading to cell cycle arrest and apoptosis.[4] The intercalative property was referred to the planar aromatic system of the acridine moiety.

In the past some authors have used partial structures of two different drugs to obtain hybrid molecules sharing the activity of the two parent compounds. For example, *m*-AMSA acridine nucleus was substituted with bisantrene (an anthracenyl bishydrazone with antitopoII activity) side chain, to identify the structural determinants for the sequence-specific recognition of the covalent topoII–DNA complexes.[5] The bisantrene/amsacrine hybrid was able to poison topoII with an intermediate activity between those of bisantrene and *m*-AMSA, but with a different base preferences of DNA cleavage.

In this way we planned to synthesize new mixed molecules to complete the structural studies in order to determine the role of the planar part in the amsacrine series of topoII inhibitors, in terms of base pair selectivity.

As new amsacrine-like compounds we projected the synthesis of a series of *N*-[4-(2′-oxo-2*H*-pyrano[2,3-*b*]quinolin-5′-ylamino)-phenyl]-methanesulfonamides (Fig. 1).

In particular, the planar and aromatic acridine moiety of AMSA has been replaced by the analogous 2-oxo-2*H*-pyrano[2,3-*b*]quinoline system. In fact, the 2-pyrone (2*H*-pyran-2-one) moiety, a six-membered cyclic unsaturated ester which shares chemical and

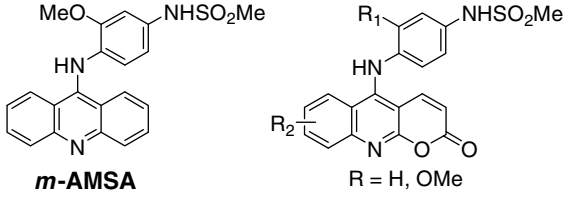

Figure 1. Chemical structures of *m*-AMSA (on the left) and of newly synthesized analogs (on the right).

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doi:10.1016/j.bmc.2008.11.072

physical properties reminiscent of alkene and aromatic compounds, is present in several natural compounds, such as coumarins and furocoumarins, with a very well documented biological activities.[6,7] In particular furocoumarins exhibited interesting DNA intercalative properties with good association constants[8,9] and thus we supposed that the replacement of one benzene of the acridine nucleus with 2-pyrone ring allowed to maintain favorable intercalative features due to the planarity of the pyranoquinolinone system.

2. Results and discussion

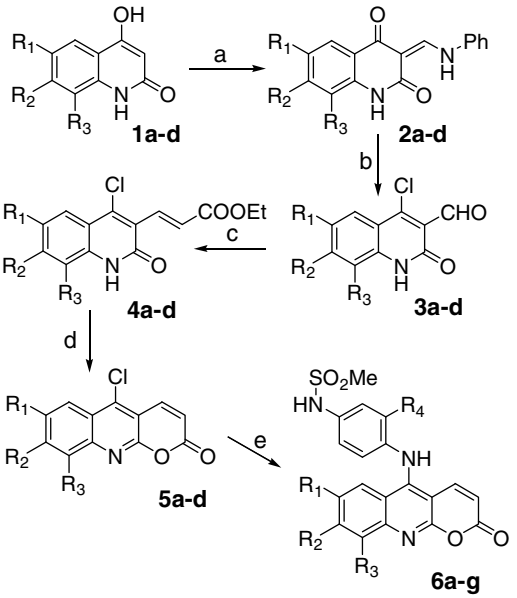
2.1. Molecular modelling studies

To assess the structure of the ligand–DNA complex, molecular modelling investigations were performed with *m*-AMSA and *m*-AMSA analog **6e**. These calculations show that the intercalation of these ligands into DNA is an exergonic process. In Figure 2 the best docking pose of *m*-AMSA (on the top) and **6e** (on the bottom) intercalated into [poly(dGdC)]$_2$ duplex is shown.

Docking studies have shown that the intercalative requirements are favourable for both *m*-AMSA and **6e** analog. Indeed, the theoretical energies of intercalation support that both compounds could be efficiently accommodated into the GC intercalation site ($\Delta E_{\text{int}-m\text{-AMSA}} = -11.2$ kcal/mol vs $\Delta H_{\text{int}-\mathbf{6e}} = -10.5$ kcal/mol).

2.2. Chemistry

The title compounds were prepared firstly synthesizing the aromatic tricyclic nucleus and then functionalizing it with the AMSA side chain, that is (4-aminophenyl)methanesulfonamide or (4-amino-3-methoxyphenyl)methanesulfonamide. The tricyclic pyranoquinolinone system was built condensing the pyrone ring on the appropriate quinolinone nucleus (Scheme 1), identifying 4-chloro-3-formylquinolones as key intermediates. These crucial synthons were obtained from 3-anilinomethylenequinolin-2,4-dione intermediates, using a known synthetic approach,[10] but with improved yields.



	R_1	R_2	R_3	R_4 (only for **6**)
a	H	H	H	H
b	OMe	H	H	H
c	H	OMe	H	H
d	H	H	OMe	H
e	H	H	H	OMe
f	OMe	H	H	OMe
g	H	H	OMe	OMe

Scheme 1. Reagents and conditions: (a) aniline, trimethyl orthoformate, ethyleneglycol, 175 °C; (b) $POCl_3$, DMF, K_2CO_3, rt; (c) (ethoxycarbonylmethylene)triphenylphosphorane, *N*,*N*-diethylaniline, 190 °C; (d) PPA, 150 °C; (e) 4'-aminomethanesulfonanilide, DMF, reflux.

Starting 4-hydroxyquinolin-2-ones[11] (**1a–d**) were reacted with trimethyl orthoformate and aniline to give 3-phenylaminomethylenquinolin-2,4-diones (**2a–d**), which were directly transformed into 4-chloro-3-formylquinolin-2-ones (**3a–d**) by phosphorus oxy-

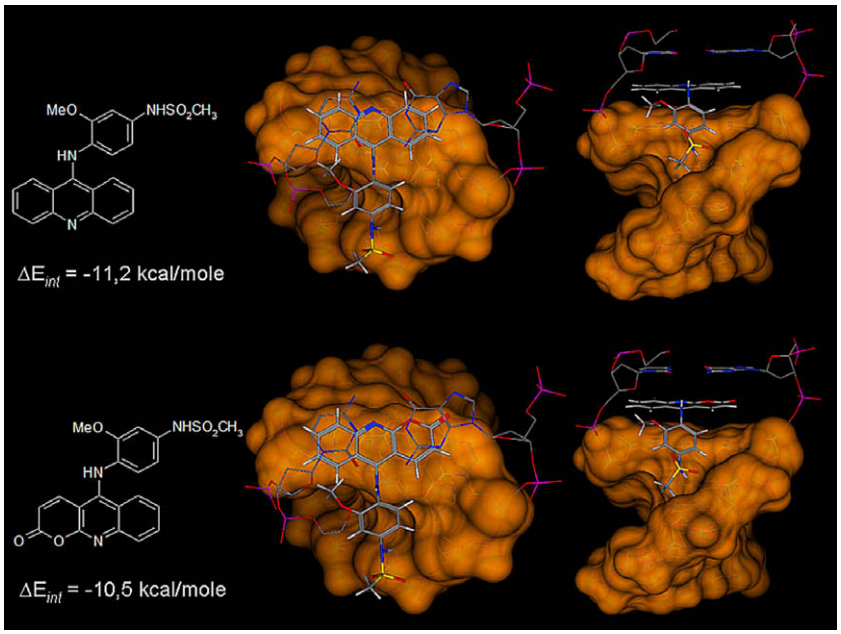


Figure 2. Molecular models of *m*-AMSA (on the top) and *m*-AMSA analog **6e** (on the bottom) intercalated into [poly(dGdC)]$_2$ duplex (see Section 4 for details); ds DNA is represented by its Connolly's surface. Intercalation binding energies (ΔE_{int}) are reported in kcal/mol.

chloride in DMF at room temperature. Subsequent Wittig olefination[12] of derivatives **3** with (ethoxycarbonylmethylene)-triphenyl-phosphorane in *N*,*N*-diethylaniline afforded the ethyl 3-(4′-chloro-2′-oxoquinolin-3′-yl)-acrylates (**4a–d**), which were cyclized with PPA at 150 °C to 5-chloro-2*H*-pyrano[2,3-*b*]quinolin-2-ones (**5a–d**). The 5-chloropyranoquinolinones **5** were finally coupled with the 4′-aminomethanesulfonanilides to provide the desired compounds **6a–g**.

2.3. Biological activity

The AMSA analogs **6a–g** and the tricyclic base compounds **5a–d** were examined for their cytotoxic properties in a panel of four human tumor cell lines containing examples of ovarian (2008), cervix (A431), lung (A549), breast (MCF-7) cancer and leukaemia (HL60). For comparison purpose, the cytotoxicity of *m*-AMSA was evaluated under the same experimental conditions. IC_{50} values, calculated from the dose–survival curves obtained after 72 h drug treatment from MTT test, are shown in the Table 1. All the derivatives showed a very low or no activity in all tumor cell lines.

To evaluate the ability of the new derivatives to form a molecular complex with DNA, flow linear dichroism (LD) experiments were performed. In detail, salmon testes DNA was incubated in the presence of increasing amounts of test compounds and the LD spectra were recorded in the 230–400 nm region. In Figure 3 the spectra of DNA (trace a) and compound **6e** (trace b) at [drug]/[DNA] = 0.08 is reported as an example, with *m*-AMSA (trace c) as reference compound. Compound **6e** was choosen as it is the *m*-AMSA analog in the newly synthesized series, but all the compounds show the same behaviour. The DNA spectrum shows a strong negative signal at 260 nm, typical of the macromolecule. It is known that for compounds able to intercalate between base pairs the appearance of a further negative LD signal at higher wavelengths (300–500 nm), where only the chromophore belonging to the added drug can absorb, occurs. The spectra obtained in the presence of the new compounds show none significant variation with respect to the DNA spectrum in this spectral range, while *m*-AMSA induces a significant negative dichroic signal in the same experimental conditions. Thus, these results suggest for the considered chromophore the inability to give rise to an efficient intercalative mode of binding with the macromolecule.

3. Conclusion

A series of new amsacrine-like derivatives was prepared and evaluated with the aim to study the influence of the tricyclic planar nucleus on the biological activity. As clearly described, our preliminary biological evaluation has shown that the replacement of the acridine moiety with the analogous 2-oxo-2*H*-pyrano[2,3-*b*]quinoline system drastically reduced both their anticancer activity and their propensity to intercalate with double stranded DNA, despite the small structural difference between the parent *m*-AMSA and the pyranoquinolinone derivatives.

Even though computational study revealed that the structures of *m*-AMSA and synthesized analogs were nearly superimposable and both could be easily inserted in DNA groove, our present efforts did not yield compounds suitable for therapeutic purposes, but gave evidence that the integrity of the acridine nucleus in the amsacrine class of anticancer agents is crucial for the biological activity.

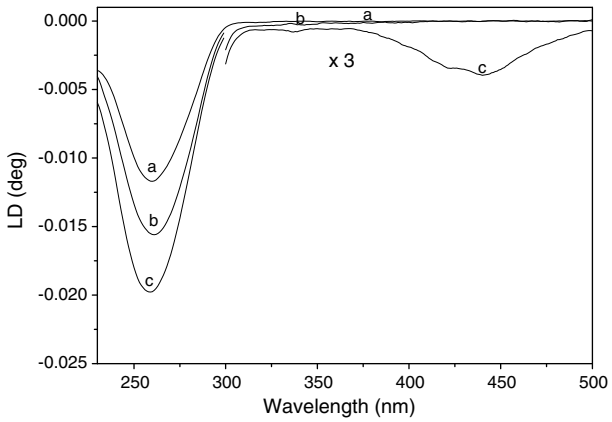


Figure 3. Linear flow dichroism spectra for DNA alone (a) and in the presence of compound **6e** (b) and *m*-AMSA (c). [DNA] = 1.5×10^{-3} M, [drug]/[DNA] = 0.08.

4. Experimental

4.1. Computational methodologies

All modeling studies were carried out on a 16 CPU (Intel Core™2 Quad CPU 2.40 GHz) linux cluster.

Geometry optimization studies were performed using the Molecular Operating Environment (MOE, ver. 2007.09) suite.[13] All structures were fully optimized without geometry constraints using RHF/AM1 semiempirical calculations. The software package MOPAC (ver.7),[14] implemented in MOE suite, was utilized for all quantum mechanical calculations.

Table 1
Cytotoxicity on human tumor cell lines[a]

Compound	IC_{50} (μM) ± S.D				
	A431	A549	2008	MCF-7	HL60
5a	54.33 ± 2.58	62.91 ± 2.60	52.6 ± 3.69	>100	39.53 ± 3.87
5b	96.99 ± 7.15	>100	>100	>100	>100
5c	>100	53.40 ± 3.55	>100	50.12 ± 2.13	60.32 ± 2.11
5d	55.31 ± 2.81	75.42 ± 2.59	60.62 ± 2.11	54.14 ± 1.25	37.12 ± 3.61
6a	94.14 ± 5.07	>100	>100	>100	92.10 ± 2.90
6b	55.63 ± 3.25	>100	68.24 ± 4.56	60.61 ± 3.15	47.28 ± 2.06
6c	92.73 ± 2.03	>100	>100	>100	>100
6d	>100	>100	>100	>100	>100
6e	>100	>100	>100	>100	>100
6f	85.69 ± 4.17	>100	>100	>100	95.23 ± 3.21
6g	>100	81.52 ± 2.66	>100	>100	>100
Amsacrine	5.89 ± 1.20	3.52 ± 1.31	4.31 ± 0.53	5.13 ± 0.50	1.15 ± 0.49

[a] IC_{50} values were calculated by probit analysis ($P < 0.05$, χ^2 test). Cells ($3–8 \times 10^4$ mL^{-1}) were treated for 72 h with increasing concentrations of tested compounds. Cytotoxicity was assessed by MTT test. S.D. = standard deviation.

4.1.1. Duplex intercalation site preparation

The present study involved the use of consensus dinucleotide intercalation geometry d(GpC) initially obtained using Nucleic Acid MOdeling Tool (NAMOT2, ver. 2) software.[15] d(GpC) Intercalation sites was contained in the centre of a decanucleotide duplex of $d(5'\text{-GCGCG-}3')_2$ sequence. Decamer in B-form was built using the 'DNA Builder' module of MOE.[13] The decanucleotide was minimized using Amber94 all-atom force field, implemented by MOE modeling package, until the rms value of Truncated Newton method (TN) was <0.1 kcal mol^{-1} $Å^{-1}$. To model the effects of solvent more directly, a set of electrostatic interaction corrections are used. MOE suite implemented a modified version of GB/SA contact function described by Still and co-authors.[16] These terms model the electrostatic contribution to the free energy of solvation in a continuum solvent model.

4.1.2. Molecular docking protocol

m-AMSA and its analogs were docked into both intercalation sites using flexible MOE-Dock methodology. The purpose of MOE-Dock is to search for favorable binding configurations between a small, flexible ligand and a rigid macromolecular target. Searching is conducted within a user-specified 3D docking box, using 'tabù search' protocol and MMFF94 force field. Charges for *m*-AMSA and its analogs were imported from the MOPAC output files. MOE-Dock performs a user-specified number of independent docking runs (55 in the presented case) and writes the resulting conformations and their energies to a molecular database file. The resulting intercalative complexes were subjected to MMFF94 all-atom energy minimization until the rms of conjugate gradient was <0.1 kcal mol^{-1} $Å^{-1}$. GB/SA approximation has been used to model the electrostatic contribution to the free energy of solvation in a continuum solvent model.

4.2. Chemistry

Melting points were determined on a Gallenkamp MFB-595-010M melting point apparatus and are uncorrected. Analytical TLC was performed on pre-coated 60 F_{254} silica gel plates (0.25 mm; Merck) developing with a $CHCl_3$/MeOH mixture (9:1) unless otherwise indicated. Preparative column chromatography was performed using silica gel 60 (0.063–0.100 mm; Merck), eluting with $CHCl_3$. ^{1}H NMR spectra were recorded on a Bruker AMX300 spectrometer with TMS as internal standard. HRMS spectra were obtained using an ESI-TOF Mariner 5220 (Applied Biosystem) mass spectrometer (in the positive ion mode). Elemental analyses were obtained on all intermediates and are within ±0.4% of theoretical values. Starting 4-hydroxyquinolinones **1a–d** were prepared according to literature methods.[11]

4.2.1. General procedure for 3-phenylaminomethylenquinolin-2,4-diones (2)

A mixture of **1**[10] (20.0 mmol), aniline (1.82 mL, 20.0 mmol), and trimethyl orthoformate (2.19 mL, 20.0 mmol) in ethyleneglycol (20 mL) was heated to 70 °C under stirring. The temperature was increased to 175 °C (10 °C/5 min), then kept at 175 °C for 2 h. After cooling the solid was collected and washed with EtOH to give **2**.

4.2.1.1. 3-Phenylaminomethylenquinolin-2,4-dione (2a). Yield 60%; mp 267 °C; ^{1}H NMR (DMSO-d_6) δ 10.87 (br s, 1H, –NH), 8.89 (br s, 1H, –CH), 7.96 (dd, 1H, J = 7.6, 1.2 Hz, 5-H), 7.59–7.45 (m, 5H, phenyl protons), 7.29 (t, 1H, J = 7.6 Hz, 6-H), 7.18 (d, 1H, J = 7.6 Hz, 8-H), 7.13 (td, 1H, J = 7.6, 1.2 Hz, 7-H). Anal. Calcd for $C_{16}H_{12}N_2O_2$: C, 72.72; H, 4.58; N, 10.60. Found: C, 72.75; H, 4.55; N, 10.63.

4.2.1.2. 3-Phenylaminomethylen-6-methoxyquinolin-2,4-dione (2b). Yield 65%; mp 297 °C; ^{1}H NMR (DMSO-d_6) δ 12.93 (d, 1H, J = 12.5 Hz, –NH), 10.80 (s, 1H, –NH), 8.89 (d, 1H, J = 12.5 Hz, –CH), 7.60–7.30 (m, 5H, phenyl protons), 7.42 (d, 1H, J = 2.8 Hz, 5-H), 7.20 (dd, 1H, J = 8.8, 2.8 Hz, 7-H), 7.13 (d, 1H, J = 8.8 Hz, 8-H), 3.79 (s, 3H, $-OCH_3$). Anal. Calcd for $C_{17}H_{14}N_2O_3$: C, 69.38; H, 4.79; N, 9.52. Found: C, 69.41; H, 4.82; N, 9.50.

4.2.1.3. 3-Phenylaminomethylen-7-methoxyquinolin-2,4-dione (2c). Yield 87%; mp 297 °C; ^{1}H NMR (DMSO-d_6) δ 12.66 (d, 1H, J = 13.3 Hz, –NH), 10.81 (s, 1H, –NH), 8.80 (d, 1H, J = 13.3 Hz, –CH), 7.88 (d, 1H, J = 8.7 Hz, 5-H), 7.51–7.28 (m, 5H, phenyl protons), 6.73 (dd, 1H, J = 8.7, 2.4 Hz, 6-H), 6.67 (d, 1H, J = 8.7 Hz, 8-H), 3.82 (s, 3H, $-OCH_3$). Anal. Calcd for $C_{17}H_{14}N_2O_3$: C, 69.38; H, 4.79; N, 9.52. Found: C, 69.42; H, 4.78; N, 9.52.

4.2.1.4. 3-Phenylaminomethylen-8-methoxyquinolin-2,4-dione (2d). Yield 78%; mp 202 °C; ^{1}H NMR (DMSO-d_6) δ 12.50 (d, 1H, J = 13.3 Hz, –NH), 9.84 (s, 1H, –NH), 9.00 (d, 1H, J = 13.3 Hz, –CH), 7.70–7.12 (m, 8H, aromatic protons), 3.96 (s, 3H, $-OCH_3$). Anal. Calcd for $C_{17}H_{14}N_2O_3$: C, 69.38; H, 4.79; N, 9.52. Found: C, 69.37; H, 4.80; N, 9.49.

4.2.2. General procedure for 4-chloro-3-formylquinolin-2-ones (3)

To a solution of **2** (10.0 mmol) in anhydrous DMF (25 mL) $POCl_3$ (1.4 mL, 15.0 mmol) was slowly added and the mixture was stirred at room temperature for 20 h. The mixture was poured into ice and water (250 mL), the precipitate was collected and crystallized from toluene to give **3**.

4.2.2.1. 4-Chloro-3-formylquinolin-2-one (3a). Yield 98%; mp 273 °C; ^{1}H NMR (DMSO-d_6) δ 12.45 (s, 1H, –NH), 10.31 (s, 1H, CHO), 8.11 (dd, 1H, J = 7.8, 1.4 Hz, 5-H), 7.73 (td, 1H, J = 7.8, 1.1 Hz, 6-H), 7.40 (dd, 1H, J = 7.8, 1.1 Hz, 8-H), 7.37 (td, 1H, J = 7.8, 1.4 Hz, 7-H). Anal. Calcd for $C_{10}H_6ClNO_2$: C, 57.85; H, 2.91; Cl, 17.08; N, 6.75. Found: C, 57.82; H, 2.93; Cl, 17.10; N, 6.73.

4.2.2.2. 4-Chloro-3-formyl-6-methoxyquinolin-2-one (3b). Yield 99%; mp 262 °C; ^{1}H NMR (DMSO-d_6) δ 12.39 (s, 1H, –NH), 10.32 (s, 1H, CHO), 7.45 (dd, 1H, J = 8.2, 2.6 Hz, 7-H), 7.41 (d, 1H, J = 2.6 Hz, 5-H), 7.37 (d, 1H, J = 8.2 Hz, 8-H), 3.86 (s, 3H, $-OCH_3$). Anal. Calcd for $C_{11}H_8ClNO_3$: C, 55.60; H, 3.39; Cl, 14.92; N, 5.89. Found: C, 55.60; H, 3.42; Cl, 14.89; N, 5.93.

4.2.2.3. 4-Chloro-3-formyl-7-methoxyquinolin-2-one (3c). Yield 78%; mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 12.29 (s, 1H, –NH), 10.28 (s, 1H, CHO), 8.02 (d, 1H, J = 9.2 Hz, 5-H), 6.99 (dd, 1H, J = 9.2, 2.4 Hz, 6-H), 6.86 (d, 1H, J = 2.4 Hz, 8-H), 3.88 (s, 3H, $-OCH_3$). Anal. Calcd for $C_{11}H_8ClNO_3$: C, 55.60; H, 3.39; Cl, 14.92; N, 5.89. Found: C, 55.61; H, 3.43; Cl, 14.92; N, 5.90.

4.2.2.4. 4-Chloro-3-formyl-8-methoxyquinolin-2-one (3d). Yield 92%; mp 210 °C; ^{1}H NMR (DMSO-d_6) δ 11.49 (s, 1H, –NH), 10.08 (s, 1H, CHO), 7.46 (dd, 1H, J = 7.8, 1.5 Hz, 5-H), 7.14 (dd, 1H, J = 7.8, 1.5 Hz, 7-H), 7.12 (t, 1H, J = 7.8 Hz, 6-H), 3.71 (s, 3H, $-OCH_3$). Anal. Calcd for $C_{11}H_8ClNO_3$: C, 55.60; H, 3.39; Cl, 14.92; N, 5.89. Found: C, 55.60; H, 3.40; Cl, 14.91; N, 5.87.

4.2.3. General procedure for ethyl 3-(4′-chloro-2′-oxoquinolin-3′-yl)-acrylates 4

A mixture of **3** (12.0 mmol), (ethoxycarbonylmethylene)triphenylphosphorane (5.0 g, 14.4 mmol) and *N,N*-diethylaniline (60 mL) was heated at 190 °C under nitrogen for 4 h. After cooling the precipitate was collected, washed with diethyl ether and crystallized from MeOH to give **4**.

4.2.3.1. Ethyl 3-(4′-chloro-2′-oxoquinolin-3′-yl)-acrylates (4a). Yield 70%; mp 210 °C; ^{1}H NMR ($CDCl_3$-*d*) δ 11.02 (s, 1H, –NH), 8.11 (d, 1H, *J* = 15.8 Hz, 3-H), 8.09 (dd, *J* = 7.5, 1.1 Hz, 5′-H), 7.63 (td, 1H, *J* = 7.5, 1.1 Hz, 7′-H), 7.61 (d, 1H, *J* = 15.8 Hz, 2-H), 7.34 (td, 1H, *J* = 7.5, 1.1 Hz, 6′-H), 7.32 (dd, 1H, *J* = 7.5, 1.1 Hz, 8′-H), 4.31 (q, 2H, *J* = 7.1 Hz, CH_2CH_3), 1.37 (t, 3H, *J* = 7.1 Hz, CH_2CH_3). Anal. Calcd for $C_{14}H_{12}ClNO_3$: C, 60.55; H, 4.36; Cl, 12.77; N, 5.04. Found: C, 60.53; H, 4.38; Cl, 12.80; N, 5.01.

4.2.3.2. Ethyl 3-(4′-chloro-6′-methoxy-2′-oxoquinolin-3′-yl)-acrylates (4b). Yield 75%; mp 279 °C; ^{1}H NMR ($CDCl_3$-*d*) δ 12.33 (s, 1H, –NH), 7.94 (d, 1H, *J* = 15.7 Hz, 3-H), 7.78–7.74 (m, 2H, 7′-H and 8′-H), 7.55 (d, 1H, *J* = 15.7 Hz, 2-H), 7.38 (d, 1H, *J* = 2.2 Hz, 5′-H), 4.21 (q, 2H, *J* = 7.1 Hz, CH_2CH_3), 3.86 (s, 3H, –OCH_3), 1.27 (t, 3H, *J* = 7.1 Hz, CH_2CH_3). Anal. Calcd for $C_{15}H_{14}ClNO_4$: C, 58.55; H, 4.59; Cl, 11.52; N, 4.55. Found: C, 58.54; H, 4.56; Cl, 11.56; N, 4.57.

4.2.3.3. Ethyl 3-(4′-chloro-7′-methoxy-2′-oxoquinolin-3′-yl)-acrylates (4c). Yield 59%; mp 294 °C; ^{1}H NMR ($CDCl_3$-*d*) δ 12.24 (s, 1H, –NH), 7.92 (d, 1H, *J* = 9.2 Hz, 5′-H), 7.91 (d, 1H, *J* = 15.4 Hz, 3-H), 7.47 (d, 1H, *J* = 15.4 Hz, 2-H), 6.97 (dd, 1H, *J* = 9.2, 2.4 Hz, 6′-H), 6.87 (d, 1H, *J* = 2.4 Hz, 8′-H), 4.20 (q, 2H, *J* = 7.1 Hz, CH_2CH_3), 3.88 (s, 3H, –OCH_3), 1.26 (t, 3H, *J* = 7.1 Hz, CH_2CH_3). Anal. Calcd for $C_{15}H_{14}ClNO_4$: C, 58.55; H, 4.59; Cl, 11.52; N, 4.55. Found: C, 58.57; H, 4.58; Cl, 11.55; N, 4.52.

4.2.3.4. Ethyl 3-(4′-chloro-8′-methoxy-2′-oxoquinolin-3′-yl)-acrylates (4d). Yield 48%; mp 262 °C; ^{1}H NMR ($CDCl_3$-*d*) δ 9.36 (s, 1H, –NH), 8.08 (d, 1H, *J* = 15.8 Hz, 3-H), 7.65 (d, 1H, *J* = 8.1 Hz, 5′-H), 7.61 (d, 1H, *J* = 15.8 Hz, 2-H), 7.25 (t, 1H, *J* = 8.1 Hz, 6′-H), 7.06 (d, 1H, *J* = 8.1 Hz, 7′-H), 4.29 (q, 2H, *J* = 7.1 Hz, CH_2CH_3), 4.01 (s, 3H, –OCH_3), 1.34 (t, 3H, *J* = 7.1 Hz, CH_2CH_3). Anal. Calcd for $C_{15}H_{14}ClNO_4$: C, 58.55; H, 4.59; Cl, 11.52; N, 4.55. Found: C, 58.55; H, 4.57; Cl, 11.56; N, 4.55.

4.2.4. General procedure for 5-chloro-2*H*-pyrano[2,3-*b*]quinolin-2-ones 5

A mixture of **4** (5.0 mmol) and PPA (10.0 g) was heated at 150 °C under nitrogen for 2 h. After cooling the solution was poured into ice and water (500 mL). The solid was collected, washed with water and crystallized from MeOH to give **5**.

4.2.4.1. 5-Chloro-2H-pyrano[2,3-b]quinolin-2-one (5a). Yield 84%; mp 210 °C; ^{1}H NMR ($CDCl_3$-*d*) δ 8.31 (dd, 1H, *J* = 8.1, 1.1 Hz, 6-H), 8.25 (d, 1H, *J* = 9.8 Hz, 4-H), 8.09 (d, 1H, *J* = 8.1 Hz, 9-H), 7.97 (td, 1H, *J* = 8.1, 1.1 Hz, 8-H), 7.69 (td, 1H, *J* = 8.1, 1.1 Hz, 7-H), 6.63 (d, 1H, *J* = 9.8, 3-H). Anal. Calcd for $C_{12}H_6ClNO_2$: C, 62.22; H, 2.61; Cl, 15.31; N, 6.05. Found: C, 62.25; H, 2.64; Cl, 15.29; N, 6.02.

4.2.4.2. 5-Chloro-7-methoxy-2H-pyrano[2,3-b]quinolin-2-one (5b). Yield 21%; mp 251 °C; ^{1}H NMR (DMSO-d_6) δ 8.35 (d, 1H, *J* = 9.8 Hz, 4-H), 7.94 (d, 1H, *J* = 9.2 Hz, 9-H), 7.63 (dd, 1H, *J* = 9.2, 2.7 Hz, 8-H), 7.51 (d, 1H, *J* = 2.7 Hz, 6-H), 6.75 (d, 1H, *J* = 9.8 Hz, 3-H), 3.98 (s, 3H, –OCH_3). Anal. Calcd for $C_{13}H_8ClNO_3$: C, 59.67; H, 3.08; Cl, 13.55; N, 5.35. Found: C, 59.70; H, 3.11; Cl, 13.54; N, 5.32.

4.2.4.3. 5-Chloro-8-methoxy-2H-pyrano[2,3-b]quinolin-2-one (5c). Yield 77%; mp 237 °C; ^{1}H NMR (DMSO-d_6) δ 8.32 (d, 1H, *J* = 9.7 Hz, 4-H), 8.20 (d, 1H, *J* = 9.2 Hz, 6-H), 7.42 (dd, 1H, *J* = 9.2, 2.4 Hz, 7-H), 7.40 (d, 1H, *J* = 2.4 Hz, 9-H), 6.67 (d, 1H, *J* = 9.7 Hz, 3-H), 3.99 (s, 3H, –OCH_3). Anal. Calcd for $C_{13}H_8ClNO_3$: C, 59.67; H, 3.08; Cl, 13.55; N, 5.35. Found: C, 59.69; H, 3.07; Cl, 13.57; N 5.36.

4.2.4.4. 5-Chloro-9-methoxy-2H-pyrano[2,3-b]quinolin-2-one (5d). Yield 55%; mp 225 °C; ^{1}H NMR ($CDCl_3$-*d*) δ 8.23 (d, 1H, *J* = 9.8 Hz, 4-H), 7.86 (dd, 1H, *J* = 8.2, 1.0 Hz, 6-H), 7.60 (t, 1H, *J* = 8.2 Hz, 7-H), 7.23 (dd, 1H, *J* = 8.2, 1.0 Hz, 8-H), 6.64 (d, 1H, *J* = 9.8 Hz, 3-H), 4.10 (s, 3H, –OCH_3). Anal. Calcd for $C_{13}H_8ClNO_3$: C, 59.67; H, 3.08; Cl, 13.55; N, 5.35. Found: C, 59.65; H, 3.10; Cl, 13.59; N, 5.30.

4.2.5. General procedure for *N*-[4-(2′-oxo-2*H*-pyrano[2,3-*b*]quinolin-5′-ylamino)-phenyl]-methane-sulfonamides 6

A solution of **5** (1.0 mmol), *N*-(4-aminophenyl)-methanesulfonamide or *N*-(4-amino-3-methoxyphenyl)methanesulfonamide[17] (1.0 mmol) and DMF (10 mL) was refluxed until starting product disappeared (4–6 h, TLC). After cooling the mixture was diluted with water (50 mL), the precipitate was collected, washed with water, and purified by column chromatography to give **6**.

4.2.5.1. N-[4-(2′-oxo-2H-pyrano[2,3-b]quinolin-5′-ylamino)-phenyl]-methanesulfonamide (6a). Pale yellow needles (MeOH); yield 42%; mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 11.94 (s, 1H, –$NHSO_2CH_3$), 10.20 (br s, 1H, –NH), 8.28 (d, 1H, *J* = 9.6 Hz, 4′-H), 7.39 (t, 1H, *J* = 8.3 Hz, 8′-H), 7.36 (d, 1H, *J* = 8.3 Hz, 9′-H), 7.33 (s, 4H, 2-H, 3-H, 5-H and 6-H), 6.72 (t, 1H, *J* = 8.3 Hz, 7′-H), 6.70 (d, 1H, *J* = 9.6 Hz, 3′-H), 6.53 (d, 1H, *J* = 8.3 Hz, 6′-H), 3.07 (s, 3H, –SCH_3); ^{13}C NMR (DMSO-d_6) δ 162.71 (C-2′), 159.11 (C-10′a), 145.27 (C-9′a), 139.45 (C-1 or C-4), 139.10 (C-1 or C-4), 137.48 (C-4′), 135.28 (C-5′), 130.85 (C-8′), 129.80 (C-3 and C-5), 125.97 (C-9′), 120.77 (C-6′ or C-7′), 120.01 (C-2 and C-6), 119.65 (C-3′), 116.70 (C-6′ or C-7′), 112.07 (C-5′a), 109.87 (C-4′a), 38.80 (–SCH_3); HRMS (ESI) calcd for $C_{19}H_{16}N_3O_4S$ $(M+1)^+$ 382.0862, found 382.0926. Anal. Calcd for $C_{19}H_{15}N_3O_4S$: C, 59.83; H, 3.96; N, 11.02; S, 8.41. Found: C, 59.78; H, 3.90; N, 11.05; S, 8.37.

4.2.5.2. N-[4-(7′-Methoxy-2′-oxo-2H-pyrano[2,3-b]quinolin-5′-ylamino)-phenyl]methanesulfonamides (6b). Pale yellow needles (MeOH); yield 10%; mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 11.86 (s, 1H, –$NHSO_2CH_3$), 10.11 (s, 1H, –NH–), 8.29 (d, 1H, *J* = 9.6 Hz, 4′-H), 7.38 (s, 4H, 2-H, 3-H, 5-H and 6-H), 7.28 (d, 1H, *J* = 9.0 Hz, 9′-H), 7.10 (dd, 1H, *J* = 9.0, 2.6 Hz, 8′-H), 6.71 (d, 1H, *J* = 9.6 Hz, 3′-H), 6.14 (d, 1H, *J* = 2.6 Hz, 6′-H), 3.27 (s, 3H, –OCH_3), 3.12 (s, 3H, –SCH_3); ^{13}C NMR (DMSO-d_6) δ 162.81 (C-2′), 158.75 (C-10′a), 152.65 (C-7′), 144.76 (C-9′a), 139.00 (C-1), 137.63 (C-4′), 135.26 (C-5′), 133.95 (C-4′), 130.06 (C-3 and C-5), 120.15 (C-2 and C-6), 119.72 (C-3′), 117.85 (C-8′ and C-9′), 112.35 (C-5′a), 110.18 (C-4′a), 108.60 (C-6′), 54.66 (–OCH_3), 38.88 (–SCH_3); HRMS (ESI) calcd for $C_{20}H_{18}N_3O_5S$ $(M+1)^+$ 412.0962, found 412.1049. Anal. Calcd for $C_{20}H_{17}N_3O_5S$: C, 58.38; H, 4.16; N, 10.21; S, 7.79. Found: C, 58.42; H, 4.14; N, 10.26; S, 7.85.

4.2.5.3. N-[4-(8′-Methoxy-2′-oxo-2H-pyrano[2,3-b]quinolin-5′-ylamino)-phenyl]-methanesulfonamides (6c). Pale yellow needles (MeOH); yield 15%; mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 11.80 (s, 1H, –$NHSO_2CH_3$), 10.08 (s, 1H, –NH–), 8.23 (d, 1H, *J* = 9.5 Hz, 4′-H), 7.36 (d, 2H, *J* = 9.1 Hz, 2-H and 6-H or 3-H and 5-H), 7.33 (d, 2H, *J* = 9.1 Hz, 2-H and 6-H or 3-H and 5-H), 6.83 (d, 1H, *J* = 2.3 Hz, 9′-H), 6.61 (d, 1H, *J* = 9.5 Hz, 3′-H), 6.43 (d, 1H, *J* = 9.6 Hz, 6′-H), 6.33 (d, 1H, *J* = 9.6 Hz, 7′-H), 3.74 (s, 3H, –OCH_3), 3.10 (s, 3H, –SCH_3); ^{13}C NMR (DMSO-d_6) δ 162.75 (C-2′), 160.69 (C-8′), 159.37 (C-10′a), 145.60 (C-9′a), 141.62 (C-5′), 138.90 (C-1), 137.66 (C-4′), 135.40 (C-4), 129.81 (C-3 and C-5), 127.67 (C-7′), 119.95 (C-2 and C-6), 118.14 (C-3′), 109.48 (C-9′), 107.67 (C-4′a), 105.76 (C-5′a), 99.30 (C-6′), 55.30 (–OCH_3), 40.10 (–SCH_3); HRMS (ESI) calcd for $C_{20}H_{18}N_3O_5S$ $(M+1)^+$ 412.0962, found 412.0941. Anal. Calcd for $C_{20}H_{17}N_3O_5S$: C, 58.38; H, 4.16; N, 10.21; S, 7.79. Found: C, 58.40; H, 4.12; N, 10.25; S, 7.80.

4.2.5.4. N-[4-(9′-Methoxy-2′-oxo-2H-pyrano[2,3-b]quinolin-5′-ylamino)-phenyl]-methanesulfonamides (6d). Pale yellow needles (MeOH); yield 33%; mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 10.87

(s, 1H, –*NH*SO_2CH_3), 10.08 (s, 1H, –NH–), 8.29 (d, 1H, *J* = 9.6 Hz, 4′-H), 7.34 (s, 4H, 2-H, 3-H, 5-H and 6-H), 7.06 (d, 1H, *J* = 8.4 Hz, 6′-H), 6.71 (d, 1H, *J* = 9.6 Hz, 3′-H), 6.69 (t, 1H, *J* = 8.4 Hz, 7′-H), 6.14 (d, 1H, *J* = 8.4 Hz, 8′-H), 3.88 (s, 3H, –OCH_3), 3.09 (s, 3H, –SCH_3); ^{13}C NMR (DMSO-d_6) δ 162.73 (C-2′), 158.72 (C-10′a), 146.14 (C-9′), 145.46 (C-9′a), 138.86 (C-1), 137.58 (C-4′), 135.46 (C-4), 129.88 (C-5′), 129.63 (C-3 and C-5), 120.44 (C-7′), 119.94 (C-2 and C-6), 119.82 (C-3′), 117.63 (C-5′a), 112.55 (C-6′ or C-8′), 111.22 (C-6′ or C-8′), 110.23 (C-4′a), 56.17 (–OCH_3), 38.85 (–SCH_3); HRMS (ESI) calcd for $C_{20}H_{18}N_3O_5S$ $(M+1)^+$ 412.0962, found 412.0892. Anal. Calcd for $C_{20}H_{17}N_3O_5S$: C, 58.38; H, 4.16; N, 10.21; S, 7.79. Found: C, 58.40; H, 4.12; N, 10.25; S, 7.80.

4.2.5.5. N-[4-(2′-Oxo-2H-pyrano[2,3-b]quinolin-5′-ylamino)-3-methoxyphenyl]methanesulfonamide (6e). pale yellow needles (MeOH); yield 18%; mp >300 °C; ^{1}H NMR (CD_3OD-d_4) δ 8.45 (d, 1H, *J* = 9.5 Hz, 4′-H), 7.44 (td, 1H, *J* = 8.1, 1.8 Hz, 8′-H), 7.34 (dd, 1H, *J* = 8.1, 1.6 Hz, 9′-H), 7.25 (d, 1H, *J* = 8.6 Hz, 5-H), 7.13 (d, 1H, *J* = 1.9 Hz, 2-H), 7.05 (dd, 1H, *J* = 8.1, 1.8 Hz, 6′-H), 6.94 (dd, 1H, *J* = 8.6, 1.9 Hz, 6-H), 6.82 (td, 1H, *J* = 8.1, 1.6 Hz, 7′-H), 6.79 (d, 1H, *J* = 9.5 Hz, 3′-H), 3.66 (s, 3H, –OCH_3), 3.10 (s, 3H, –SCH_3); ^{13}C NMR (DMSO-d_6) δ 162.12 (C-2′), 159.10 (C-10′a), 155.19 (C-3), 145.64 (C-9′a), 140.73 (C-1), 139.27 (C-4), 137.53 (C-4′), 131.05 (C-5′), 130.29 (C-8′), 124.62 (C-9′), 124.02 (C-5), 121.13 (C-6′ or C-7′), 119.54 (C-3′), 116.78 (C-6′ o C-7′), 112.14 (C-6), 111.39 (C-5′a), 109.46 (C-4′a), 103.59 (C-2), 55.70 (–OCH_3), 39.00 (–SCH_3); HRMS (ESI) calcd for $C_{20}H_{18}N_3O_5S$ $(M+1)^+$ 412.0967, found 412.1066. Anal. Calcd for $C_{20}H_{17}N_3O_5S$: C, 58.38; H, 4.16; N, 10.21; S, 7.79. Found: C, 58.44; H, 4.10; N, 10.21; S, 7.81.

4.2.5.6. N-[4-(7′-Methoxy-2′-oxo-2H-pyrano[2,3-b]quinolin-5′-ylamino)-3-methoxyphenyl]-methane-sulfonamide (6f). Pale yellow needles (MeOH); yield 12%; mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 11.86 (s, 1H, –*NH*SO_2CH_3), 9.57 (s, 1H, –NH–), 7.52 (d, 1H, *J* = 9.8 Hz, 4′-H), 7.27 (d, 1H, *J* = 9.0 Hz, 5-H), 7.24 (dd, 1H, *J* = 9.0, 2.5 Hz, 6-H), 7.12 (d, 1H, *J* = 8.3 Hz, 9′-H), 6.96 (d, 1H, *J* = 2.2 Hz, 6′-H), 6.85 (dd, 1H, *J* = 8.3, 2.2 Hz, 8′-H), 6.74 (d, 1H, *J* = 2.5 Hz, 2-H), 6.61 (d, 1H, *J* = 9.8 Hz, 3′-H), 3.70 (s, 3H, –OCH_3), 3.63 (s, 3H, –OCH_3), 2.98 (s, 3H, –SCH_3); ^{13}C NMR (DMSO-d_6) δ 158.74 (C-2′), 156.49 (C-10′a), 154.31 (C-7′), 151.16 (C-3), 148.24 (C-9′a), 135.08 (C-1), 133.07 (C-5′), 131.45 (C-4′), 131.32 (C-4), 122.80 (C-9′), 122.18 (C-8′), 118.95 (C-3′), 117.42 (C-5), 112.32 (C-6), 112.06 (C-5′a), 107.34 (C-4′a), 104.64 (C-2), 101.44 (C-6′), 55.35 (–OCH_3), 55.14 (–OCH_3), 38.74 (–SCH_3); HRMS (ESI) calcd for $C_{21}H_{20}N_3O_6S$ $(M+1)^+$ 442.1067, found 442.1017. Anal. Calcd for $C_{21}H_{19}N_3O_6S$: C, 57.13; H, 4.34; N, 9.52; S, 7.26. Found: C, 57.15; H, 4.30; N, 9.55; S, 7.22.

4.2.5.7. N-[4-(9′-Methoxy-2′-oxo-2H-pyrano[2,3-b]quinolin-5′-ylamino)-3-methoxyphenyl]-methane-sulfonamide (6g). Pale yellow needles (EtOAc); yield 10%; mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 10.84 (s, 1H, –*NH*SO_2CH_3), 9.95 (s, 1H, –NH–), 8.30 (d, 1H, *J* = 9.5 Hz, 4′-H), 7.23 (d, 1H, *J* = 8.5 Hz, 5-H), 7.09 (d, 1H, *J* = 8.6 Hz, 6′-H), 7.03 (d, 1H, *J* = 1.9 Hz, 2-H), 6.94 (dd, 1H, *J* = 8.5, 1.9 Hz, 6-H), 6.73 (t, 1H, *J* = 8.6 Hz, 7′-H), 6.69 (d, 1H, *J* = 9.5 Hz, 3′-H), 6.35 (d, 1H, *J* = 8.6 Hz, 8′-H), 3.88 (s, 3H, –OCH_3), 3.60 (s, 3H, –OCH_3), 3.12 (s, 3H, –SCH_3); ^{13}C NMR (DMSO-d_6) δ 162.16 (C-2′), 158.70 (C-10′a), 155.19 (C-3), 146.11 (C-9′), 145.88 (C-9′a), 140.83 (C-1), 137.60 (C-4′), 130.36 (C-5), 129.42 (C-5′), 124.03 (C-4), 120.79 (C-7′), 119.70 (C-3′), 116.24 (C-6), 112.61 (C-5′a), 111.42 (C-6′ or C-8′), 111.34 (C-6′ or C-8′), 109.77 (C-4′a), 103.57 (C-2), 56.14 (–OCH_3), 55.72 (–OCH_3), 39.27 (–SCH_3); HRMS (ESI) calcd for $C_{21}H_{20}N_3O_6S$ $(M+1)^+$ 442.1067, found 442.1008. Anal. Calcd for $C_{21}H_{19}N_3O_6S$: C, 57.13; H, 4.34; N, 9.52; S, 7.26. Found: C, 57.14; H, 4.30; N, 9.51; S, 7.21.

4.3. Biology

4.3.1. Experiments with human cells

Compounds were dissolved in dimethyl sulfoxide just before the experiments; calculated amounts of drug solution were added to the growth medium containing cells to a final solvent concentration of 0.5% which had no discernible effect on cell killing.

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and *m*-amsacrine were obtained from Sigma Chemical Co., St. Louis, USA.

4.3.2. Cell cultures

Human lung (A549) and breast (MCF-7) carcinoma cell lines along with promyelocytic leukemia (HL60) cells were obtained by ATCC (RocKville, MD). 2008 Human ovarian cancer cell line was kindly provided by Prof. G. Marverti (Dept. of Biomedical Science of Modena University, Italy) and A431 human cervix carcinoma were kindly provided by Prof. Zunino (Division of Experimental Oncology B, Istituto Nazionale dei Tumori, Milan, Italy). Cell lines were maintained in the logarithmic phase at 37 °C in a 5% carbon dioxide atmosphere using the following culture media: (i) RPMI-1640 medium (Euroclone, Celbio, Milan, Italy) containing 10% foetal calf serum (Biochrom-Seromed GmbH&Co, Berlin, Germany) and supplemented with 25 mM HEPES buffer, L-glutamine and with antibiotics penicillin (50 units mL^{-1}) and streptomycin (50 μg mL^{-1}) for 2008, A431, MCF7 and HL60 cells; (ii) F-12 Ham's (Sigma Chemical Co.) containing 15% foetal calf serum, penicillin (50 units mL^{-1}) and streptomycin (50 μg mL^{-1}) for A549 cells.

4.3.3. Cytotoxicity assay

The growth inhibitory effect towards tumor cell lines was evaluated by means of MTT (tetrazolium salt reduction) assay.[18] Briefly, between 3 and 8×10^{-3} cells, dependent upon the growth characteristics of the cell line, were seeded in 96-well microplates in growth medium (100 μL) and then incubated at 37 °C in a 5% carbon dioxide atmosphere. After 24 h, the medium was removed and replaced with a fresh one containing the compound to be studied at the appropriate concentrations. Quadruplicate cultures were established for each treatment. Seventy-two hours later, each well was treated with 10 μL of a 5 mg mL^{-1} MTT saline solution, and after 5 h of incubation, 100 μL of a sodium dodecylsulfate (SDS) solution in HCl 0.01 M was added. After overnight incubation, the inhibition of cell growth induced by the tested complexes was detected by measuring the absorbance of each well at 540 nm using a Camberra–Packard microplate reader. Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted versus drug concentration. IC_{50} values represent the drug concentrations that reduced the mean absorbance at 540 nm to 50% of those in the untreated control wells.

4.3.4. Statistical analysis

All the values are the means ± SD of not less than five measurements. IC_{50} values were calculated by the Probit model. Statistical analysis was performed using Chi-square test. $P < 0.05$ was considered statistically significant.

4.3.5. Experiments with nucleic acid

Salmon testes DNA was purchased from Sigma Chemical Company. The concentration was determined using extinction coefficient 6600 M^{-1} cm^{-1} at 260 nm.

4.3.6. Linear flow dichroism

Linear dichroism (LD) measurements were performed on a Jasco J500A circular dichroism spectropolarimeter converted for LD and

equipped with an IBM PC and a Jasco J interface. Linear dichroism is defined as:

$$LD_{(\lambda)} = A_{\parallel(\lambda)} - A_{\perp(\lambda)}$$

where $A_{\parallel(\lambda)}$ and $A_{\perp(\lambda)}$ correspond to the absorbances of the sample when polarized light is oriented parallel or perpendicular to the flow direction, respectively. The orientation is produced by a device designed by Wada and Kozawa[19] at a shear gradient of 500–700 rpm and each spectrum was accumulated four times. A solution of salmon testes DNA (1.5×10^{-3} M) in ETN buffer (containing 10 mM Tris, 10 mM NaCl, and 1 mM EDTA, pH 7) was used. Spectra were recorded at 25 °C at [drug]/[DNA] ratios: 0, 0.02, 0.04, 0.08.

Acknowledgements

The present work has been carried out with financial supports of the University of Padova (Italy) and the Italian Ministry for University and Research (MIUR), Rome (Italy). We thanks the Molecular Modeling Section (MMS) of our Department for the modeling studies.

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