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Novel tetra-acridine derivatives as dual inhibitors of topoisomerase II and the human proteasome

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ABSTRACT

Acridine derivatives, such as amsacrine, represent a well known class of multi-targeted anti-cancer agents that generally interfere with DNA synthesis and inhibit topoisomerase II. But in addition, these tricyclic molecules often display secondary effects on other biochemical pathways including protein metabolism. In order to identify novel anti-cancer drugs, we evaluated the mechanism of action of a novel series of bis- and tetra-acridines. As expected, these molecules were found to interact with DNA and inhibit the topoisomerase II-mediated DNA decatenation. Interestingly when tested on human tumour cells either sensitive (HL-60) or resistant (HL-60/MX2) to topoisomerase II inhibitors, these molecules proved equi-cytotoxic against the two cell lines, suggesting that they do not only rely on topoisomerase II inhibition to exert their cytotoxic effects. In order to identify alternative targets, we tested the capacity of acridines **1–9** to inhibit the proteasome machinery. Four tetra-acridines inhibited the proteasome *in vitro*, with IC_{50} values up to 40 times lower than that of the reference proteasome inhibitor lactacystin. Moreover, unlike peptide aldehydes used as reference inhibitors for the proteasome, these new acridine compounds demonstrated a good selectivity towards the proteasome, when tested against four unrelated proteases. A cellular assay based on the degradation of a proteasome protein substrate indicated that at least two of the tetra-acridines maintained this proteasome inhibition activity in a cellular context. This is the first report of tetra-acridines that demonstrate dual topoisomerase II and proteasome inhibition properties. This new dual activity could represent a novel anti-cancer approach to circumvent certain forms of tumour resistance.

1. Introduction

Acridine derivatives have a long history for the treatment of human diseases, in particular for parasite infections and cancers [1,2]. Amsacrine (*m*-AMSA) for instance has been used over the past three decades to treat patients suffering from acute leukaemia and its anti-cancer activity can be attributed to a potent and selective inhibition of topoisomerase II. A large

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Abbreviations: *m*-AMSA, amsacrine; TL, trypsin-like; PGPH, peptidyl glutamyl peptide hydrolase; CTL, chymotrypsin-like; kDNA, kinetoplast DNA; CPI, calpain peptide inhibitor; RFU, relative fluorescence unit; RLU, relative luminescence unit; HIV, human immunodeficiency virus

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variety of acridine derivatives has been synthesized and, in some cases, promising results were obtained prompting the development of new acridine-based drugs as anti-cancer or anti-parasitic agents. This is the case for a series of recently described 9-amino-2-methoxyacridines and their bis- and tetra-acridine analogues which demonstrated marked anti-proliferative properties against *Leshmania infantum* [3]. These compounds are considered to be multi-targeted drugs, interacting with DNA and inhibiting topoisomerase II (considered as the main target), but with secondary effects on other biochemical pathways including protein metabolism [3]. It has been reported that the anthracycline derivative aclacinomycin A, which is also a topoisomerase II-interfering DNA intercalating agent, is capable of inhibiting the chymotrypsin-like activity of the purified mammalian 20S proteasome [4]. From all these data we postulated that the proteasome cell machinery could represent an alternative target for these acridines. The proteasome is a multimeric complex possessing three endoproteolytic activities, named after their cleavage specificities on peptides, trypsin-like (TL), post-acidic (or caspase-like, or peptidyl glutamyl peptide hydrolase, PGPH), and chymotrypsin-like (CTL). This multi-protein complex is responsible for the degradation of the majority of proteins in the cells, including regulators of the cell cycle and apoptosis, making it a potential target for cancer treatment [5–7]. Among the recently studied anti-cancer targets, the proteasome gained much interest through the development and commercialisation of Velcade™ (Bortezomib, PS-341, from Millennium Pharmaceuticals), a peptidic boronic acid which demonstrated anti-tumour activities through the specific inhibition of the chymotrypsin-like endoprotease of the proteasome. Velcade™ was approved in 2003 for the treatment of multiple myeloma [8,7].

With this in mind and with the aim to identify new anti-cancer compounds, we evaluated the mechanism of action of a series of nine novel bis- and tetra-acridine derivatives. Their structures are given in Table 1. Because of their acridine-based structure we first evaluated their DNA interaction along with their topoisomerase II inhibition properties. Inhibition of topoisomerase II-mediated DNA decatenation was observed but cytotoxicity measurements performed with cancer cell lines sensitive or resistant to reference topoisomerase inhibitors indicated that topoisomerase II could not be considered as the exclusive target for these molecules. Looking for alternative targets, we identified that some of these molecules also function as proteasome inhibitors, providing therefore opportunities to explain the cytotoxic activities of these novel acridines.

2. Materials and methods

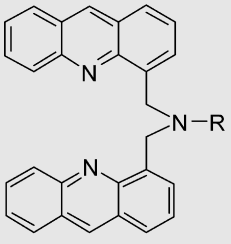
2.1. Materials and chemicals

The acridine derivatives are summarized in Table 1, and were synthesized in the UMR CNRS 6178 Symbio using published methods [9,3].

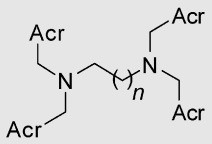
The fluorescent peptides: Methoxysuccinyl-Leu-Leu-Arg-AMC (AMC = 7-amino-4-methyl-coumarin), Z-Leu-Leu-Glu-AMC, Succinyl-Leu-Leu-Val-Tyr-AMC, used to reveal the trypsin-like, PGPH, and chymotrypsin-like activities, respectively, were purchased from NeoSystem. The calpain peptide inhibitor (CPI) was purchased from Calbiochem. Leupeptine, calpain, chymotrypsin, trypsin, and cathepsin B were purchased from Sigma–Aldrich. The chymotrypsin inhibitor, aryl ester coumarin, was synthesized according to a previously described procedure: compound **3l** in Ref. [10].

Table 1 – Chemical structures of studied bis- and tetra-acridine derivatives

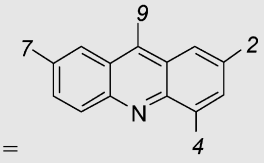
Bis-acridines
compound **1**, R = CN
compound **2**, R = C_3H_7



Tetra-acridines
compounds **3-9**



Acr =



Compound	Substituent			Linkage position on the acridine (Acr)
	9	7	*n*	
3	H	H	1	4
4	H	H	3	4
5	H	H	5	4
6	H	H	7	4
7	NH_2	H	1	4
8	Cl	Br	3	4
9	H	H	7	2

The human topoisomerase IIα was expressed in yeast using an expression vector kindly provided by Dr. J.L. Nitiss (Memphis, TN, USA) and purified according to a published method [11,12].

2.2. Cell lines and culture conditions

DLD1, HL-60, and HL-60–MX2 cells were obtained from the ATCC repository, and cultivated in RPMI 1640 medium supplemented with 10% foetal calf serum, 100 units/ml penicillin and 100 units/ml streptomycin (Invitrogen). All cells were maintained at 37 °C in a humidified incubator with an atmosphere of 5% CO_2.

2.3. Purification of the human proteasome

Our procedure was adapted from published methods and modified as follows [13]. All steps were conducted at +4 °C with solutions buffered at pH 7.5. A pellet of 5×10^9 human HeLa cells (Henogen sa), was lysed in two pellets volume buffer containing 25 mM Hepes, 1 mM DTT, 5 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.5% NP40, 1 mM ATP, 100 μM Na_3VO_4. This lysate was frozen 10 min at −80 °C before centrifugation 3 h at 100,000 × *g*. The supernatant was diluted twice in buffer A containing 10% glycerol, 30 mM Tris–HCl, 1 mM ATP, 5 mM $MgCl_2$, 5 mM NaF, 2 mM DTT, 1 mM EDTA, 100 μM Na_3VO_4, to which 10 mM NaCl was added. This sample was loaded, at a flow rate of 0.7 ml/min, onto a 70 ml DEAE column (GE Healthcare). The column was washed by five column volumes buffer A containing 10 mM NaCl, followed by five column volumes buffer A containing 100 mM NaCl. The proteins were eluted with five column volumes of a 100–300 mM NaCl gradient in buffer A at a flow rate of 2 ml/min. Fractions of 5 ml were collected for subsequent protein quantitation and proteasome *in vitro* assay. The fractions containing at least 50% of the maximal activity observed were pooled and separated on a Heparine Sepharose column. The pool from the DEAE column was first dialysed against buffer H containing 10% glycerol, 50 mM Hepes, 1 mM ATP, 5 mM $MgCl_2$, 5 mM NaF, 1 mM DTT, and 100 μM Na_3VO_4, then loaded, at a flow rate of 0.2 ml/min, onto a 10 ml HiTrap Heparin HP Sepharose column (GE Healthcare). The column was then washed, at a flow rate of 0.5 ml/min, with five column volumes of buffer H, then the proteins were eluted with five column volumes of a 0–1.2 M NaCl gradient in buffer H. Fractions of 5 ml were collected for subsequent protein quantitation and proteasome *in vitro* assay. The fractions containing at least 50% of the maximal activity observed were pooled and glycerol was added to reach 20% final before freezing at −80 °C.

2.4. In vitro proteasome assay

This fluorescent assay is based on a described methodology [14]. Each proteasome activity was measured by the cleavage of the fluorescent peptides: Methoxysuccinyl-Leu-Leu-Arg-AMC, Z-Leu-Leu-Glu-AMC, or Succinyl-Leu-Leu-Val-Tyr-AMC, used to reveal the trypsin-like, the PGPH, and the chymotrypsin-like activities, respectively. In a 96-well plate, 100 μM of each substrate was incubated in a 200 μl reaction containing 30 mM Tris–HCl, 1 mM ATP, 5 mM $MgCl_2$, 5 mM NaF, 1 mM DTT, and 100 μM Na_3VO_4, pH 7.5. The reaction was initiated by the addition of 3–9 μg of proteasome to get equal specific activities with the various peptides and developed for 90 min at 37 °C. The fluorescence coming from the cleaved AMC is then measured with a 380 nm excitation wavelength and a 460 nm emission wavelength on a SpectraMax Gemini spectrofluorimeter (Molecular Devices). The measures are expressed in RFUs (Relative Fluorescence Units) and collected by the SoftMaxPro software (Molecular Devices). IC_{50} values were calculated as the compound dose required to inhibit 50% of the enzymatic activity obtained in the presence of DMSO alone.

2.5. In vitro proteases assays

Calpain was tested against 100 μM succ-LLVY-AMC peptide in 20 mM Tris–HCl, 2 mM $CaCl_2$, 2 mM DTT, pH 8; cathepsin B was tested against 40 μM Z-RR-AMC in 100 mM sodium acetate, 5 mM EDTA, pH 5.5; chymotrypsin was tested against 100 μM succ-LLVY-AMC in 200 mM Tris–HCl, 20 mM $CaCl_2$, pH 8; trypsin was tested against 100 μM Z-LLR-AMC in 200 mM Tris–HCl, 20 mM $CaCl_2$, pH 8. All enzymes were incubated at room temperature for 30 min. The fluorescence coming from the cleaved AMC is then measured with a 380 nm excitation wavelength and a 460 nm emission wavelength on a SpectraMax Gemini spectrofluorimeter (Molecular Devices).

2.6. In vitro DNA interaction assay

Inhibition of EtBr–DNA interaction was evaluated according to published procedures [15,16]. Test compounds were incubated for 10 min at room temperature with 20 μM calf thymus DNA (Sigma) in the presence of EtBr in 2 mM acetate buffer pH 5.0 containing 10 mM NaCl, 0.1 mM NaEDTA. Inhibition of bisbenzimide–DNA interaction was monitored as described earlier [17]. Test compounds were incubated for 10 min at room temperature with 10 μM calf thymus DNA in the presence of either bisbenzimide (Hoechst 33258, Sigma), or picogreen in case of fluorescence interference between the test compound and bisbenzimide, in 10 mM Tris–HCl buffer pH 7.5 containing 150 mM NaCl. The final solvent concentration used was 10% and experiments were performed in duplicate in 96-well fluorescence microwell plates. Fluorescence was read on a SpectraMax Gemini spectrofluorimeter (Molecular Devices). With EtBr, excitation was at 546 nm and emission at 595 nm, while an excitation at 354 nm and emission at 450 nm was used with bisbenzimide, and an excitation at 480 nm and emission at 520 nm was used with picogreen. Background fluorescence due to interactions with either DNA or each of the individual chromophores alone was subtracted from the fluorescence detected in the presence of both DNA and the respective fluorochrome. Results are expressed as a percentage of the control fluorescence in the presence of the solvent alone, converted into IC_{50} values.

2.7. kDNA decatenation activity of topoisomerase II

Eighteen microliters of buffer A (50 mM Tris, 120 mM KCl, 0.5 mM DTT, 0.5 mM ATP, 10 mM $MgCl_2$, pH 8.0) containing 200 ng of kDNA (TOPOgen) and one unit of the human

recombinant topoisomerase IIα (the amount of enzyme which resulted in the complete decatenation of 200 ng of kDNA) were added to 2 μl of either solvent (DMSO) or a solution of the tested drug. After a 30 min incubation, the reaction mixture was analysed on a 1% agarose gel and run at 35 mA for 2 h in Tris borate EDTA (TBE) buffer. Gels were stained with EtBr and DNA was visualised using a Molecular Imager (Bio-Rad).

2.8. Proteasome cellular assay

The development of the DLD1 Luc–PEST cell line is described elsewhere (Fig. 3) and [18]. The stably transfected DLD1 Luc–PEST cells were cultured in MEM medium supplemented with 5% foetal calf serum and fongizone. Twenty-four hours prior treatment, 10^3 cells were platted in each well of a 96-wells plates. Compounds were dissolved in DMSO prior dilution in fresh medium at the appropriate concentration. Cells were then treated for 18 h with 100 μl of 10^{-6}, 5×10^{-5} or 10^{-5} M solutions. Plates were then washed once with PBS and cells lysed with 50 μl of the 1X Passive lysis buffer (Promega) for 5 min at room temperature before freezing. After addition of 100 μl of luciferase assay reagent (Promega) in each well, luciferase activity, measured as RLUs (Relative Luminescence Units) was then quantified at room temperature using a luminometer (Berthold). Luciferase accumulation factors (AF) were defined as the ratio between RLUs in treated cells over RLUs in untreated cells.

2.9. ATPLite cytotoxicity assay

4×10^4 HL-60 and HL-60/MX2 cells were seeded in 96-well plates. After 24 h various compound concentrations were added to the cells, and 0.1% DMSO added to the control cells giving the 100% of cell proliferation. After 72 h incubation, the ATP released from the viable cells was dosed using the ATPLite kit (Perkin-Elmer). IC_{50} values were determined as the compound dose required to reduced the ATP concentration, i.e. the cell number, to 50% of that obtained in control cells treated with DMSO alone.

3. Results

3.1. DNA interaction and topoisomerase II inhibition

DNA binding was investigated by a fluorescence competition assay using two complementary probes: ethidium bromide as a reference intercalating agent and bisbenzimide (Hoechst 33258) as a standard DNA minor groove binder. The probe was incubated with DNA in the presence of increasing concentrations of the test compound and the concentration needed to reduce the fluorescence by 50% was determined for each molecule and the two probes. The data are collated in Table 2. This competition assay is an indirect means to evaluate the DNA binding capacity of a new molecule. As expected, both amsacrine and aclacinomycin A were able to compete for DNA binding by ethidium (EtBr) or bisbenzimide (Table 2). The DNA-binding properties of these two reference topoisomerase II inhibitors has been largely documented [19–21]. In contrast, the bis-acridine derivative **1** did not show any significant binding as it was unable to displace the fluorescent probes from their binding sites. The other bis-acridine **2** exhibited a weak competition with bisbenzimide with an IC_{50} value of 44 μM, but not with ethidium. The tetra-acridines **3**–**9** proved to be much more efficient in this assay, with the amino derivative **7** being the most potent and the halogeno derivative **8** being the weakest DNA binder in the series. Unsurprisingly, these tetra-acridines must be considered as potent DNA binders. Parenthetically, lactacystin, a reference proteasome inhibitor, was also tested in this assay, but no binding to DNA could be detected.

Table 2 – *In vitro* DNA interaction

Compound	IC50 values for DNA interaction[a]	
	EtBr	Bisbenzimide
m-AMSA	12	6
Mitoxantrone	0.3	0.3
Aclacinomycin A	1.7	1.2
Lactacystin	>100	>100
1	>100	>100[b]
2	>100	44
3	4	7
4	5	5
5	65	5
6	6	>10[c]
7	2	0.8
8	70	30
9	9	>10[c]

[a] IC_{50} values in μM.
[b] Compound evaluated against picogreen due to interference with the bisbenzimide fluorescence.
[c] Those compounds were not evaluated at 100 μM due to a limited water solubility.

Next we explored inhibition of topoisomerase IIα by the acridines, using the capacity of the human enzyme to decatenate kinetoplast DNA (kDNA) into monomers. The same reference drugs, amsacrine and mitoxantrone (positive controls) and lactacystin (negative control) were used. IC_{50} values for topoisomerase II-mediated decatenation are collated in Table 3, and gels illustrating the activity of amsacrine, lactacystin, **3** and **4** are given in Fig. 1. The tetra-acridines

Table 3 – Inhibition of human topoisomerase IIα-mediated decatenation of kinetoplast DNA (kDNA)

Compound	IC_{50} [a]
m-AMSA	30
Mitoxantrone	2
Aclacinomycin A	2
Lactacystin	>100
1	6.5
2	>100
3	5.7
4	4.3
5	66
6	8.4
7	0.9
8	0.2
9	0.4

[a] IC_{50} values in μM.

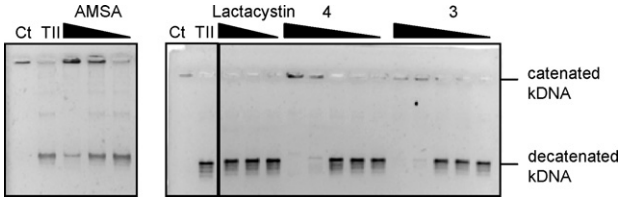


Fig. 1 – Inhibition of human topoisomerase IIα-mediated decatenation of kinetoplast DNA (kDNA). Ct, control catenated kDNA; TII, kDNA incubated with topoisomerase II; AMSA; Lacta; 4; 3; kDNA incubated with topoisomerase II in the presence of 100, 32, 10 μM AMSA; 100, 10, 1 μM lactacystin; 100, 32, 10, 1 μM 4, or 3, respectively. The products of the reactions were separated on agarose gel and visualised through ethidium bromide.

along with bisacridine **1** were all active in inhibiting topoisomerase II-mediated decatenation, with IC_{50} values ranging from 0.2 to 66 μM for compounds **8** and **5**, respectively. These results show that, in addition to DNA interaction, these acridines hold, in their vast majority, the ability to inhibit topoisomerase II activity.

3.2. *Topoisomerase II-dependent cytotoxicity*

At first sight, topoisomerase II could be invoked as a potential target for the tetra-acridines. To address this question at the cellular level, we compared the capacity of the compounds to inhibit proliferation of HL-60 and HL-60/MX2 human leukaemia cells. The HL-60/MX2 cell line has been selected for its resistance to the anti-cancer drug mitoxantrone which is a well established topoisomerase II poison. Resistance of these cells to mitoxantrone is characterized by a down-regulation of topoisomerase II activity, limiting the toxic effects of DNA damages [22,23]. HL-60/MX2 cells exhibit a marked resistance to mitoxantrone and a cross-resistance to amsacrine, with relative resistance indexes of 100 and 43, respectively (Table 4). The use of HL-60 and HL-60/MX2 cells thus represent a robust method to identify drugs that interfere with topoisomerase II at the cellular level. This assay is fairly specific as, in sharp contrast to mitoxantrone, the proteasome inhibitor lactacystin proved almost equitoxic towards the two cell lines (Table 4). This indicates that both cell lines are similarly sensitive to proteasome inhibition. The bis- and tetra-acridines demonstrated cytotoxicity to the two cell lines, with IC_{50} values in the micromolar range, with the exception of **7** and **9** which are considered as non-cytotoxic ($IC_{50} > 10$ μM; they could not be tested at a higher concentration due to their limited solubility in water). The other bis- and tetra-acridines appeared to be equitoxic to HL-60 and HL-60/MX2, with relative resistance ratios ranging from 0.5 to 1.4, i.e. considerably lower than those measured with amsacrine and mitoxantrone (Table 4). Under these circumstances, it is inconceivable that topoisomerase II represents a primary target for these compounds. At least one other target must be implicated to account for the potent cytotoxic activities of these molecules. The same conclusion also applies for aclacinomycin A which is also toxic to the topoisomerase II-deficient HL-60/MX2 cells and to the parental HL-60 cells.

Table 4 – IC_{50} values for proliferation of HL-60 and HL-60/MX2

Compound	IC_{50} [a]		
	HL-60	HL-60/MX2	RRI[b]
m-AMSA	0.34	13	43
Mitoxantrone	0.004	0.4	100
Aclacinomycin A	0.06	0.03	0.5
Lactacystin	2.9	3.7	1.3
1	13	15	1.2
2	3.7	4.1	1.1
3	1.9	2.6	1.4
4	3.3	4.7	1.4
5	2.6	1.7	0.7
6	3.7	3.0	0.8
7	>10	>10	–
8	3.5	3.0	0.9
9	>10	>10	–

[a] IC_{50} values in μM.
[b] RRI is the relative resistance index ($IC_{50}^{HL\text{-}60/MX2}/IC_{50}^{HL\text{-}60}$).

3.3. *In vitro inhibition of the proteasome*

We set up an *in vitro* assay based on the inhibition of the three proteolytic activities of a semi-purified mammalian proteasome, isolated from human HeLa cells through a two anion exchange columns purification process. Lactacystin, a known naturally occurring proteasome inhibitor selective for the chymotryspin-like activity, was used as a calibrator for this test. It gave an IC_{50} value of 7.6 μM (Table 5), in perfect agreement with the value given in the literature [24]. Aclacinomycin A, previously described as a proteasome inhibitor, was found in our test to inhibit the chymotrypsin-like activity with an IC_{50} value similar to that of lactacystin (Table 5). Here also, the IC_{50} value measured under our experimental conditions for aclacinomycin A is fully consistent with the published data [4], validating thus the assay. Among the tetra-acridines tested, compounds **4**, **5**, **6**, and **7** demonstrated *in vitro* proteasome inhibition properties. Interestingly, these compounds were found to be more potent

Table 5 – *In vitro* proteasome inhibition

Compound	IC_{50} for proteasome catalytic activities[a]		
	TL	PGPH	CTL
m-AMSA	>100	>100	>100
Aclacinomycin A	>10	>10	7.0
Lactacystin	>100	>100	7.6
1	>10	>10	>10
2	>10	>10	>10
3	>10	>10	>10
4	5	1	0.2
5	>10	1	0.4
6	>10	7	3
7	>10	2	0.8
8	>10	>10	>10
9	>10	>10	>10

[a] IC_{50} values in μM.

than lactacystin, with IC_{50} values 2–40-fold lower (Table 5). Like the vast majority of the proteasome inhibitors identified to date, these acridines exhibited a selectivity towards the chymotrypsin-like activity. The dose response curves for compound **4** and lactacystin on the chymotrypsin-like activity, as shown in Fig. 2A, highlight its higher potency compared to lactacystin. The length *N* (number of methylene units, where $N = (n + 1)CH_2$, Table 1) of the alkyl chain between the two dimeric acridines units seems to play an important role for proteasome inhibition. Compound **3** ($N = 2$) is practically inactive in this assay and the longer analogues **5** ($N = 6$) and **6** ($N = 8$) are less potent than **4** ($N = 4$). But unlike lactacystin, compound **4** failed to inhibit completely the chymotrypsin-like activity, leaving a plateau of 30% residual activity at the highest doses tested (Fig. 2A). At higher doses, this compound exhibited a significant PGPH inhibition around 60%. A potent inhibition of CTL activity was also observed with **7**, but not with **8** and **9**. The marked inhibition of the PGPH activity by compounds **4**–**7** clearly distinguishes these acridines from lactacystin and aclacinomycin A which are both inactive against PGPH. Amsacrine did not exhibit any proteasome inhibition activity, even at the highest dose tested of 100 μM (Table 5). A Lineweaver–Burk analysis for the most active compound in the series, tetra-acridine **4**, revealed a complex inhibition mechanism (Fig. 2B). At the lower dose of 0.1 μM, the inhibitor acts as a classical competitor towards the CTL substrate LLVY-AMC. But at the higher dose of 0.4 μM, the inhibition turns into an uncompetitive mode. Since at these higher doses, **4** also inhibits significantly the PGPH activity, we may hypothesize that this binding exerts a negative allosteric effect on CTL activity, lowering its affinity towards **4** (Fig. 2B). This inhibitory activity exhibited by tetra-acridine binding to PGPH would also explain why at high inhibitor doses, the CTL activity is no longer inhibited, and retains a residual peptide cleavage activity. Similar results were obtained with **5** arguing that this is a property of this series of molecules (data not shown). Overall, these data indicate that in addition to DNA interaction and topoisomerase II inhibition, these acridine derivatives possess an *in vitro* proteasome inhibition property.

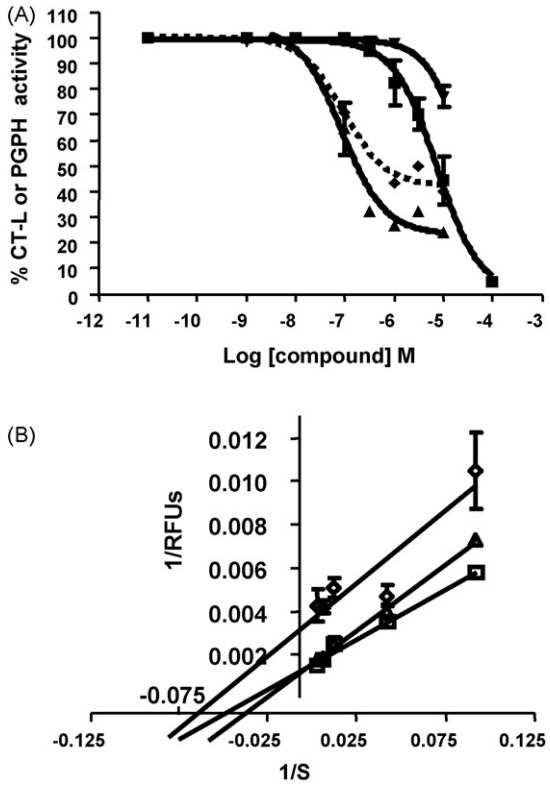


Fig. 2 – Proteasome inhibition mechanism by acridines 3 and 4. (Panel A) *In vitro* **inhibition of CTL (plain lines), and PGPH (dotted line) activities by 3 and 4 compared to lactacystin. Inhibition of the 26S CTL activity was measured** *in vitro* **in response to a dose range of lactacystin (■), 3 (▼), and 4 (▲). Inhibition of PGPH activity was also measured in response to a dose range of 4 (◆). Results are given as percentage of proteolytic activity relative to a control incubated with DMSO alone. (Panel B) Enzymatic study of proteasome inhibition by 4. Lineweaver–Burk plot for CTL inhibition where S and RFUs represent the succ-LLVY-AMC substrate concentration in μM and the fluorescence, in relative fluorescence units (RFUs), released from this substrate, respectively; control (□), 0.1 μM (△), and 0.4 μm (◇) of 4.**

A few structure–activity relationships can be inferred from this set of data. Among the compounds tested only the tetra-acridines were able to inhibit significantly the proteasome *in vitro* at doses below 10 μM. Neither the bis-acridines nor amsacrine demonstrated such an activity, suggesting that this effect is not the property of the acridine moiety itself, but a larger assembled structure may be required. Proteasome inhibition is not a general feature of acridine, but is restricted to a few tetra-acridine structures. As mentioned above, the length of the methylene linker joining the bis-acridine units has a direct impact on the activity. A length of three methylene units (as in compound **4**) appears as the most favourable linker chain. The substitution on the acridine rings also contribute to the activity. The addition of a 9-amino group on the acridine moiety confers proteasome inhibition properties (compare compounds **3** and **7**). In contrast, the addition of 9-chlorine and 7-bromine atoms on the acridine moiety resulted in a complete loss of activity (compare compounds **4** and **8**). The same substitutions are also detrimental to DNA binding but not to topoisomerase II inhibition. In general the structural requirements are relatively distinct for topoisomerase II versus proteasome inhibition. A good topoisomerase II inhibitor does not necessarily correspond to a proteasome inhibitor (e.g. compound **3**) and *vice versa* (e.g. compound **5**). Finally, it is worth mentioning that apparently the site of linkage of the acridine rings to the linker plays a significant role. Compound **6** with the acridines attached to the linker via position 4 inhibits the CTL and PGPH activities of the proteasome whereas the related analogue **9** with the acridines attached to the linker via position 2 is totally inactive. At first sight, 4-substituted acridines will provide more potent inhibitors than the corresponding 2-substituted analogues, but additional molecules will be needed to confirm this preliminary observation.

In a previous report [4], it has been proposed that a hydrophobic non-polar residue in aclacinomycin A plays a direct role in the interaction with and inhibition of the CTL activity of the proteasome. Hydrophobic interactions with tyrosine and/or phenylalanine residues have been suggested.

Table 6 – *In vitro* selectivity of acridine derivatives towards four proteases

Compound	IC_{50} [a]			
	Calpain	Chymotrypsin	Cathepsin B	Trypsin
m-AMSA	>10	>10	>10	>10
Aclacinomycin A	>10	>10	>10	>10
Lactacystin	>10	>10	>10	>10
1	>10	>10	2	>10
2	>10	>10	>10	>10
3	>10	10	6.7	>10
4	>10	>10	>10	>10
5	>10	>10	>10	>10
6	>10	>10	>10	>10
7	>10	6.2	>10	>10
8	>10	>10	>10	>10
9	>10	>10	>10	>10
Leupeptine	ND[b]	ND	0.0022	0.21
CPI[c]	0.0028	ND	ND	ND
Aryl ester Coumarin	ND	0.0047	ND	ND

[a] IC_{50} values in μM.
[b] Not determined.
[c] Calpain peptide inhibitor.

By analogy, a similar role for the hydrophobic acridine moieties in the tetra-acridines can be proposed. In the near future, it will be interesting to replace the acridine moieties of **4** with other heterocycles.

3.4. *Proteasome selectivity*

The inhibitory potential of the bis- and tetra-acridines was evaluated against four enzymes, two serine proteases, trypsin and chymotrypsin, and two cysteine proteases, calpain and cathepsin B. The assays were calibrated using known inhibitors of these proteases, i.e. leupeptine for trypsin and cathepsin B, calpain peptide inhibitor (CPI) for calpain, and a previously described aryl ester coumarin for chymotrypsin, i.e. compound **3l** in Ref. [10]. IC_{50} values were calculated for compounds giving more than 50% inhibition at, or below 10 μM (Table 6). Only a moderate inhibition of cathepsin B and/or chymotrypsin was identified for compounds **1**, **3** and **7**. The reference compounds lactacystin and aclacinomycin A and the most potent proteasome inhibitors **4**, **5**, and **6**, did not show significant proteases inhibition at 10 μM (Table 5). These data indicate that there is no correlation between the proteasome inhibition potency and the targeting of other proteases. In other words, compounds **4**, **5**, and **6** present a marked selectivity for the proteasome over these four other proteases.

3.5. *Proteasome inhibition in cells*

To evaluate the inhibition of the proteasome by the acridine derivatives at the cellular level we set up an assay based on the inhibition of the degradation of a proteasome-targeted protein (Fig. 3). Human colon cancer DLD1 cells were stably transfected with a luciferase gene fused to the proteasome recognition sequence PEST derived from the ornithine decarboxylase protein. In parallel, a clone expressing the wild type luciferase protein was constructed as a control cell line for proteasome-independent inhibition of luciferase activity. This PEST–luciferase fusion has been reported to be efficiently degraded by the proteasome [25]. Proteasome inhibition in these cells by a known reference inhibitor like lactacystin induced a PEST–luciferase accumulation while the luciferase level was unaffected in cells transfected with the control construct (Table 7; Fig. 4). Among the acridine derivatives, only **4** and **6** gave significant PEST–luciferase accumulation without affecting the control luciferase level, with a significant accumulation factor above 3 for concentrations of 5 and 10 μM (Table 7; Fig. 4). Nevertheless, one cannot rule out the possibility that **3**, inhibiting the proteasome *in vitro* at higher doses compared to **4** and **6**, would also inhibit the cellular

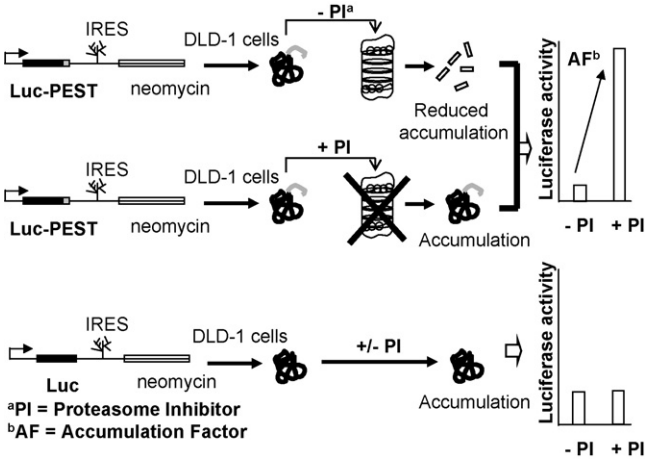


Fig. 3 – Diagram of the cellular assay for proteasome inhibition. The destabilised luciferase–PEST, stably transfected in human cancer DLD1 cells, is preferentially degraded by the proteasome compared to the non destabilised luciferase. As a consequence, inhibition of the proteasome is measured through an accumulation of luciferase–PEST, while the luciferase level is hardly affected.

Table 7 – Proteasome inhibition in DLD1 luciferase–PEST cells

Compound	Accumulation factors[a] (AF)		
	1 μM	5 μM	10 μM
Lactacystin	+	+	+
m-AMSA	–	–	–
Aclacinomycin A	–	–	–
1	–	–	–
2	–	–	–
3	–	–	–
4	–	+	+
5	–	–	–
6	–	+	+
7	–	–	–
8	–	–	–
9	–	–	–

[a] + refers to a luciferase accumulation factor considered to be significantly higher in DLD1 treated cells vs. untreated cells, i.e. an accumulation factor ≥3.

proteasome at doses above 10 μM, or that luciferase accumulation requires a more pronounced proteasome inhibition that would be achieved at higher doses of **3**. Compound **5**, like aclacinomycin A, potently inhibits the proteasome *in vitro* but failed to do so in cells, at least for concentrations up to 10 μM (higher concentrations could not be tested in cells due to restricted water solubility and the sensitivity of the cellular assay to DMSO used to solubilise these compounds). These results demonstrated that at least two tetra-acridine derivatives, **4** and **6**, inhibit the proteasome both *in vitro* and *in cellulo*.

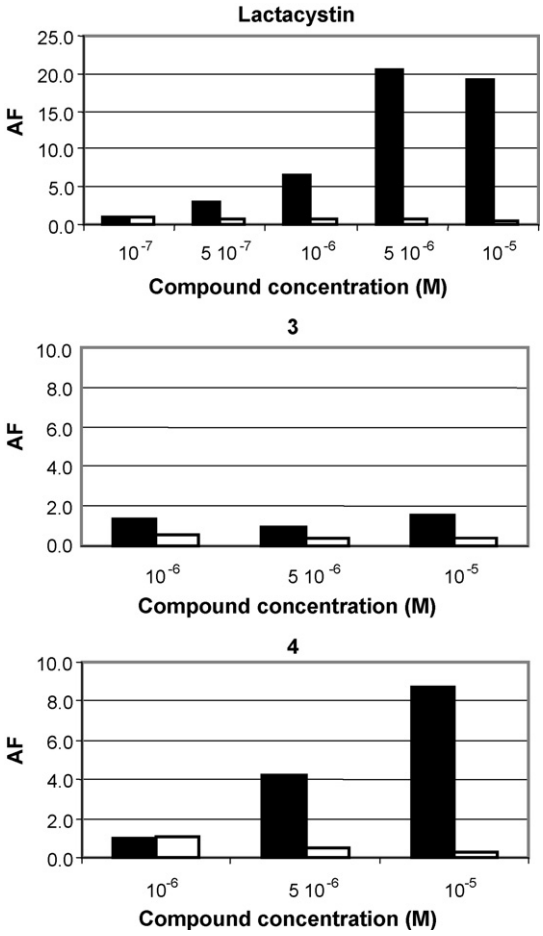


Fig. 4 – Luciferase accumulation in response to treatment with acridines 3 and 4. DLD1 luciferase–PEST (■) and DLD1 luciferase (□) cells were treated for 18 h with increasing doses of either lactacystin, 3 or 4. The luciferase accumulation factors (AF) in each cell line and for each compound dose were calculated and reported on these histograms.

4. Discussion

We have identified a novel series of acridine derivatives that can interact with DNA and, for a majority of them, inhibit topoisomerase II-mediated decatenation of DNA. These compounds are cytotoxic to HL-60 human leukaemia cells and maintain an equally potent cytotoxicity when the topoisomerase II activity of these cells is down-regulated. HL-60/MX2 resistant to the topoisomerase II poison mitoxantrone and cross-resistant to amsacrine, are not resistant to the acridine derivatives tested here, suggesting that topoisomerase II is not the unique or primary target of these compounds. Searching for alternative targets, we identified the proteasome as a potential receptor for these molecules. When tested on a purified proteasome *in vitro*, four of these compounds demonstrated inhibitory properties, and at least two of them were found to inhibit the proteasome in cells. In addition, these molecules appeared selective for the proteasome, without any significant inhibition of four other proteases, such as calpain, trypsin, cathepsin B and chymotrypsin. These molecules seem to be more selective than peptide aldehydes which inhibit cathepsin B and calpain in addition to the proteasome [26] and data not shown. Of course, additional targets other than the proteasome cannot be excluded for these novel acridines, but nevertheless the present findings open new directions for a more rational design of acridine-containing proteasome inhibitors. The branched structure of these compounds may also be of interest to build pharmacological tools to study the catalytic activities of the proteasome and identify proteins partners and substrates. In addition to being anti-cancer targets, it was reported that topoisomerase II and the proteasome are indeed essential for protozoan parasite survival [27–32]. Accordingly, the proteasome inhibitor lactacystin, and the HIV protease inhibitors indinavir and saquinavir, were shown to exhibit antileishmanial activity [33,32]. Thus, these data in addition to our findings shed light on previously described bis- and tetra-acridines possessing antileishmanial activity [3]. One can propose that this activity results, as shown here, from the dual inhibition of topoisomerase II and the proteasome.

An other interesting link between topoisomerase II and proteasome can be established. Ogiso et al. [34] demonstrated that proteasome inhibition can restore topoisomerase IIα protein level in human cancer cells resistant to topoisomerase II-targeted drugs like etoposide and doxorubicin. Topoisomerase II restoration was associated with a reversal of the resistance to the drugs-induced cytotoxicity. It can be proposed that our proteasome-inhibiting tetra-acridines

would induce a restoration of the topoisomerase II level which in turn would increase the sensitivity of the HL-60/MX2 resistant cell line to the topoisomerase II inhibition potency of the tetra-acridines. This sensitisation is also likely to take place in hypoxic and/or glucose-deprived cells, mimicking tissues found in rapidly growing tumours, since such cells have a decreased level of topoisomerase II that can be prevented by using lactacystin as a proteasome inhibitor [34]. The identification of drugs that target both topoisomerase II and the proteasome may thus be of a major interest in cancer chemotherapy. This is certainly a pharmacological route to follow in the near future.

Resistance to some topoisomerase II poisons, including acridine derivatives like amsacrine, can involve mutation and/or down-regulation of topoisomerase II activity, a phenotype known as atypical multi-drug resistance (at-MDR) [35–39]. Such tumours resistant to topoisomerase II inhibitors would likely remain sensitive to proteasome-interacting acridines, as those presented in this study. Such compounds inhibiting two validated cytotoxic targets, i.e. topoisomerase II and the proteasome, would represent a new interesting class of anti-cancer drugs compensating for resistance mediated by topoisomerase II down-regulation, and maintaining an anti-tumour activity in cancers having developed a resistance to topoisomerase II inhibitors. The present study provides novel opportunities to design molecules susceptible to interfere with two oncogenic targets at the same time, namely topoisomerase II and the proteasome. If anti-cancer activities are validated *in vivo* (compound **4** is currently tested in xenograft models), the dual topoisomerase II/proteasome targeting may well represent a promising new anti-cancer strategy. Indirectly, this study also suggests that drug regimen combining a proteasome inhibitor with a topoisomerase II poison should be considered further.

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