Eur. J. Med. Chem. 35 (2000) 827−837

S0223523400001707/FLA

Original article

Synthesis and antiproliferative activity of some variously substituted acridine and azacridine derivatives

Maria Grazia Ferlin, Cristina Marzano, Gianfranco Chiarelotto, Francarosa Baccichetti, Franco Bordin*

Department of Pharmaceutical Sciences of Padua University, Centro di Studio sulla Chimica del Farmaco e dei Prodotti Biologicamente Attivi del CNR, (associated with the National Institute for the Chemistry of Biological Systems), Padova, Italy

Received 1 July 1999; accepted 28 March 2000

Abstract – A group of 9-substituted acridine and azacridine derivatives (m-AMSA analogues) were synthesised following classical procedures as potential antitumour agents with inhibitory effects on DNA topoisomerase II. Some were found to have noticeable cytotoxicity against human HL-60 and HeLa cells grown in culture. Their non-covalent interactions with calf thymus DNA have been studied using fluorescence quenching. We evaluated DNA damage produced by the tested compounds by means of DNA filter elution and protein precipitation techniques. Catalytic studies carried out with purified topoisomerase confirmed these agents as antitopoisomerase inhibitors. Chemotherapy of solid-tumour-bearing mice with tested compounds allowed an aza-analogue (compound **IIIb**), as potent as m-AMSA but less toxic towards the host, to be recognised. © 2000 Éditions scientifiques et médicales Elsevier SAS

amsacrine / topoisomerase II inhibitors / DNA damage

1. Introduction

During the last 20–30 years a large number of derivatives belonging to the general class of anilinoacridines have been prepared and evaluated extensively as antimalarial [1, 2], antileishmanial [3], antitrypanosomal [4, 5] and anticancer agents [6–8].

Among these compounds, amsacrine (m-AMSA) has shown high antileukaemic activity [9]. This was attributed later to topoisomerase II-mediated cleavage of double-stranded DNA by a mechanism which appears to be common to DNA-intercalating agents [10, 11], and which has now become a clinically useful drug [12, 13]. In the field of chemotherapeutics, the further development of m-AMSA as lead compound has yielded several subsets of many analogues, which permit consistent SAR and QSAR findings [14–17]. Aza analogues were prepared as non-classical bioisosters of tumour inhibiting electro-deficient nitro-substituted 9-(2′-methoxy-4′-methansolfonamido-anilino)-acridines, and some isomeric benzo-naphthyridine derivatives showed antileukaemic activities comparable to those of the parent compound, but with lower potency [18, 19].

As part of our program on polyetherocyclic compounds showing anti-topoisomerase activity, we were interested in preparing new aza-analogues with the aim of evaluating the influence of a substituted tricyclic ring on their biological activity. As a first approach, we planned the compound having the benzo[b] [1, 5]naphthyridine bi-substituted nucleus, as in the antimalarial azacridine [20], and the same side chain (o-methoxy-p-methansolfonamido-aniline) as m-AMSA.

In the present work we describe the synthesis and results of some preliminary in vitro and in vivo biological tests of both the novel N-isosteric compound and the known corresponding m-AMSA analogous derivative taken as a comparison [16]. At the same time we were also interested in testing the available corresponding 9-chloro- and 9-phenoxy- intermediates with the aim of gaining a greater knowledge of their possible biological activity than has been found in the literature [21].

* Correspondence and reprints: bordin@dsfarm.unipd.it

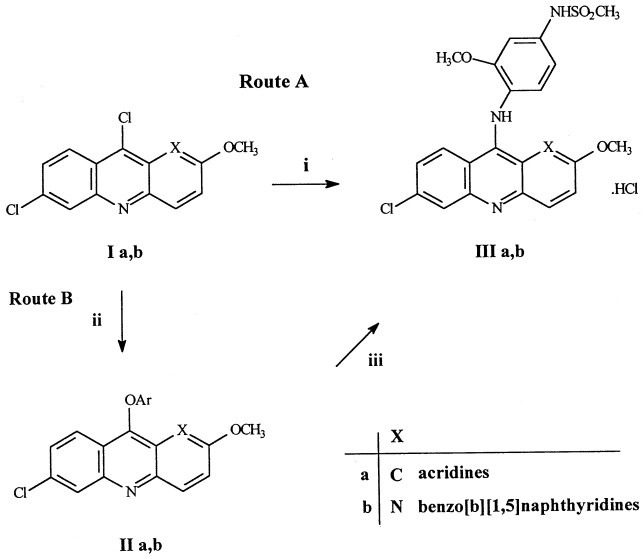

Figure 1. Reagents and conditions: A) Direct method, i: absolute alcohol, $NH_2Ar(o\text{-}OCH_3)(p\text{-}NHSO_2CH_3)$, reflux; B) Indirect method, ii: phenol, NaOH, 120–130 °C; iii: phenol, $NH_2Ar(o\text{-}OCH_3)(p\text{-}NHSO_2CH_3)$. HCl, 130–140 °C.

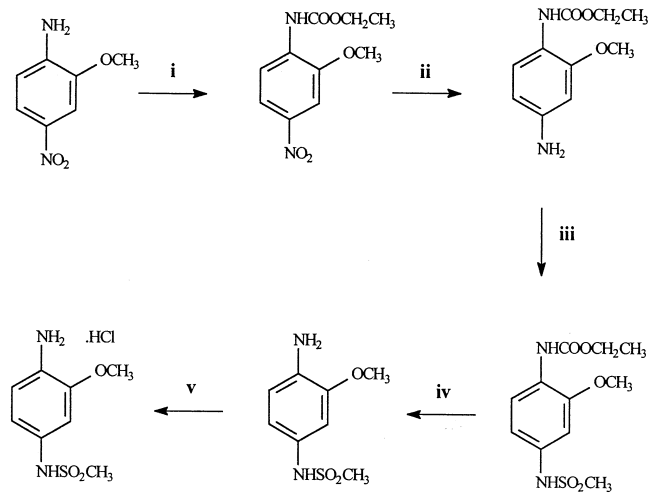

Figure 2. Reagents and conditions: i: $ClCOOCH_2CH_3$, THF, reflux; ii: H_2, Pd/C; ii: CH_3SO_2Cl, pyridine; iv: NaOH 20%, reflux; v: HCl, absolute ethanol.

2. Chemistry

Compounds **IIIa,b** were prepared starting from commercial 2-methoxy-6,9-dichloro-derivatives **Ia,b** following classical synthetic procedures: routes A and B shown in *figure 1*. Acridine derivatives have already been described [16]. Direct nucleophylic substitution of the chlorine in the 9 position of both acridine and azacridine rings by aniline derivative afforded desired chlorides **IIIa,b** in good yields (route A).

Indirectly, (route B) compounds **IIIa,b** were also obtained, but in higher yields than the above by the reaction between phenoxy-derivatives **IIa,b** and o-methoxy-p-methansolfonamido-aniline chloride in phenol.

Intermediate phenoxy-derivatives were prepared from **Ia,b** by heating with phenate, isolated and purified [22].

Figure 2 describes the known pathway to o-methoxy-p-methansolfonamido-aniline in which the original amine protecting acetyl group [8] is replaced by an ethyl-formate group. This replacement was found to be particularly efficient in the cases of both protection and deprotection because of the mild reaction conditions applied, which produced higher yields. The starting p-nitro-m-methoxy-aniline was easily transformed in urethanic derivative and after carrying out catalytic reduction and mesylation of the amine group, basic hydrolysis of the ethyl-carbamate protecting group finally produced the target compound in good yields.

3. Biology

9-Substituted acridine and azacridine (m-AMSA analogues) were investigated for their affinity to DNA in vitro. They were submitted to screening for antiproliferative activity and DNA damage to mammalian cells. Antitumour activity was also tested in solid-tumour-bearing mice. In all biological studies, m-AMSA was used as reference compound.

4. Results

4.1. Interaction with DNA in vitro

Spectophotometric analysis of the interactions between drug and DNA revealed several isosbestic points, thus suggesting only one mode of binding (data not shown). Unfortunately the changes in absorption spectra of the free and DNA-bound compounds were too weak to permit correct determinations of DNA binding by UV spectroscopy. As m-AMSA-analogues showed high emission intensities in the characteristic acridine region (400–500 nm) of the UV spectra, we assessed their ability to bind to nucleic acids by fluorescence titrations, as described by Gimenez-Arnau et al. [23]. *Figure 3* reports the quenching of fluorescence of tested compounds at

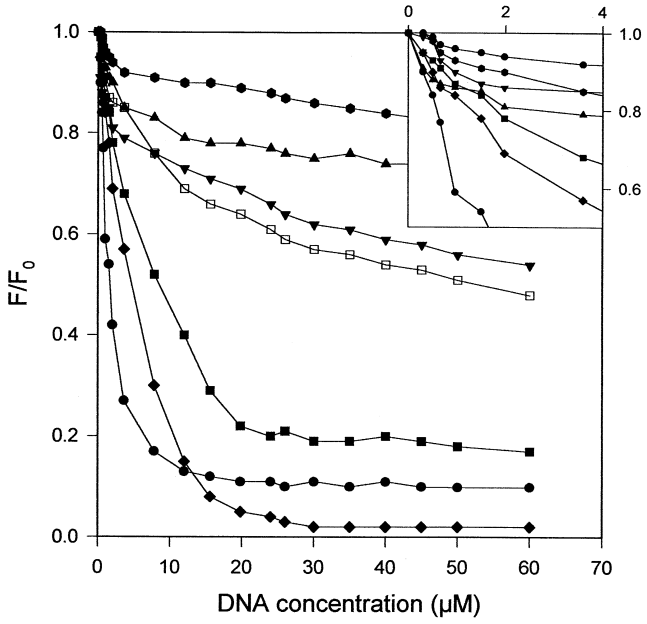

Figure 3. Fluorescence of m-AMSA analogues as a function of calf thymus DNA concentration. F/F_0 is the relative fluorescence, where F is the fluorescence intensity of the ligand quenched with DNA and F_0 is the fluorescence intensity of free ligand (see materials and methods). The symbols are: **Ia** (▲), **Ib** (◆), **IIa** (●), **IIb** (■), **IIIa** (▼), **IIIb** (●), m-AMSA (□) was used as reference.

constant concentrations with increasing concentrations of DNA. The observed fluorescence intensities were affected differently by the presence of calf thymus DNA. However, the fluorescence of each derivative promptly decreases on addition of DNA up to a certain ratio between drug and DNA, and is followed by stabilisation of fluorescence at higher DNA/drug ratios. The evident quenching of fluorescence in the case of compounds **Ib**, **IIb** and **IIIb** suggests that aza-acridines are strong DNA-binding ligands showing DNA affinities significantly greater than those of m-AMSA. The 9-anilinoacridine derivative **IIIa**, with the same side chain of m-AMSA shows an affinity for binding with DNA very similar to that of m-AMSA. In the same order of magnitude were the affinities between DNA and acridine **Ia** or phenoxyacridine **IIa**, but their fluorescences were also quenched less in the presence of high amounts of DNA.

4.2. Cytotoxic activity

m-AMSA analogues were examined for their cytotoxic properties. The dye exclusion test was performed on human leukaemia HL-60 cells and the results from this set of experiments are summarised in terms of IC_{50} values in table I. For the purposes of comparison, the cytotoxicity of m-AMSA was evaluated under the same conditions. Both derivatives **Ia** and **Ib**, lacking the 9-aromatic substituent, were poorly effective. Of the 9-phenoxy derivatives, only **IIb** exhibited a significant effect while **IIa** was much less cytotoxic.

Table I. Detection of cell lethality.

Compound	Dye exclusion HL-60 cells[a] $IC_{50} \pm SE$ (μM)	Colony formation HeLa cells[b] $IC_{50} \pm SE$ (μM)	[^{3}H]TdR uptake Ehrlich cells[c] $IC_{50} \pm SE$ (μM)
Ia	34.9 ± 0.09	3.8 ± 0.11	37.66 ± 0.54
Ib	22.7 ± 0.10	2.44 ± 0.04	21.80 ± 0.33
IIa	18.5 ± 0.12	2.7 ± 0.17	33.49 ± 0.29
IIb	5.1 ± 0.17	0.9 ± 0.07	13.38 ± 0.21
IIIa	1.43 ± 0.14	0.52 ± 0.01	12.4 ± 1.67
IIIb	1.14 ± 0.08	0.49 ± 0.03	8.77 ± 0.10
m-AMSA	1.15 ± 0.01	0.75 ± 0.06	10.5 ± 0.22

IC_{50} values were determined by probit analysis. Linearity of the thus computed regressions was checked by the χ^2 test. ($P = 0.05$). SE = standard error. [a] Cell viability was determined by Trypan blue dye exclusion in HL-60 cells (1×10^6.mL^{-1}) after 12 h incubation in the presence of increasing concentrations of m-AMSA analogues. [b] Clonal growth capacity of HeLa cells cultivated in vitro was evaluated after 3 h incubation in the presence of different concentrations of m-AMSA analogues. [c] Ehrlich cells were incubated with tritiated thymidine and exposed to increasing concentrations of m-AMSA analogues for 1 h. Acid insoluble radioactivity was then determined. Each experimental condition was checked in at least triplicate samples.

Methanesulfonamidoaniline derivatives **IIIa** and **IIIb** appeared to be the most potent derivatives with IC_{50} values ranging from 0.1–0.4 μM very similar to those of m-AMSA.

Similar findings were observed measuring the inhibition of [^{3}H]TdR incorporation in mouse ascite cells treated with increasing concentrations of tested m-AMSA analogues. The IC_{50} values are reported in *table I.*

Compounds **Ib**, **Ia** and **IIa** induced a moderate inhibition of DNA synthesis. An evident increase in activity was obtained with derivatives **IIb**, **IIIa** and **IIIb** which are more, or at least as effective, as amsacrine.

The clonogenic assay was used to determine human adenocarcinoma HeLa cell survival following exposure to various concentrations of tested compounds. The drug inhibitory effect on cell proliferation was concentration dependent (data not shown). *Table I* shows the results obtained in terms of IC_{50} values. Again, compounds **IIb**, **IIIa** and **IIIb** were the most potent derivatives, provoking a strong inhibition of colony forming ability.

Table II. Detection of DNA damage in HeLa cells.

Treatment	Cross-linking Coefficient ± SE[a]	Elution rate ($\times 10^2 \pm$ SE[b])
Control	1	1.55 ± 0.15
IIb	1.59 ± 0.26	1.89 ± 0.16
IIIa	2.09 ± 0.22	2.53 ± 0.18
IIIb	2.83 ± 0.19	3.26 ± 0.20
m-AMSA	2.42 ± 0.21	2.88 ± 0.23

[a] Hela cells were incubated for 3 h in the presence of m-AMSA analogues (10 μM) and then processed for potassium–SDS precipitation assay [24]. The cross-linking coefficient is the ratio between the fraction of DNA precipitated together with proteins for treated and control samples. [b] HeLa cells, treated for 3 h with m-AMSA analogues were then submitted to neutral elution [25]. Elution rate values were calculated as described in Experimental protocols. Statistical evaluations were carried out by the F test of variance ($P < 0.01$ compared with control).

Noticeably, the IC_{50} values of both compounds **IIIa** and **IIIb** were significantly lower than those of the reference compound, m-AMSA.

4.3. Detection of DNA damage

4.3.1. DNA–protein cross-links

HeLa cells labelled with ^{3}H-thymidine were incubated for 3 h with the analogues of amsacrine (10 μM) and then processed for the protein precipitation assay as already described [24]. *Table II* shows the data concerning the three most potent cytotoxic m-AMSA analogues (**IIb**, **IIIa** and **IIIb**). m-AMSA was tested in the same experimental conditions as reference. Treatment with all tested compounds significantly increased the amount of ^{3}H-DNA precipitated together with proteins in comparison with untreated controls. The cross-linking coefficients were all similar to those of *m*-AMSA and for compound **IIIb** the value was even slightly higher. Dose–response relationships were observed for the formation of DPCs after exposure to the tested compounds (data not shown).

4.3.2. Double-strand breaks

The induction of double-strand breaks (DSBs) into DNA of HeLa cells was assayed by means of neutral elution. The rate of elution depends on the molecular size of polynucleotide chains. Undamaged double helix DNA eluted slowly and when DSBs were introduced into DNA elution, kinetics increased [25]. *Table II* summarises the results when performed with the three most potent cytotoxic m-AMSA analogues (**IIb**, **IIIa** and **IIIb**). Also in these experiments, m-AMSA was used as reference. Treatment of cells for 3 h in complete growth medium in the presence of each agent (15 μM) yielded a substantial number of breaks and the frequency of DSB clearly correlated to cytotoxic potency. Moreover, most disappeared within 30 min after drug removal (data not shown). This phenomenon has been previously described with topoisomerase inhibitors [26].

4.4. Inhibition of topoisomerase II activity

The three most potent cytotoxic m-AMSA analogues, i.e. derivatives **IIb**, **IIIa** and **IIIb** were assayed for their ability to inhibit the activity of topoisomerase II. We performed the relaxation test of supercoiled DNA of PM2 phage and electrophoresis on agarose gel. In this cell-free assay, 0.125 μg of supercoiled DNA was incubated with increasing concentrations (from 10–160 μM) of drug plus purified topoisomerase II from *Drosophila melanogaster* embryos. *Figure 4* reports results from an electrophoresis gel where each of the three m-AMSA analogues was tested at a lower concentration (10 μM).

Lane 1 shows the electrophoretic pattern of PM2 DNA, where the two bands correspond to the relaxed (slower) and supercoiled (faster) forms, respectively. Lane 2 shows the effect of topoisomerase II activity, where the supercoiled form completely disappeared. Lanes 3–5 show the inhibition of topoisomerase II induced by compounds **IIb**, **IIIa** and **IIIb**, respectively. There was a similar inhibition of topoisomerase II activity by each of the three derivatives since the bands corresponding to the supercoiled form of PM2 DNA were comparable to those of the control sample.

The figure also displays the migration pattern obtained from experiments with m-AMSA (10 μM), used in the same experimental conditions as a reference (lane 6).

4.5. Antitumour activity

The antitumour activity of m-AMSA analogues against solid tumour was studied in Ehrlich carcinoma-bearing mice. We only tested the derivatives that exhibited better antiproliferative activity against cell lines in culture, i.e. derivatives **IIb**, **IIIa** and **IIIb**, amsacrine was used as reference compound. The results of these in vivo experiments are reported in *table III*. Chemotherapy of solid-tumour-bearing mice with 10 $mg.kg^{-1}$ of derivatives **IIb**, **IIIa** and **IIIb** resulted in significant tumour volume reduction. In particular compound **IIIb** appeared approximately as potent as amsacrine, inducing about 40% inhibition in tumour growth. However, chemotherapy with amsacrine appeared more toxic toward the host, killing one third of treated animals, while mice inoculated with compound **IIIb** rapidly recovered lost body weight.

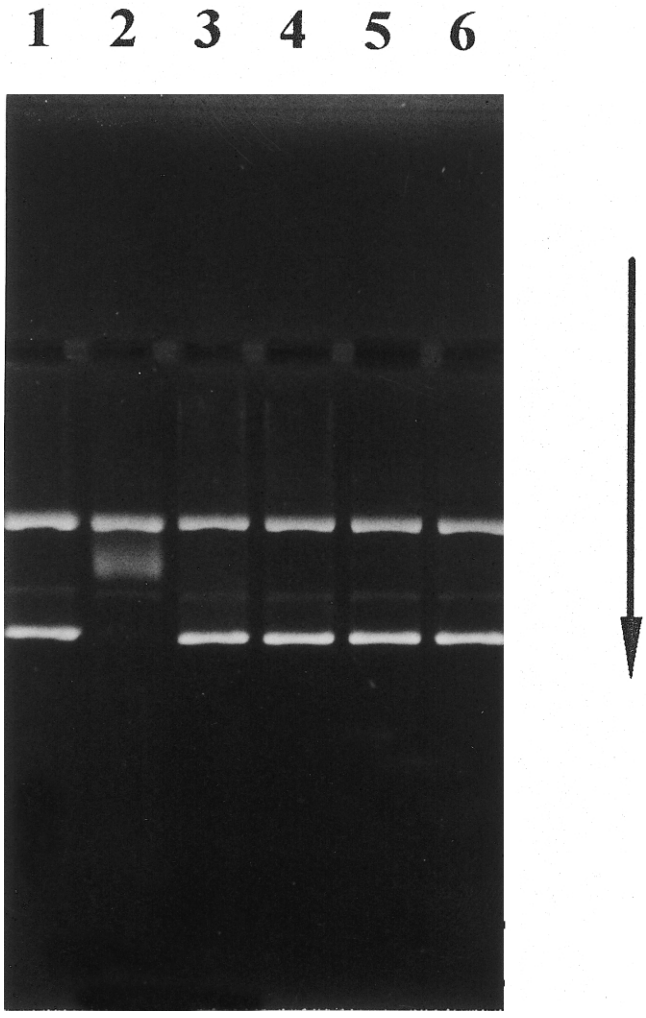

Figure 4. Inhibition of topoisomerase II activity studied by the relaxation assay. PM2 DNA was incubated in the presence of topoisomerase II and different concentrations of m-AMSA analogues. Agarose gel electrophoresis of the samples is shown. Lane 1: PM2 DNA alone, which migrates as two bands; lane 2: PM2 DNA incubated in the presence of topoisomerase II, showing only the relaxed form (the slower band); lane 3–5: PM2 DNA incubated in the presence of topoisomerase II and 10 μM compounds **IIb**, **IIIa** and **IIIb**, respectively. Lane 6: PM2 DNA incubated in the presence of topoisomerase II and m-AMSA used as reference (10 μM). The arrow indicates gel mobility.

5. Discussion

A large number of the 9-anilinoacridine class of antitumour drugs have been prepared and tested for in vivo activity [7–9]. Among them, some nitro m-AMSA congeners have shown good tumour inhibitory activity

Table III. Antitumour activity against Ehrlich carcinoma*.

Compound	Average tumour weight (g ± SE)	Inhibition of tumour growth (% ± SE)
Control	0.097 ± 0.013	
IIb	0.067 ± 0.02[a]	30.48 ± 9.1
IIIa	0.064 ± 0.020[b]	34.16 ± 10.6
IIIb	0.059 ± 0.013[a]	38.87 ± 8.5
m-AMSA	0.043 ± 0.01[a]	45.52 ± 10.6

* Mice bearing Ehrlich carcinoma were treated intraperitoneally with 10 $mg.kg^{-1}$ m-AMSA analogues as described in Experimental protocols. Average weight in grams was measured on day 7. Statistical evaluations were performed by F test of variance. SE = standard error. [a] $P < 0.01$. [b] $P < 0.1$.

[7, 18, 19]. Because of the similar electronic effects of both a ring nitrogen and a nitro substituent, the corresponding aza-bioisosters were also synthesised and studied, proving effective [7], even if the presence of a –N= function did not always provide an improvement in their biological activity [18]. As part of our program on polyetherocyclic compounds showing antitopoisomerase activity, we planned the synthesis of an m-AMSA aza-analogue having bisubstituted the tricyclic nucleus (compound **IIIb**). As synthetic intermediate we obtained the corresponding phenoxy-derivative (**IIb**). With the aim of investigating the effect of this 1-azaisosterism in the 2-methoxy-6-chloro-acridinic nucleus, we studied the biological activity of aza analogues **Ib**, **IIb** and **IIIb** compared with the corresponding acridinic derivatives (compounds **Ia**, **IIa** and **IIIa**). All studies were performed considering m-AMSA as reference compound.

Studying DNA binding in vitro of the tested compounds by fluorescence titrations we observed that acridine derivatives showed a fluorescence behaviour very similar to that of m-AMSA; conversely, the 1-aza-acridines exhibited a strong binding affinity towards DNA. Their DNA-binding properties are essentially similar, in spite of the noticeable differences in the nature of the 9-substituent. The presence of two hydrophobic groups in the acridine skeleton, like in the 6,9-dichlorosubstituted aza-analogue **Ib**, highly enhances the affinity of the tricyclic ring toward DNA.

Various in vitro methods were used to evaluate cellular damage following exposure to the tested AMSA-analogues. The dye exclusion test was carried out as an indicator of cell membrane integrity, while the incorporation of radioactive DNA precursors was an index correlating inhibition of DNA synthesis with cell death after drug exposure. Finally, as these kinds of biological tests evaluate metabolic arrest, a clonogenic assay was carried out to assess reproductive cellular death. The

Table IV. Analysis of compounds **Ia–IIIb**.

Compound	Formula	Analysis[a]	pK_a [b]
Ia	$C_{14}H_9NOCl_2$	C, H, N, Cl	6.02
Ib	$C_{13}H_8N_2OCl_2$	C, H, N, Cl	7.55
Iia	$C_{20}H_{14}NO_2Cl$	C, H, N, Cl	5.54
Iib	$C_{19}H_{13}N_2O_2Cl$	C, H, N, Cl	5.88
IIIa	$C_{22}H_{20}N_3O_4Cl$ S·HCl	C, H, N, Cl, S	5.75
IIIb	$C_{21}H_{19}N_4O_4Cl$ S·HCl	C, H, N, Cl, S	4.51

[a] Analyses for elements indicated were within ± 0.4% of the theoretical figures for the formula quoted. [b] Ionisation constants were measured as detailed in ref. [23]. pK_a of m-AMSA = 7.19 [23].

results suggested that compounds **IIb**, **IIIa** and **IIIb** were the most potent derivatives, provoking strong antiproliferative effects. In particular, for compounds **IIIa** and **IIIb** the IC_{50} values were similar or significantly lower than those of the reference compound, m-AMSA. These results, combined with those regarding DNA affinity, show that a correlation between DNA–drug binding and drug antiproliferative activity could not be detected. In fact, compound **Ib**, which appears as a strong DNA-binding ligand, was poorly effective in all biological tests. Moreover, it is well-known that m-AMSA forms weak DNA intercalation complexes [21].

Previous studies have suggested that pK_a values for substituted acridines are an important determinant of activity [16]. In particular it has been pointed out that derivatives with high pK_a values do not provide antiproliferative activity [18]. It has also been seen that the introduction of a 1-aza function in the acridinic nucleus provides very little base weakening effect [18]. On the basis of pK_a values detected (*table IV*), we could suppose that the neighbouring presence of the 1-aza function and the 2-methoxylic group in the pyridinic ring, as in derivative **IIIb**, lowers the pK_a values, presumably due to the ready formation of a resonant cation stabilising the charge. It has usually been considered that the cation of chemotherapeutic acridines provides observed biological activity, thus more highly ionised acridines normally prove more effective.

To investigate the mechanism of action of the new derivatives, we studied the damage induced in DNA in HeLa cells in vivo. Using the potassium–SDS protein precipitation assay, we observed that derivatives **IIb**, **IIIa** and **IIIb** are capable of forming covalent DPC, similarly to m-AMSA. Neutral elution studies have given evidence that derivatives **IIb**, **IIIa** and **IIIb** induce DSB to an equivalent extent. However, DSB disappeared quickly after drug removal. This phenomenon has been previously described for topoisomerase II inhibitors acting in mammalian cells where DNA breaks re-seal with a mismatch of the DNA strands after drug removal [26]. We found a strong correlation between the ability of these m-AMSA analogues to induce topoisomerase II-mediated strand breaks in intact HeLa cells and cytotoxicity, thus suggesting that DNA topoisomerase is the intracellular target for these compounds.

Performing some experiments in vitro with topoisomerase II from embryos of *Drosophila melanogaster*, we observed that derivatives **IIb**, **IIIa** and **IIIb** inhibit the activity of this enzyme at very low concentrations. Keeping in mind the results regarding the formation of non-covalent complexes with calf thymus DNA, the data obtained on purified enzyme suggest that, for this class of compounds, a relationship between the capacity to inhibit topoisomerase activity and DNA binding properties does not exist.

All the data regarding DNA damage suggest that the side chain on the 9 position of the acridine skeleton is essential for biological activity with little difference between the anilino and phenoxy rings. In all probability, these compounds also bind by intercalation of the acridine or aza-acridine chromophore between the base pairs with the side chain lodging in the minor groove and may interact with enzymes such as topoisomerase as already described for the acridinylamino-sulfonanilides [27].

Finally, to detect the antitumour properties of m-AMSA analogues, we tested them against a solid tumour in mice, and then compared with those of m-AMSA. Our data show that compound **IIIb** was similarly effective against murine tumour as m-AMSA, but was much less toxic toward the host, and this feature is regarded as extremely important among potential antitumour drugs.

6. Experimental protocols

6.1. Chemistry

Melting points were determined on a Gallenkamp MFB 595010M/B capillary melting point apparatus and are not corrected. Infrared spectra were recorded on a Perkin-Elmer 1760 FTIR spectrometer as potassium bromide pressed disks. Values are expressed in cm^{-1}. ^{1}H-NMR spectra were recorded on a Varian Gemini apparatus (200 MHz) using the indicated solvents: chemical shifts are reported in δ (ppm) downfield from tetramethylsilane as internal reference. *J* values are given in hertz (Hz). In the case of multiplets, the chemical shift quoted is measured from the approximate centre. Integrals corresponded satisfactorily to those expected on the basis of compound structure. Elemental analyses were performed in the Microanalytical Laboratory of the Department of

Pharmaceutical Sciences of the University of Padova, using a Perkin-Elmer Elemental Analyser Model 240B. Results fell within the range ± 0.4% with respect to calculated values. Column flash chromatography was carried out on Merck silica gel (250–400 mesh ASTM) and reactions were monitored by analytical thin-layer chromatography (TLC) using Merck silica gel 60 F-254 glass plates. Solutions were concentrated in a rotary evaporator under reduced pressure. Starting compounds **Ia,b** and m-methoxy-p-nitro-aniline were purchased from Aldrich Chimica and Janssen Chimica (now Acros), respectively.

6.1.1. General procedure
for the direct synthesis of anilino derivatives: route A

A solution obtained dissolving equimolar amounts of the starting tricyclic compound and o-methoxy-p-methansolfonamido-aniline in 80–150 mL of absolute ethanol, was refluxed for 2.5–3 h until starting materials disappeared (TLC, ethylacetate/n-hexane 7:3). On standing and cooling a red–orange precipitate formed which was filtered and dried (40–50% yields). Purification by repeated crystallisation afforded pure chlorides.

6.1.1.1. 2-Methoxy-6-chloro-9-(2′-methoxy-4′-methansolfonamido-aniline)-acridine chloride **IIIa**

Prepared from compound **Ia** following the general procedure A as red microcrystals. Yield 50%. M.p. 307–309 °C dec. (absolute ethanol) lit. [16] 300–303 °C. R.f. 0.56. ^{1}H-NMR (CD_3OD) δ: 3.09 (s, 3H, CH_3), 3.61 (s, 3H, 3′-OCH_3), 3.72 (s, 3H, 2-OCH_3), 7.05 (m, 2H, HC-3′ and HC-5′), 7.42 (dd, 1H, *J* = 2.2 and 9.5 Hz, HC-3), 7.49 (d, 1H, *J* = 8.9 Hz, HC-6′), 7.56 (d, 1H, *J* = 2.5 Hz, HC-1), 7.68 (dd, 1H, *J* = 2.6 and 9.3 Hz, HC-7), 7.83 (d, 1H, *J* = 9.7 Hz, HC-4), 7.88 (d, 1H, 1.6 Hz, HC-5), 8.19 (d, 1H, *J* = 9.6 Hz, HC-8).

6.1.1.2. 2-Methoxy-7-chloro-10-(2′-methoxy-4′-methansolfonamido-aniline)-benzo[b] [1, 5]naphthyridine chloride **IIIb**

Prepared from compound **Ib** following the general procedure A as orange crystals. Yield 40%. M.p. 270–273 °C (absolute ethanol). R.f. 0.61. ^{1}H-NMR (CD_3OD), δ: 3.09 (s, 3H, CH_3), 3.66 (s, 3H, 2-OCH_3), 4.01 (s, 3H, 3′-OCH_3), 6.03 (dd, 1H, *J* = 2.4 and 8.3 Hz, HC-5′), 7.08 (d, 1H, *J* = 2.2 Hz, HC-3′), 7.38 (dd, 1H, *J* = 2.1 and 9.5 Hz, HC-9), 7.49 (d, 1H, *J* = 8.5 Hz, HC-6′), 7.54 (d, 1H, *J* = 9.2 Hz, HC-3), 7.9 (m, 2H, HC-2′ and HC-8), 8.19 (d, 1H, *J* = 9.2 Hz, HC-4).

6.1.2. General procedure
for the indirect synthesis of anilino-derivatives: route B

6.1.2.1. Preparation of phenoxy derivatives

A solution of phenol and NaOH was stirred at 120–130 °C for 1 h. The methoxy-chloro-substituted starting compound was then added and the reaction mixture was stirred at the above temperature for about 2 h. After incomplete cooling, the melted mixture was poured into a vigorously stirred 2 N aqueous solution of NaOH to give a yellow suspension which was collected and dried. Purification by crystallisation followed.

6.1.2.2. 2-Methoxy-6-chloro-9-phenoxy-acridine **IIa**

Prepared from **Ia** in good yields. R.f. 0.92 (ethylacetate/n-hexane 1:1). ^{1}H-NMR (CD_3OD) δ: 3.61 (s, 3H, OCH_3), 6.87 (m, 3H), 7.07 (m, 1H), 7.18 (d, 1H, *J* = 2.9 Hz, HC-1), 7.32 (m, 2H), 7.5 (m, 2H), 8.07 (m, 3H), 8.14 (d, 1H, *J* = 1.6 Hz, HC-5).

6.1.2.3. 2-Methoxy-7-chloro-10-phenoxy-benzo[b] [1, 5]naphthyridine **IIb**

Prepared from compound **Ib** as yellow powder. Yield 83%. M.p. 185–187 °C (benzene/petroleum ether 40–60 °C). R.f. 0.92 (ethylacetate/ n-hexane 1:1). ^{1}H-NMR (CD_3OD) δ: 3.52 (s, 3H, OCH_3), 6.99 (m, 2H), 7.05 (d, 1H, *J* = 10 Hz, HC-3), 7.31 (m, 2H), 7.38 (d, 1H, *J* = 3.3 Hz), 7.69 (dd, 1H, *J* = 2.2 and 10 Hz), 8.27 (d, 1H, *J* = 1.9 Hz), 8.32 (d, 1H, *J* = 9.2 Hz), 8.39 (d, 1H, *J* = 9.4 Hz).

6.1.2.4. Preparation of 9-aniline-derivatives

A mixture of phenoxy derivative and phenol was melted by heating at 80 °C, and then a stoichiometric amount of 2-methoxy-4-methansolfonamido-aniline chloride was added. Gradually the temperature of the reaction mixture was then raised to 130–140 °C and maintained for about 3 h. After incomplete cooling the melted mixture was poured into vigorously stirred anhydrous ether giving a flaky dark red suspension. After standing and cooling at –20 °C for 2 days a microcrystalline product separated which was collected and dried (55–65%). This raw material was crystallised from absolute ethanol to afford pure chlorides **IIIa,b**.

6.1.3. N-(2-Methoxy-4-nitro-benzen)-ethylcarbamate

1.4 mL (12 mM) of ethylchloroformiate was added to a solution of commercial 2-methoxy-4-nitro-aniline (2 g, 12 mM) in 50 mL dry THF and the mixture was refluxed until the starting compound disappeared at TLC analysis (ethylacetate/n-hexane 7:3). The yellow solution was evaporated to dryness giving a crystalline yellow product (95% yield). $C_9H_{12}O_5N_2$. M.p. 154–156 °C. R.f. 0.45

(n-hexane/ethylacetate 5:5). ^{1}H-NMR (deutero-acetone) δ: 1.29 (t, 3H, J = 7.1 Hz, CH_3), 4.06 (s, 3H, OCH_3), 4.23 (q, 2H, J = 7.1 Hz, CH_2), 7.82 (d, 1H, $J_{3,5}$ = 2.6 Hz, HC-3), 7.92 (dd, 1H, $J_{5,3}$ = 2.6 and $J_{5,6}$ = 9.2 Hz, HC-5), 8.21 (sa, 1H, NH), 8.31 (d, 1H, $J_{6,5}$ = 9.2 Hz, HC-6).

6.1.3.1. N-(2-Methoxy-4-amino-benzen)-ethylcarbamate

Easily air oxidable intermediate compound. Yield 90%. R.f. 0.6 (n-hexane/ethylacetate 5:5).

6.1.3.2. N-(2-Methoxy-4-methansolfonamido-benzen)-ethylcarbamate

$C_{11}H_{16}N_2O_5$. Yield 75%. M.p. 139–141 °C. R.f. 0.75 (ethylacetate/n-hexane 7:3). ^{1}H-NMR (deutero-acetone) δ: 1.26 (t, 3H, J = 7 Hz, CH_3), 2.96 (s, 3H, SO_2CH_3), 3.88 (s, 3H, OCH_3), 4.16 (q, 2H, J = 7 Hz, CH_2), 6.9 (dd, 1H, $J_{5,3}$ = 2.5 Hz and $J_{5,6}$ = 8.7 Hz, HC-5), 7.04 (d, 1H, $J_{3,5}$ = 2.5 Hz, HC-3), 7.58 (sa, 1H, uretanic NH), 7.94 (d, 1H, $J_{6,5}$ = 8.6 Hz, HC-6), 8.4 (sa, 1H, amidic NH).

6.1.3.3. 2-Methoxy-4-methansolfonamido-aniline

A solution of the uretanic derivative in aqueous 20% NaOH was refluxed for a useful period of time (TLC). After cooling at room temperature the reaction mixture was extracted with diethylic ether and the organic layer was separated, dried over Na_2SO_4, and evaporated in vacuo to dryness to give the aniline compound [8] in 94% yield. Crystallisation from ethanol afforded pure compound.

6.1.3.4. 2-Methoxy-4-methansolfonamido-aniline chloride

An ethanolic solution of aniline derivative was saturated with HCl dry gas and after a night at –15 °C, a crystalline yellow product was filtered and dried. $C_8H_{12}O_3N_2S$.HCl.

6.2. Biological methods

6.2.1. Chemicals

Calf thymus DNA (Cat. D 1501) and tetrapropylammonium hydroxide (1 M aqueous solution) were obtained from Sigma Chemical Co, St Louis, MO, USA.

Compounds were dissolved in dimethyl sulfoxide (DMSO) (4.5 mM) and the solutions were stored at –20 °C. Just before the experiments, a calculated amount of drug solution was added to phosphate buffer saline (PBS) or to the growth medium to a final solvent concentration of 0.5%, which had no discernible effect on cell killing. ^{3}H-Thymidine (4.77 Tbq.mM^{-1}) was obtained from Amersham International Inc., UK. Proteinase K was obtained from Boehringer Mannheim GmbH (Germany).

6.2.2. Interaction with DNA in vitro

6.2.2.1. UV determinations

Absorption spectra were recorded using a Kontron UVIKON-930 UV-Visible spectrophotometer. Increasing micro-volumes of a DNA solution (1 mg.mL^{-1}), having the same above-mentioned drug concentrations, were added to a fixed volume of an aqueous drug solution ($3.0–5.0 \times 10^{-5}$ M). The additions were performed up to a high DNA/drug ratio. An equal amount of DNA was also added to the reference cell.

6.2.2.2. Fluorescence determinations

The formation of a molecular complex with DNA was investigated by fluorimetric measurements according to Gimenez-Arnau et al. [23]. The experiments were carried out by means of a spectrophotofluorimeter, Kontron Instruments model SFM-25. Molar concentrations of aqueous solutions of calf thymus DNA were determined spectrophotometrically using 6600 as extinction coefficient (M^{-1}.cm^{-1}) at 260 nm and expressed in base pairs. Fluorescence emission spectra were performed using an excitation wavelength of 380 nm and fluorescence intensity values were recorded at the emission maximum of the tested compounds. Spectra were obtained by adding an aqueous drug solution ($3.0–5.0 \times 10^{-6}$ M), with micro-volumes of a DNA solution (1 mg.mL^{-1}) at the same drug concentration. During the titrations we recorded the values of F, the fluorescence intensity of the ligand quenched by DNA, and F_0, the fluorescence intensity of free ligand. We then calculated F/F_0 as relative fluorescence.

6.2.3. Cell cultures

HeLa cells (kindly provided by Prof. F. Majone, Dept. of Biology of Padua University, Italy) were grown as monolayers in Nutrient Mixture F12 Ham medium (Sigma Chemical Co.) supplemented with 10% foetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel). Trypsin (0.25%, Boehringer Mannheim) was routinely used for subcultures.

HL-60 cells were grown in RPMI-1640 medium (Whittaker Bioproduct, Walkersville, MD, USA) containing 5% foetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel), supplemented with 25 mM HEPES buffer and l-glutamine. Both HeLa and HL60 media were supplemented with antibiotics, penicillin (50 units.mL^{-1}) and streptomycin (50 μg.mL^{-1}), and cell growth was accomplished at 37 °C in a 5% carbon dioxide atmosphere.

6.2.3.1. Clonogenic survival

1.5–2 × 10^5 HeLa cells were seeded in 60 mm Petri dishes in growth medium (4 mL). After 24 h the medium was removed and replaced with a fresh one containing the compound to be studied at the appropriate concentrations. Cells were then incubated for 3 h. Triplicate cultures were established for each treatment. The dishes were then washed with PBS and 200 cells from each treated and untreated culture were then seeded in complete growth medium. After 7 days incubation colonies were stained and counted, discarding colonies with less than 50 cells. The efficiency of clonal growth, that is the ratio between the number of colonies formed and the number of cells seeded, was then calculated and used to normalise the cytotoxicity induced by the drugs. In each experiment, triplicate samples of untreated cells were plated as controls and plating efficiency ranged from 88–92%.

6.2.3.2. Non-clonogenic assay

Cytotoxicity against human leukaemia HL-60 cells was studied using the trypan blue dye exclusion test [28]. Cells at a concentration of 2×10^5.mL^{-1} were incubated for 3 h in the presence of different concentrations of the compound to be tested. Cells were then incubated for 4 min with 0.25% trypan blue (Sigma Chemical Co, St Louis, MO, USA) and 5% foetal calf serum. Viable cells were identified by their ability to exclude dye, whereas the dye diffuses into non-viable cells. At least 100 cells were counted for each experimental point recorded. The controls were triplicate samples of untreated cells and their viability ranged from 92–98%.

6.2.4. DNA synthesis in Ehrlich cells

Ehrlich ascite tumour cells (Lettrè strain, from Heidelberg) were routinely transferred by injecting intraperitoneally 2 × 10^6 cells per animal into NCL mice. The tumour cells, collected on the 6–7th day after transplant, were processed as already described [29].

2 × 10^7 cells.mL^{-1} in Hank's solution containing the compound to be studied were incubated at 37 °C for 60 min. ^{3}H-thymidine (40 KBq.mL^{-1}), in a small volume of the same medium, was then added and the cells were further incubated at 37 °C for 30 min. The acid-insoluble fraction was precipitated by adding 5% ice-cold trichloroacetic acid and then filtered through Whatman GF/C filters. After several washings with cold 1% trichloroacetic acid, the filters were dried and counted. The results were calculated as the percentage of radioactivity incorporated into the DNA of untreated control cells (about 3–6 MBq; quadruplicate samples). Filtrations were carried out by a Sample Manifold apparatus (Millipore Corp., Bedford, MA, USA).

6.2.5. DNA damage

6.2.5.1. Detection of double-strand breaks

DNA double-strand breaks (DSB) were detected by neutral elution carried out according to Kohn [25].

HeLa cells in exponential growth were labelled by overnight incubation in the presence of ^{3}H-thymidine (7.4 KBq.mL^{-1}). The radioactive medium was removed and replaced by a fresh one containing the compound to be studied or 0.5% dimethyl sulfoxide for the controls. In both cases, the cells were incubated for 3 h in the dark. The cells were then washed with PBS.

About 0.5–1.0 × 10^6 treated ^{3}H-cells were deposited on a polycarbonate filter (pores 2 μm in diameter, Nucleopore Corp. Pleasanton, CA, USA) in a Swinnex-25 filter holder (Millipore Corp. Bedford, MA, USA) and immediately lysed with 2% sodium dodecylsulfate (SDS), 0.1 M glycine, 0.025 M EDTA, pH 9.6, (5 mL). Thereafter, the solution was allowed to flow through by gravity. 2 mL of the same solution containing 0.5 mg.mL^{-1} of proteinase K was then gently poured onto the filter, followed by 40 mL of the eluting solution, (EDTA 0.02 M, SDS 0.1%, and tetrapropylammonium hydroxide pH 9.6). The elution was carried out by a Gilson Minipuls peristaltic pump, at a flow of 0.03–0.04 mL.min^{-1}. The fractions were collected with a Gilson fraction collector (approximately 3.5 mL per fraction) and the radioactivity in each fraction was then determined.

The results obtained with the neutral elution assay were expressed as K values according to the formula [30]:

$$K = \frac{V}{-\ln(r)}$$

where K is the average elution rate constant of DNA, r is the fraction of DNA retained on filter and V is the eluted volume. The formula reflects the assumption of a first-order kinetics for DNA elution, as a first approximation [25].

6.2.5.2. Potassium–SDS precipitation assay

Precipitation of DNA covalently associated with proteins was performed as described previously [24]. Briefly, HeLa cells in exponential growth were labelled by overnight incubation in the presence of ^{3}H-thymidine as described above for filter elution. Cells (1–2 × 10^6 cells.mL^{-1}, 0.1 mL aliquots) were incubated for 3 h in the dark with the tested compounds, washed twice with PBS and then lysed with 0.1 mL of SDS 2%, 1 mM EDTA, pH 7.5. The samples were mixed energetically for 10 s, warmed for 10 min at 60 °C. 0.5 mL of 200 mM KCl and 20 mM Tris-HCl, pH 7.5, were added and the mixtures were cooled in an ice-bath for 5 min. The precipitates

formed were collected at 4 °C by an Eppendorf centrifuge and suspended in 1 mL of 100 mM KCl. These mixtures were heated again and the above procedure repeated. Finally, the pellets were dissolved in 1 mL aliquots of water and the solutions and corresponding supernatants were counted. The results obtained with protein precipitation were expressed in terms of cross-linking coefficient (CC), a parameter which is proportional to the number of DNA–protein cross-links (DPC). It is defined as follows:

$$CC = \frac{F_t}{F_c}$$

where F_t and F_c represent the fractions of radioactivity precipitated together with proteins in the treated and control samples, respectively. Generally, in the control cells the total radioactivity of a sample was about 5–6 MBq and only 0.4–0.7% of this amount was recovered together with proteins in the precipitated fraction.

6.2.6. Inhibition of topoisomerase II activity

The inhibition of topoisomerase II activity was studied using a purified enzyme from *Drosophila melanogaster* embryos (USB, from Amersham Italia S.r.l.) [31]. Aliquots of 0.125 μg of PM2 DNA (from Boehringer Mannheim) were incubated for 15 min at 30 °C in the presence of two units of topoisomerase II (one unit is defined as the activity capable of relaxing 0.3 μg of super-coiled DNA) in the presence of reaction buffer (10 mM Tris-HCl, pH 7.9; 50 mM NaCl; 50 mM KCl; 5 mM $MgCl_2$; 0.1 mM EDTA; 15 $\mu g.mL^{-1}$ bovine serum albumin (BSA); 1 mM ATP). Aliquots of 2 μL of a DMSO solution containing tested compound (4.5 mM) were added in order to reach final concentrations of 10, 20, 40 and 160 μM. A suitable amount of reaction buffer was then added to each sample to reach the final volume of 20 μL. The reaction was blocked by adding EDTA 7 mM (3 μL) containing 0.77% of SDS. 2 μL of bromophenol blue containing 15% glycerol were added to the samples and electrophoresis was carried out on 0.7% agarose gel containing TAE (40 mM Tris-sodium acetate, pH 8.2, 1 mM EDTA) for 90 min. The gel was stained for 1 h in aqueous ethidium bromide 0.5 $\mu g.mL^{-1}$ and then photographed using a Polaroid camera.

6.2.7. Radiochemical determinations

Radioactivity measurements were performed using Ultima Gold (Packard Instruments, Meriden, CT, USA) as scintillation fluid. All determinations were carried out by a Packard Tri-Carb 1900 TR spectrometer. Isotope counting was accomplished automatically on the basis of quenching curves obtained using ^3H-radioactivity standards.

6.2.8. Antitumour activity

Antitumour tests were carried out as described [32] using groups of 10 Swiss mice (20 ± 3 g body weight) and Ehrlich carcinoma. The tumour was maintained by weekly transplantation into recipient mice. For the experiments, about 5×10^8 tumour cells were implanted subcutaneously in the right axillary region of mice. This transplant yielded the development of a tumour in the insertion place. Even 10 days after transplant, these tumours appeared to be solid and well delimited, necrosis and ascite free. The day after transplant, the drugs were administered i.p. every day ($10\ mg.kg^{-1}$), for 5 days. At day 7 after transplant the animals were sacrificed, the tumours removed and their weight determined. The control received the same amount of physiological solution.

6.2.9. Statistical evaluations

Cell lethality (dye exclusion, clonal growth and thymidine uptake) was assayed in the presence of increasing drug concentrations (triplicate samples). IC_{50} values were calculated by probit analysis using the χ^2 test to check the linearity of the thus obtained regressions.

Experimental data related to DNA damage in HeLa cells and antitumour activity against Ehrlich carcinoma were submitted to statistical analysis by the F test of variance.

Acknowledgements

Part of this work has been supported by MURST.

References

[1] Chavalitshewinkoo P., Wilairat P., Gamage S.A., Denny W.A., Figgitt D.P., Ralph R., Antimicrob. Agents Chemother. 37 (1993) 403–406.

[2] Gamage S.A., Tepsiri N., Wilairat P., Wojcik S.J., Figgitt D.P., Ralph R., Denny W.A., J. Med. Chem. 37 (1994) 1486–1494.

[3] Mauel J., Denny W.A., Gamage S.A., Ransijn A., Wojcik S.J., Figgitt D.P., Ralph R., Antimicrob. Agents Chemother. 37 (1993) 991–996.

[4] Gamage S.A., Figgitt D.P., Wojcik S.J., Ralph R., Ransijn A., Mauel J., et al., J. Med. Chem. 40 (1997) 2634–2642.

[5] Figgitt D.P., Denny W.A., Chavalitshewinkoo P., Wilairat P., Ralph R., Antimicrob. Agents Chemother. 36 (1992) 1644–1647.

[6] Atwell G.J., Cain B.F., Seelye R.N., J. Med. Chem. 15 (1972) 611–615.

[7] Cain B.F., Seelye R.N., Atwell G.J., J. Med. Chem. 17 (1974) 922–930.

[8] Cain B.F., Atwell G.J., Denny W.A., J. Med. Chem. 18 (1975) 1110–1117.

[9] Cain B.F., Atwell G.J., Denny W.A., J. Med. Chem. 19 (1976) 772–777.

[10] Nelson E.M., Tewey K.M., Leu L.F., Proc. Natl. Acad. Sci. USA 81 (1984) 1361–1365.

[11] Pommier Y., Zwelling L.A., Kao-Shan C.S., Wanng-Peng J., Bradly M.O., Cancer Res. 45 (1985) 3143–3149.

[12] Cain B.F., Atwell G.L., Eur. J. Cancer 10 (1974) 539–549.

[13] Arlin Z., Cancer Treat. Rep. 67 (1983) 967–970.

[14] Figgitt D.P., Denny W.A., Gamage S.A., Ralph R.K., Anti-Cancer Drug Des. 9 (1994) 199–208.

[15] Baguley B.C., Denny W.A., Atwell G.L., Cain B.F., J. Med. Chem. 24 (1981) 170–177.

[16] Denny W.A., Cain B.F., Atwell G.L., Hansch C., Panthananickal A., Leo A., J. Med. Chem. 25 (1982) 276–315.

[17] Gao H., Denny W.A., Garg R., Hansch C., Chem. Biol. Interact. 116 (1998) 157–180.

[18] Denny W.A., Atwell G.J., Cain B.F., J. Med. Chem. 20 (1977) 1242–1246.

[19] Denny W.A., Atwell G.J., Cain B.F., J. Med. Chem. 22 (1979) 1453–1460.

[20] Besly D.M., Goldberg A.A., J. Am. Med. Assoc. 151 (1954) 2448–2455.

[21] Su T.-L., Chou T.-C., Kim J.Y., Huang J.-T., Ciszewska G., Ren W.-Y., Otter G.M., Sirotnak F.M., Watanabe K.A., J. Med Chem. 38 (1995) 3226–3235.

[22] Duprè D.J., Robinson F.A., J. Chem. Soc. (1945) 549–551.

[23] Gimenez-Arnau E., Missalidis S., Stevens M.F., Anti-Cancer Drug Des. 13 (1998) 431–451.

[24] Zhitkovich A., Costa M., Carcinogenesis 13 (1992) 1485–1489.

[25] Kohn K.W., Pharmaco. Ther. 49 (1991) 55–77.

[26] Holm C., Covey J.M., Kerrigan D., Pommier Y., Cancer Res. 49 (1989) 6365–6366.

[27] Chen K.-X., Gresh N., Pullman B., Nucleic Acids Res. 16 (1988) 3061–3073.

[28] Durkin W.J., Ghanta V.K., Balch C.M., Davis D.W., Hiramoto R.N., Cancer Res. 39 (1979) 402–407.

[29] Bordin F., Carlassare F., Conconi M.T., Capozzi A., Majone F., Guiotto A., Baccichetti F., Photochem. Photobiol. 55 (1992) 221–226.

[30] Noviello E., Aluigi M.G., Cimoli G., Rovini E., Mazzoni A., Parodi S., De Sessa F., Russo P., Mutation Res. 311 (1994) 21–29.

[31] Tao-Shih H., Methods Enzymol. 199 (1983) 161–170.

[32] E.O.R.T.C screening group, Eur. J. Cancer 8 (1972) 185–196.