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Synthesis and biological evaluation of indazolo[4,3-*bc*]-[1,5]naphthyridines(10-aza-pyrazolo[3,4,5-*kl*]acridines): a new class of antitumor agents

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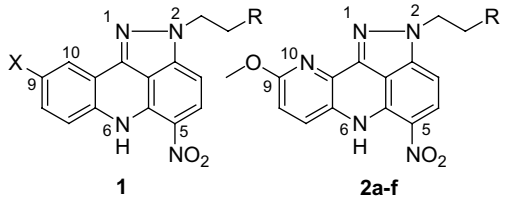
Abstract—A series of potential DNA-binding antitumor agents, 2-[ω-(alkylamino)alkyl]-9-methoxy-5-nitro-2,6-dihydroindazolo[4,3-*bc*][1,5]naphthyridines (**2a**–**f**), 10-aza derivatives of PZA, has been prepared by condensation of 9-chloro-2-methoxy-6-nitro-5,10-dihydrobenzo[*b*][1,5]naphthyridin-10-one (**6**) with the appropriate (ω-aminoalkyl)hydrazine in tetrahydrofuran/methanol. Compound **6** was obtained by heating at 100 °C in H_2SO_4 **5**, yielded by the condensation of 2,6-dichloro-3-nitrobenzoic acid (**4**) and 6-methoxy-3-pyridinamine (**3**). The non-covalent DNA-binding properties of **2** have been examined using a fluorometric technique. In vitro cytotoxic potencies of these derivatives against human hormone-refractory prostate adenocarcinoma cell line (PC-3) are described and compared to that of parent drug PZA. We selected the most cytotoxic target derivatives **2c**,**d**, the in vitro inactive **2f**, and reference compound PZA to investigate whether in vitro treatment with these drugs was able to induce necrotic and/or apoptotic cell death. To this purpose, we evaluated the percentage of apoptotic cells in PC-3 treated with the target compounds **2c**,**d**,**f** and reference compound PZA, by Annexin V staining and Propidium iodide (PI)/Annexin V, biparametric flow cytometric analysis and agarose gel electrophoresis.

1. Introduction

In the class of DNA-intercalating anticancer drugs, structurally characterized by the presence of a planar or semi-planar chromophore portion possibly capable of intercalation into DNA, the acridine and the 9-acridone chromophores play an important role.[1,2] Often, the acridine/acridone ring system is fused with a five- or six-membered heterocyclic ring to yield interesting polycyclic derivatives.[1,2] Among them, a noticeable example is constituted by the 5-nitropyrazolo[3,4,5-*kl*]-acridines (**1**, Fig. 1).[3] The 2-[3-(dimethylamino)propyl]-9-methoxy-5-nitro-2,6-dihydropyrazolo[3,4,5-*kl*]acridine (PZA; **1a**: R = $CH_2N(CH_3)_2$, X = OMe) combines the DNA-binding properties of acridine with the reducible nitro group responsible for hypoxic cell selectivity.



1 **2a-f**

Figure 1. Structures and ring numbering of parent 5-nitropyrazolo-[3,4,5-*kl*]acridines **1** and of target 5-nitroindazolo[4,3-*bc*][1,5]naphthyridines (5-nitro-10-aza-pyrazolo[3,4,5-*kl*]acridines) **2a**–**f**.

Recent studies suggest that PZA might be a dual inhibitor of DNA topoisomerase I and DNA topoisomerase II that exerts its effects by diminishing the formation of topoisomerase-DNA adducts. Moreover, PZA-mediated DNA fragmentatiom, accompanied by the induction of very high molecular weight DNA fragments (0.5–1 Mb) was detected by pulsed-field gel electrophoresis in human breast cancer cells,[4] and bone marrow mononuclear cells from metastatic colorectal cancer

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patients.[5] Consistent with these mechanisms of action, PZA exhibits broad spectrum antitumor activity in preclinical models in vivo. In addition, this agent displays several unique properties including solid tumor selectivity, activity against hypoxic cells, and cytotoxicity in non-cycling cells. PZA also retains full activity against cells that are resistant to other agents on the basis of overexpression of P-glycoprotein or the multidrug resistance-associated protein (MRP) and with loss of p53 function.[6] Moreover, recently, the combination of PZA with conventional anticancer agents such as doxorubicin, etoposide, and topotecan increased the cytotoxicity against drug-resistant tumor cells.[7]

PZA has been studied in phase I trials in adults and children, and underwent broad phase II trials.[8] However, these phase II studies of PZA in patients with previously treated colorectal cancer, pancreatic cancer, transitional cancer of the bladder, germ cell, and renal cancer have shown no objective responses, whereas myelosuppression was prominent. In contrast, evidence of clinical activity has been seen in platinum-sensitive ovarian cancer and hormone refractory prostate cancer. In the later case, 1 of 17 patients with androgen-independent prostate cancer shows a 96% decrease in the prostatic specific antigen level (PSA) accompanied by improvement of bone scan.[9]

In the continual search of new classes of anticancer agents, we decided to further investigate a novel chromophore constituted by an aza-acridine skeleton fused with a five-membered heterocyclic ring to form the new heterocycle indazolo[4,3-*bc*][1,5]naphthyridine. As shown in Figure 1, the target 2-[ω-(alkylamino)alkyl]-9-methoxy-5-nitro-2,6-dihydroindazolo[4,3-*bc*][1,5]naphthyridines (2-[ω-(alkylamino)alkyl]-9-methoxy-5-nitro-2,6-dihydro-10-aza-pyrazolo[3,4,5-*kl*]acridines) (**2a**–**f**) represent the 10-aza analogues of the parent 5-nitropyrazolo[3,4,5-*kl*]acridines **1**. Prompted by the above rationale, we synthesized compounds **2a**–**f** investigating some biologically relevant aspects such as (i) DNA-binding properties, (ii) cytotoxic activity, (iii) apoptotic cell death, and (iv) DNA fragmentation.

2. Results and discussion

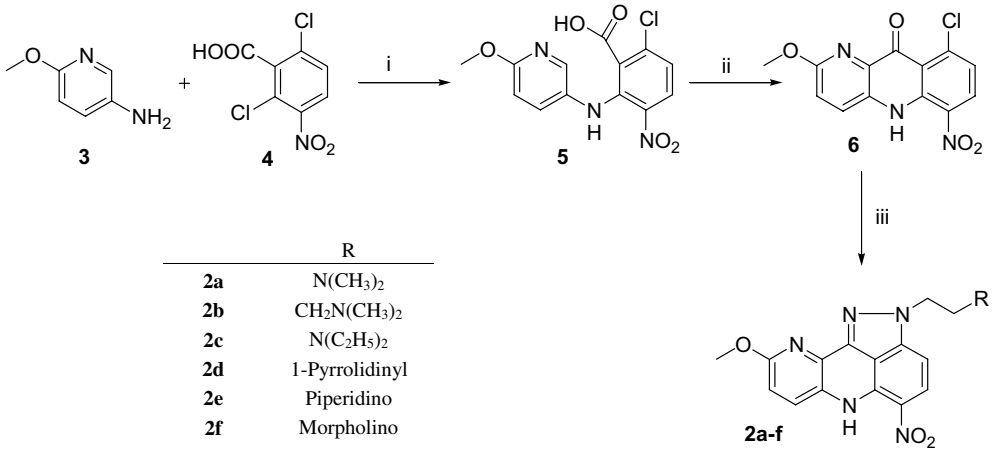
2.1. Chemistry

Scheme 1 shows the synthetic pathway leading to target derivatives **2a**–**f**. Thus, the new 6-chloro-2-[(6-methoxy-3-pyridyl)amino]-3-nitrobenzoic acid (**5**) was obtained by heating at 75 °C the 2,6-dichloro-3-nitrobenzoic acid (**4**) in 6-methoxy-3-pyridinamine (**3**). Compound **5** was heated at 100 °C in H_2SO_4, the resulting mixture was poured into crushed ice to yield the 9-chloro-2-methoxy-6-nitro-5,10-dihydrobenzo[*b*][1,5]naphthyridin-10-one (**6**) as an orange precipitate. Condensation of **6** with the appropriate (ω-aminoalkyl)hydrazine[10] in tetrahydrofuran/methanol (1:1) at room temperature afforded the desired 2-[ω-(alkylamino)alkyl]-9-methoxy-5-nitro-2,6-dihydroindazolo[4,3-*bc*][1,5]naphthyridines, or 2-[ω-(alkylamino)alkyl]-9-methoxy-5-nitro-2,6-dihydro-10-aza-pyrazolo[3,4,5-*kl*]acridines (**2a**–**f**) as orange precipitates.

To examine the DNA-binding properties and the antineoplastic activity of these agents, the free base forms of the target derivatives **2** were converted into their water-soluble hydrochlorides by the usual methods.

2.2. DNA-binding properties

(i) Binding to calf thymus DNA (CT-DNA). 'Apparent' binding constant (K_{app}) values were determined using a competitive fluorometric ethidium displacement method that has been used extensively for other DNA-binding ligands, particularly intercalants.[11–13] In this assay, the relative K_{app} affinity for CT-DNA is defined by K_{app} (drug) = $1.26/C_{50} \times K_{app}$ (ethidium), were 1.26 is the concentration (μmol) of ethidium in ethidium–DNA complex, C_{50} represents the concentration (micromole) of added compound required to reduce the fluorescence of the ethidium–DNA complex by 50%, and the K_{app} (ethidium) binding constant is taken as $10^7\,M^{-1}$.[11,13] In the present study, fluorescence displacement assays



	R
2a	$N(CH_3)_2$
2b	$CH_2N(CH_3)_2$
2c	$N(C_2H_5)_2$
2d	1-Pyrrolidinyl
2e	Piperidino
2f	Morpholino

Scheme 1. Reagents: (i) Δ; (ii) H_2SO_4; (iii) $NH_2NHCH_2CH_2R$.

were performed at pH 7 to enable comparison in biological conditions.

On these bases, the K_{app} values can be regarded as indicative of the strength and extent of binding to this 'pseudo-random' DNA sequence, but not of the mode of interaction (e.g., intercalation and/or groove binding mechanism). Table 1 shows that all compounds **2** have a significant binding with CT-DNA. The K_{app} values in the range $1.4–1.6 \times 10^7$ indicate that the target derivatives are more DNA-affinic than ethidium and a little more than parent compound PZA. The only exception is the morpholino derivative **2f**, which is the weakest ligand in the series, as we expected from previous results.[14,15] We suggested that the poor effect of **2f** is due to the protonation status of the pendant side chain, since the morpholine residue provides the least basic amine residue within this series and would be only ~10–20% protonated at pH 7.[14,15]

(ii) Binding to the synthetic polynucleotides [poly(dA–dT)]$_2$ (A–T) and [poly(dG–dC)]$_2$ (G–C). An equivalent competitive fluorometric assay method was similarly used to assess the binding of derivatives **2** and of the parent compound PZA to [poly(dA–dT)]$_2$ (A–T) and [poly(dG–dC)]$_2$ (G–C) in order to examine the possible binding site preferences. The K_{app} values determined for the compounds with each duplex are collected in Table 1, together with the binding side preferences (in parentheses, the G–C/A–T or A–T/G–C affinity ratio). The binding site selectivity is considered to be significant only for an G–C/A–T (or A–T/G–C) affinity ratio differing by more than 30% from the sequence-neutral unity value (i.e., >1.3). Also in these cases, the K_{app} values of compounds **2** are indicative of a strong binding with these synthetic DNA. For derivatives **2a**,**b**,**c**,**f**, the site-dependent behavior is significantly (**2f**) or very markedly (**2a**,**b**,**c**) G–C preferential. On the contrary, derivatives **2d**,**e** and parent compound PZA show very marked A–T binding site preference.

All together, the data on binding indicate that the aza substitution of PZA (**1a**), leading to derivative **2b**, does not affect the strength and the extent of binding, but affects the binding site preference. The nature of pendent side chain deeply influences the binding properties of the new compounds **2**.

2.3. In vitro cytotoxic activity

In vitro cytotoxic potencies of target 5-nitro-10-aza-pyrazolo[3,4,5-*kl*]acridines (**2a**–**f**) in comparison with parent 5-nitro-pyrazolo[3,4,5-*kl*]acridine (**1a**, PZA) against human hormone-refractory prostate adenocarcinoma cell line (PC-3) are reported in Table 1. The growth inhibition 50, GI_{50} (micromolar), and the total growth nhibition, TGI (micromolar), represent the drug concentration required to inhibit cell growth by 50% and by 100%, respectively, giving an idea of the cytostatic action of the drugs. The lethal concentration 50, LC_{50} (micromolar), represents the drug concentration required to kill 50% of the initial cell number, giving an idea of the cytotoxic action of the drugs. Each quoted value is the mean of triplicate experiments.

The results indicate that (a) As shown by GI_{50} values, all compounds **2** possess a good antiproliferative activity in the submicromolar range, similar, but a little inferior than, to that of parent PZA; the only exception is constituted by **2f**, which does not inhibit the cell growth of 50% even at highest concentration (10^{-4} M) tested. (b) The TGI values show that the new derivatives completely inhibit the cell growth at micromolar concentration even lower, especially **2e**, than PZA. (c) The LC_{50} values indicate that compounds **2** posses cytotoxicity similar to that of PZA, **2c**,**d** being the most potent compounds in the series. The data obtained for a limited number of derivatives do not allow us to formulate structure–activity relationships but some considerations about pendent side chain can be made: (i) The optimal distance between the two nitrogen atoms is of two methylene units, as indicated by the difference in all the three parameters, GI_{50}, TGI, and LC_{50}, in the pair **2a**,**b**. (ii) Bulky substituents at the terminal nitrogen atoms do not decrease the cytotoxicity, as can be seen from the GI_{50}, TGI, and LC_{50} values of the pair **2a**,**c**. (iii) When the distal nitrogen atom is part of an heterocycle, compounds **2d**–**f**, the best results are obtained with a pyrrolidine, but a piperidino yielded the best TGI value in the series; instead, a morpholino resulted in a complete loss of activity.

There is no quantitative correlation between DNA-binding and in vitro activity, but the lack of activity of **2f**, which possesses weakest K_{app} values, confirms that an

Table 1. DNA-binding properties and cytotoxic activity (PC-3) of target compounds **2a**–**f** in comparison with parent compound PZA[a]

Compd	$K_{app} \times 10^{-7}$ M^{-1}			Binding site preference	Cytotoxic activity		
	A–T	CT-DNA	G–C		GI_{50} (μM)	TGI (μM)	LC_{50} (μM)
2a	0.36	1.6	1.7	G–C (4.7)	0.600	11.7	63.1
2b	0.22	1.6	4.5	G–C (20)	0.891	14.1	108
2c	0.16	1.4	4.0	G–C (25)	0.562	10.7	58.9
2d	2.4	1.4	0.37	A–T (6.5)	0.380	10.0	53.7
2e	3.0	1.4	0.63	A–T (4.8)	0.646	3.40	126
2f	0.12	0.19	0.24	G–C (2.0)	N.A.[b]	N.A.[b]	N.A.[b]
PZA	1.7	1.3	0.36	A–T (4.7)	0.355	11.2	73.6

[a] For the meaning of K_{app} binding site preference, GI_{50}, TGI, and LC_{50} see text.
[b] N.A. = not active.

efficient binding is necessary, also if not sufficient, condition for cytotoxic activity. The binding site preference does not seem to play an important role on cytotoxicity.

2.4. Apoptosis assays

Recent evidence suggested that the characteristics of tumor cell death may be the most important determinant for successful chemotherapy. Apoptosis is defined by morphological and biochemical changes resulting in cell loss and has been found to be relevant to a wide spectrum of biological processes, including neoplasia and cancer chemotherapy. Concentration on the mechanisms of action of chemotherapeutic agents has allowed us to establish that most of these agents exert their biological effects by triggering apoptotic cell death. The ability of these agents to induce apoptosis in tumor cells has now become a rationale for therapeutic approaches and entertains the possibility that apoptosis may be enhanced in tumors for therapeutic benefit. Although available data demonstrate the induction of apoptosis in tumor cells, the information is limited on the correlation of apoptotic cell death to the success of chemotherapy and resistance. Recent findings have supported and confirmed the presence of chemotherapy-induced DNA fragmentation in tumor cells following treatment with PZA in vivo, however the mechanisms responsible to apoptotic and/or necrotic cell death are poorly addressed so far.[16]

Thus, we selected the most cytotoxic target derivatives **2c**,**d**, the in vitro inactive **2f**, and reference compound PZA to investigate whether in vitro treatment with these drugs was able to induce necrotic and/or apoptotic cell death. To this purpose, we evaluated the percentage of apoptotic cells in PC-3 treated with the target compounds **2c**,**d**,**f** and reference compound PZA, by Annexin V staining and Propidium iodide (PI)/Annexin V, biparametric flow cytometric analysis, and agarose gel electrophoresis.

A characteristic feature of necrotic cell death is the loss of plasma membrane integrity, whereas early during apoptosis phosphatidylserine translocates from the inner to the outer plasma membrane layer.[17] Thus, we evaluated whether the PZA treatment of PC-3 cells induce externalization of PS residues from the inner to the outer leaflet of the plasma membrane. To this end we analyzed Annexin V binding on PC-3 cells by flow cytometric analysis. As shown in Figure 2, treatment with target compound **2d** induce an early (2 h) and extensive translocation of PS. PS exposure on target

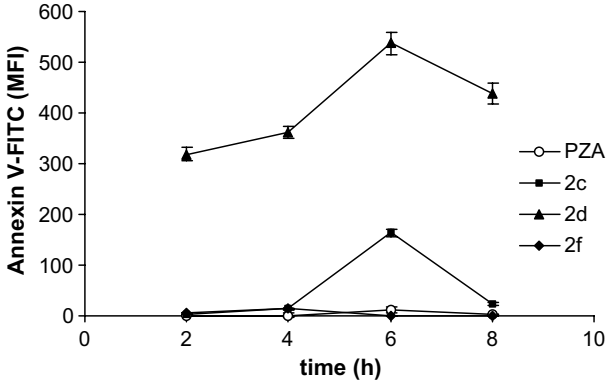


Figure 2. The expression of Annexin V on PC-3 cells, treated at different times (2, 4, 6, and 8 h) with target compounds **2c**,**d**,**f** and reference compound PZA, was evaluated by immunofluorescence and FACS analysis. Data expressed as mean fluorescence intensity (MFI) are representative of one out of three separate experiments.

compound **2d**-treated PC-3 cells increased in a time-dependent manner until 6 h and the declined at 8 h. Similar, but less intensive (2.5-fold less) PS exposure was observed on target compound **2c**-treated PC-3 cells. The target compound **2f** and the reference compound PZA did not affect PS expression on PC-3 cells.

Data obtained by Annexin V staining were further strengthened by biparametric flow cytometric analysis. Thus, 6 h after target compounds **2d** and **2c** treatment about 36% and 15% of PC-3 cells, respectively, were Annexin V^+ PI^-, whereas negligible PS expression was observed on PC-3 cells treated with the target compound **2f** and the reference compound PZA (Table 2); moreover, about 25% and 20% of the PC-3 cells treated with the target compound **2f** and the reference compound PZA, respectively, were PI^+ Annexin V^-.

2.5. DNA fragmentation assay

DNA fragmentation during apoptosis leads to extensive loss of DNA content resulting in a characteristic internucleosomal DNA ladder.[18] In accordance with cytofluorimetric analysis, agarose gel electrophoresis evidenced necrosis or apoptosis of PC-3 cells depending on the different compounds used (Fig. 3). Target compound **2d** at 50 μM concentration induced apoptosis as evidenced by a characteristic ladder pattern of DNA fragments, whereas as previously described[5] very high molecular weight (about 0.8 Mb) DNA fragments were observed in PC-3 cells treated with the reference compound PZA. In addition, PC3 cells treated with the

Table 2. Treatment of PC-3 cells for 6 h with target compounds **2c**,**d**,**f** and parent compound PZA

Compounds	PI^+ Annexin V^-	PI^- Annexin V^-	PI^- Annexin V^+	PI^+ Annexin V^+
2c	25.1 ± 1.9[a]	71.6 ± 3.2	2.3 ± 0.2	1.1 ± 0.1
2d	20.1 ± 1.5	63.0 ± 2.1	15.0 ± 0.8	1.8 ± 0.3
2f	3.8 ± 0.3	56.2 ± 2.5	36.2 ± 2.1	3.8 ± 0.4
PZA	8.2 ± 0.3	90.6 ± 2.8	0.1 ± 0.0	1.1 ± 0.1

The target compounds **2c**,**d** induce apoptotic cell death.

[a] Percent positive cells. Results are representative of one out of three separate experiments.

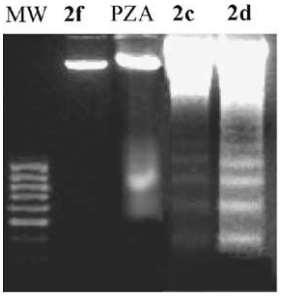


Figure 3. Analysis of DNA fragmentation from PC-3 cells, treated with target compounds **2c**,**d**,**f** and reference compound PZA at 6 h after treatment, was performed by agarose gel (1.7%) electrophoresis. MW = molecular weight. Data shown are representative of three separate experiments.

target compound **2c** showed both necrotic/apoptotic cell death as evidenced by 20% and 15% of PC-3 cells being PI^+ Annexin V^- and PI^- Annexin V^+. Neither apoptosis nor necrosis was observed in PC-3 cells treated with the compound **2f**.

Overall, these results indicate that the cytotoxic activity of target compound **2d** on androgen-independent PC3 prostate cancer cells detected by SRB assay, is the result of its ability to induce oligonucleosomal DNA fragmentation and apoptotic cell death.

3. Conclusions

It can be concluded that the 2-[ω-(alkylamino)alkyl]-9-methoxy-5-nitro-2,6-dihydroindazolo[4,3-*bc*][1,5]naphthyridines **2** represent a new class of aza-acridine derivatives endowed with noticeable antitumor properties. The 10-aza substitution of 5-nitropyrazolo[3,4,5-*kl*]acridines **1** yielded derivatives **2** endowed with enhanced DNA affinity and similar in vitro cytotoxic activity in respect to reference compound PZA, but specially, with a capacity to induce oligonucleosomal DNA fragmentation and apoptotic cell death, not present in PZA. In particular the 9-methoxy-5-nitro-2-[2-(tetrahydro-1*H*-1-pyrrolyl)ethyl]-2,6-dihydroindazolo[4,3-*bc*][1,5]-naphthyridine (**2d**), which possesses the most relevant biological characteristics in the series, can be regarded as a new lead in the field of potential anticancer derivatives. Activation of programmed cell death in cancer cells offer novel and potentially useful approaches to improving patient responses to conventional chemotherapy. Thus, the ability of this compound to early induce oligonucleosomal DNA fragmentation and apoptotic cell death of the hormone-refractory PC-3 prostate cancer cells may be particulary relevant to overcoming drug resistance or sensitize tumor cells to the effects of other antineoplastic agents.

4. Experimental

4.1. Chemistry

Melting points were determined on a Büchi 540 apparatus and are uncorrected. Thin-layer chromatography (TLC) was accomplished using plates precoated with silica gel 60 F-254 (Merck). All ^{1}H NMR spectra were recorded on a Varian VXR 300 instrument. Chemical shifts are reported as δ values (parts per million) downfield from internal Me_4Si in the solvent shown. The following NMR abbreviations are used: br (broad), s (singlet), d (doublet), t (triplet), m (multiplet), ar (aromatic proton), ex (exchangeable with D_2O). Elemental analyses were performed on a EA1108CHAZ-O elemental analyzer (Fisons Instruments).

4.1.1. 6-Chloro-2-[(6-methoxy-3-pyridyl)amino]-3-nitrobenzoic acid (5). The 2,6-dichloro-3-nitrobenzoic acid (**4**, 0.5 g, 2.12 mmol) was added in portions to 6-methoxy-3-pyridinamine (**3**, 1.05 g, 8.48 mmol) in 10 min. The resulting mixture was heated at 75 °C under stirring for 24 h. After cooling, 1 N aqueous Na_2CO_3 (5 mL) was poured in the mixture and the solid precipitated was filtered off. The solution, acidified with 2 M aqueous HCl, was extracted with $CHCl_3$ (3×30 mL). The organic layer was worked up to give **5** as a solid residue (0.55 g, 80%) pure enough, TLC eluted with $CHCl_3$/MeOH (4:1), to be used for the next step. A small sample was further purified by dissolution in methanol (2 mL) and elution with $CHCl_3$/MeOH (30:1) in a silica gel column to afford pure **5**; mp 104–105 °C; ^{1}H NMR (DMSO-d_6): δ 3.82 (s, 3H, CH_3), 6.71 (d, 1H, ar), 7.20 (d, 1H, ar), 7.38 (d, 1H, ar), 7.85 (s, 1H, ar), 8.10 (d, 1H, ar), 8.82 (s, 1H, COOH ex), 13.54 (br s, 1H, NH, ex).

4.1.2. 9-Chloro-2-methoxy-6-nitro-5,10-dihydrobenzo[*b*]-[1,5]naphthyridin-10-one (6). The 6-chloro-2-[(6-methoxy-3-pyridyl)amino]-3-nitrobenzoic acid (**5**, 100 mg, 0.31 mmol) was added of H_2SO_4 (0.5 mL) and heated at 100 °C for 30 min. The obtained mixture was poured into crushed ice to yield an orange precipitate, which was filtered, washed successively with water and methanol, and dried at room temperature. The resulting solid **6** (80 mg, 84%) pure enough, TLC eluted with $CHCl_3$/MeOH (99:1), was used for next step. A small sample was further purified by dissolution in hot methanol (1 mL) and flash elution with $CHCl_3$ in a silica gel column to afford pure **6**; mp 191–193 °C; ^{1}H NMR (DMSO-d_6): δ 3.97 (s, 3H, CH_3), 7.32 (d, 1H, ar), 7.42 (d, 1H, ar), 8.48–8.58 (m, 2H, ar), 11.62 (s, 1H, NH, ex).

4.1.3. 2-[2-(Dimethylamino)ethyl]-9-methoxy-5-nitro-2,6-dihydroindazolo[4,3-*bc*][1,5]naphthyridine (2a). Example of general procedure for the preparation of 2a–f. *N*-(2-Hydrazinoethyl)-*N*,*N*-dimethylamine[5] (0.16 g, 1.33 mmol) was added to a suspension of compound **6** (0.2 g, 0.65 mmol) in tetrahydrofuran/methanol 1:1 (7 mL). The mixture was stirred at room temperature for 3 h, the orange precipitate obtained was filtered, and washed with tetrahydrofuran/methanol 1:1. The dried crude solid obtained was suspended in $CHCl_3$ and flash chromatographed in a silica gel column eluted with $CHCl_3$/MeOH (19:1) to yield pure **2a** (78%): mp 257–258 °C; hydrochloride mp >300 °C; ^{1}H NMR (DMSO-d_6): δ 2.20 (s, 6H, $2 \times NCH_3$), 2.76 (t, 2H, CH_2-2′), 3.97 (s, 3H, OCH_3), 4.52 (t, 2H, CH_2-1′), 6.92 (d, 1H, ar), 7.03 (d, 1H, ar), 7.92 (d, 1H, ar), 8.48

(d, 1H, ar), 11.38 (s, 1H, NH, ex). Anal. Calcd for $C_{17}H_{18}N_6O_3$: C, 57.62; H, 5.12; N, 23.72. Found: C, 57.23; H, 5.09; N, 23.93.

Derivatives **2b–f** were prepared in a similar manner.

4.1.4. 2-[3-(Dimethylamino)propyl]-9-methoxy-5-nitro-2,6-dihydroindazolo[4,3-*bc*][1,5]naphthyridine (2b). (58%); mp 257–258 °C; hydrochloride 268–270 °C dec; ^{1}H NMR (DMSO-d_6): δ 1.95–2.13 (m, 2H, CH_2-2′), 2.14–2.33 (m, 8H, $2\times NCH_3$+CH_2-3′), 3.95 (s, 3H, OCH_3), 4.45 (t, 2H, CH_2-1′), 6.88 (d, 1H, ar), 7.03 (d, 1H, ar), 7.91 (d, 1H, ar), 8.46 (d, 1H, ar), 11.38 (s, 1H, NH, ex). Anal. Calcd for $C_{18}H_{20}N_6O_3$: C, 58.69; H, 5.47; N, 22.81. Found: C, 58.83; H, 5.29; N, 22.93.

4.1.5. 2-[2-(Diethylamino)ethyl]-9-methoxy-5-nitro-2,6-dihydroindazolo[4,3-*bc*][1,5]naphthyridine (2c). (58%); mp 249–250 °C; hydrochloride mp >300 °C; ^{1}H NMR (DMSO-d_6): δ 0.85 (t, 6H, $2\times NCH_3$), 2.42–2.52 (m, 4H, $2\times$diethyl CH_2), 2.90 (t, 2H, CH_2-2′), 3.98 (s, 3H, OCH_3), 4.49 (t, 2H, CH_2-1′), 6.90 (d, 1H, ar), 7.06 (d, 1H, ar), 7.95 (d, 1H, ar), 8.48 (d, 1H, ar), 11.40 (s, 1H, NH, ex). Anal. Calcd for $C_{19}H_{22}N_6O_3$: C, 59.67; H, 5.80; N, 21.98. Found: C, 59.88; H, 5.59; N, 22.13.

4.1.6. 9-Methoxy-5-nitro-2-[2-(1-pyrrolidinyl)ethyl]-2,6-dihydroindazolo[4,3-*bc*][1,5]naphthyridine (2d). (77%); mp 249–250 °C dec; hydrochloride mp 248–250 °C; ^{1}H NMR (DMSO-d_6): δ 2.58–2.70 (m, 4H, $2\times CH_2$-2″), 2.52–2.62 (m, 4H, $2\times CH_2$-3″), 3.00 (t, 2H, CH_2-2′), 3.98 (s, 3H, OCH_3), 4.58 (t, 2H, CH_2 -1′), 6.93 (d, 1H, ar), 7.04 (d, 1H, ar), 7.91 (d, 1H, ar), 8.46 (d, 1H, ar), 11.41 (s, 1H, NH, ex). Anal. Calcd for $C_{19}H_{20}N_6O_3$: C, 59.99; H, 5.30; N, 22.09. Found: C, 59.76; H, 5.48; N, 22.27.

4.1.7. 9-Methoxy-5-nitro-2-(2-piperidinoethyl)-2,6-dihydroindazolo[4,3-*bc*][1,5]naphthyridine (2e). (77%); mp 290–291 °C; hydrochloride mp >300 °C; ^{1}H NMR (DMSO-d_6): δ 1.20–1.55 (m, 6H, $2\times CH_2$-2″+CH_2-3″), 2.31–2.54 (m, 4H, 2 CH_2-1″), 2.63–2.90 (m, 2H, CH_2-2′), 3.98 (s, 3H, OCH_3), 4.42–4.63 (m, 2H, CH_2-1′), 6.88 (d, 1H, ar), 7.02 (d, 1H, ar), 7.90 (d, 1H, ar), 8.46 (d, 1H, ar), 11.40 (s, 1H, NH, ex). Anal. Calcd for $C_{20}H_{22}N_6O_3$: C, 60.90; H, 5.62; N, 21.31. Found: C, 60.67; H, 5.89; N, 21.05.

4.1.8. 9-Methoxy-2-(2-morpholinoethyl)-5-nitro-2,6-dihydroindazolo[4,3-*bc*][1,5]naphthyridine (2f). (38%); mp 242–243 °C; hydrochloride mp 265–267 °C dec; ^{1}H NMR (DMSO-d_6): δ 2.43 (t, 4H, $2\times OCH_2$), 2.81 (t, 4H, $2\times NCH_2$), 3.45 (t, 2H, CH_2-2′), 3.98 (s, 3H, OCH_3), 4.57 (t, 2H, CH_2-1′), 6.95 (d, 1H, ar), 7.05 (d, 1H, ar), 7.96 (d, 1H, ar), 8.50 (d, 1H, ar), 11.42 (s, 1H, NH, ex). Anal. Calcd for $C_{19}H_{20}N_6O_4$: C, 57.57; H, 5.09; N, 21.20. Found: C, 57.35; H, 4.80; N, 21.37.

4.2. Fluorescence binding studies

The fluorometric assays have been described previously.[19] The C_{50} values for ethidium displacement from CT-DNA, A–T, and G–C were determined using aqueous buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.0) containing 1.26 μM ethidium bromide and 1 μM CT-DNA, A–T, and G–C, respectively.[19–21]

All measurements were made in 10-mm quartz cuvettes at 20 °C using a Perkin–Elmer LS5 instrument (excitation at 546 nm; emission at 595 nm) following serial addition of aliquots of a stock drug solution (~5 mM in DMSO). The C_{50} values are defined as the drug concentrations, which reduce the fluorescence of the DNA-bound ethidium by 50%, and are calculated as the mean from three determinations.

4.3. In vitro cytotoxicity assay

Human androgen-independent prostate adenocarcinoma cell line (PC-3) was used for cytotoxicity testing in vitro using the SRB (Sulforhodamine B) assay.[22] Cells were maintained as stocks in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco). Cell cultures were passaged twice weekly using trypsin-EDTA to detach the cells from their culture flasks. The rapidly growing cells were harvested, counted, and incubated under the appropriate concentrations (7×10^5 cells/well) in 96-well microtiter plates. After incubation for 24 h, target and reference compounds dissolved in culture medium were applied to the culture wells in quadruplicate and incubated for 48 h at 37 °C in a 5% CO_2 atmosphere and 95% relative humidity. At the same time a plate is tested to value the cell population before the drug addition (T_z). Culture fixed with cold trichloroacetic acid (TCA) (J. T. Baker B.V., Deventer, Holland), were stained by 0.4% Sulforhodamine B (SRB) (Sigma–Aldrich, Milan, Italy) dissolved in 1% acetic acid. Bound stain is subsequently solubilized with 10 mM Trizma (Sigma–Aldrich, Milan, Italy), and the absorbance read on the microplate reader Dynatech Model MR 700 at a wavelength of 520 nm. The cytotoxic activity was evaluated by measuring the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation (GI_{50}), the drug concentration resulting in total growth inhibition (TGI) and the drug concentration resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning (LC_{50}). The percentage of growth inhibition was calculated as $[(T_i - T_z)/(C - T_z)]\times100$ for concentration for which $T_i \geqslant T_z$; $[(T_i - T_z)/T_z]\times100$ for concentration for which $T_i < T_z$, where T_z = absorbance time zero, C = absorbance in presence of vehicle and T_i = absorbance in presence of drug at different concentration. GI_{50}, TGI, and LC_{50} were obtained by interpolation in a graph % of growth versus Log(M). Each quoted value represents the mean of quadruplicate.

4.4. Apoptotic assays

Apoptosis of PC-3 cells treated with about $1\times LD_{50}$ of target compounds and reference was evaluated by Annexin V binding[17] and biparametric PI/Annexin V cytofluorimetric analysis as well as agarose gel electrophoresis.[18] To detect early stages of apoptosis,

the expression of Annexin V, a Ca^{2+}-dependent phospholipid-binding protein with high affinity for phosphatidylserine was employed. Moreover, simultaneous staining of cells with FITC-Annexin V and with PI, allows the discrimination of intact cells (Annexin V PI^-), early apoptotic (Annexin V^+ PI^-) and late apoptotic or necrotic cells (Annexin V^+ PI^+).[23] Apoptotic cells become Annexin$^-$ V^+ after nuclear condensation has started, but before the cells becomes permeable to PI. Briefly, 2×10^6 PC-3 cells treated with $1 \times LC_{50}$ of target compounds **2c**, **2d**, and **2f** (50 μM) and parent compound PZA (70 μM) for 6 h, were resuspended in 0.2 mL of binding buffer (10 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) in the presence of 5 μL of FITC-Annexin V (Bender MedSystem, Vienna, Austria), were incubated for 10 min at room temperature in the dark. Cells were washed, resuspended in 0.2 mL of binding buffer containing 10 μL of PI (20 μg/mL in PBS) (Molecular Probes, Eugene, OR) and then analyzed as above mentioned.

The percentage of positive cells determined over 10,000 events was analyzed on a FACScan cytofluorimeter (Becton Dickinson, San Jose', CA). Fluorescence intensity is expressed in arbitrary units on a logarithmic scale.

4.5. DNA fragmentation assay

Time course of target compounds **2c**,**d**,**f**-induced nucleosomal DNA fragmentation was performed by agarose gel electrophoresis. Briefly, 5×10^5/mL were cultured at 37 °C, 5% CO_2, treated with 50 μM of target compounds **2c**,**d**,**f** and 70 μM of reference compound PZA for different times (2, 4, 6, and 8 h). After treatment cells were washed and DNA was extracted using the Genomix Cells and Tissues Mini Preparations kit (Talent, Trieste, Italy). The DNA samples were electrophoresed on a 1.7% agarose gel and stained with ethidium bromide and acquired by a ChemiDoc (BioRad, Milano, Italy).

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