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Synthesis of Triazenoazaindoles: a New Class of Triazenes with Antitumor Activity

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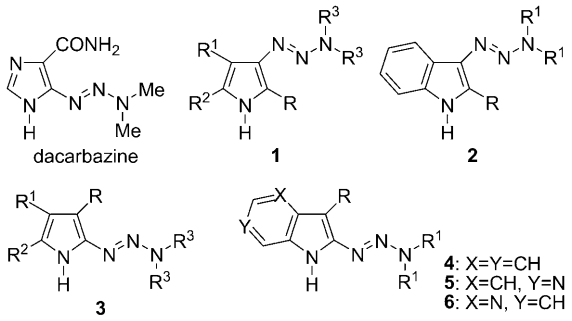
Despite improvements in the treatment and prevention of cancer, the number of new diagnoses continues to rise; this has fuelled substantial interest in the development of new and effective chemotherapeutic agents. Compounds of the triazene class, such as dacarbazine, have been used in the clinical management of many cancer types including brain, leukemia, and melanoma. A new compound class bearing a triazenoazaindole scaffold was synthesized with the aim of identifying new antiproliferative agents. Compounds **5a–g** and **6a–c** were screened against a panel of human tumor cell lines, and two of them, **5e** and **5f**, showed cytotoxicity (GI_{50} range: 2.2–8.2 μM) in all cell lines. These two compounds even maintained their cytotoxicity in some multidrug-resistant cell lines. Flow cytometry analysis demonstrated their ability to induce cell death by apoptosis with involvement of lysosomes.

Introduction

Cancer is a growing public problem estimated to have approximately seven million new incidences per year worldwide. Improvements in treatment and prevention have led to a decrease in cancer deaths, but the number of new diagnoses continues to rise. There is substantial and continuing interest in artificial molecules that bind and interact with DNA. Chemotherapeutic agents of the triazene class have been used in the clinical management of many tumors including brain, leukemia, and melanoma.[1] Dacarbazine, 5-(3,3-dimethyl-1-triazenyl)-imidazole-4-carboxamide (DTIC), has been one of the most widely used, as a single drug, to treat malignant melanoma and Hodgkin tumors resistant to the combination mustargen–oncovin–procarbazine–prednisone (MOPP) therapy.[2] The potent antitumor activity shown by dacarbazine led to the development of aryl and heteroaryl triazeno derivatives.[3] Among the derivatives belonging to the latter class of compounds, azole derivatives were the most important. In fact, triazenotriazoles,[4] –pyrazoles,[5] and -imidazoles[6] were active against several types of tumors and, within these series, the heterocyclic moiety seemed to modulate the antitumor activity: the more electron-rich the heterocyclic ring is, the higher is the potency.

Thus, we synthesized 3-triazenopyrrole of type **1**,[7] the most electron-rich of the azoles, which showed cytotoxic activity against Friend erythroleukemia cells (FLC) in the range of 1.1–3.1 μM and a mutagenic effect on *Streptomyces coelicolor*.[8] Benzocondensation on this series led to 3-triazenoindoles of type **2** which were 20–40-fold more active than the pyrrole derivatives against erythroleukemia and multidrug-resistant cells with IC_{50} values of 0.053–0.080 μM and 0.10–0.14 μM, respectively. This activity is similar to that of doxorubicin, even if there is no structural relationship.[9] Moreover, 2-triazenopyrroles of type **3** proved to be cytotoxic against leukemia, lymphoma, and carcinoma with an IC_{50} value of 3.9–21 μM[10] and



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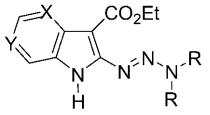
the corresponding benzofused triazenes of type **4** showed activity on breast, ovarian, and CNS cancers with IC_{50} values in the range of 23–36 μM.[11]

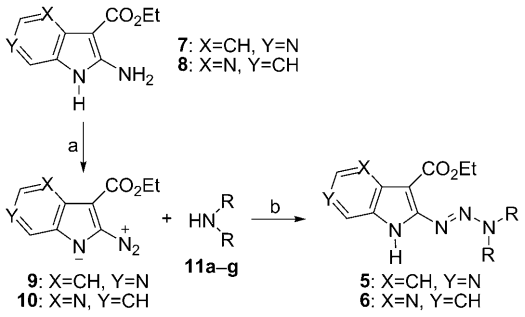
Recently, within the framework of a novel tumor targeting concept, it was demonstrated that the potency of triazenes could be enhanced by appending them to the classical structure of inhibitors of the epidermal growth factor receptor (EGFR) tyrosine kinase. This may ultimately confer new indications in refractory tumors expressing this oncogene. For example, Bcr-Abl is a constitutively activated tyrosine kinase that is known to be the cause of chronic myelogenous leukemia (CML), a malignancy of hematopoietic stem cells.[12] Moreover, it has been shown that Bcr-Abl induces resistance to cytotoxic drugs by upregulating DNA repair mechanisms. These results lend support to strategies that seek to combine a DNA damaging agent and a Bcr-Abl inhibitor into a single molecule to induce enhanced potency in CML cells.[13] It was demonstrated that some triazene derivatives inhibited the EGF receptor at the level of ATP binding.[14]

Considering the interest in triazene chemistry, and consequently in their biological properties, and the inhibitory activity of EGFR by several heterocyclic systems such as pyrroles, indoles, and diazaindoles,[15] we extended our research to include the synthesis of a new triazene class, having an azaindole nucleus. Our aim was to evaluate their anti-neoplastic activity, both as alkylating agents and potential EGFR kinase inhibitors. In particular, we focused our attention on the synthesis of 2-(3,3-dialkyltriaz-1-en-1-yl)-1*H*-pyrido[2,3-*c*]pyrroles of type **5** and 2-(3,3-dialkyltriaz-1-en-1-yl)-1*H*-pyrido[3,2-*b*]pyrroles of type **6**.

Table 1. 2-(3,3-Dialkyltriaz-1-en-1-yl)-1*H*-pyrido[2,3-*c*]pyrroles **5a–g** and 2-(3,3-dialkyltriaz-1-en-1-yl)-1*H*-pyrido[3,2-*b*]pyrroles **6a–c**.



Compd	X	Y	R	Yield [%]
5a	CH	N	Et	53
5b	CH	N	R–R: -$(CH_2)_4$-	53
5c	CH	N	*i*Pr	48
5d	CH	N	Me	46
5e	CH	N	Bu	52
5f	CH	N	Bn	49
5g	CH	N	R–R: -$(CH_2)_5$-	59
6a	N	CH	Et	45
6b	N	CH	R–R: -$(CH_2)_4$-	43
6c	N	CH	*i*Pr	43



Scheme 1. Synthesis of 2-(3,3-dialkyltriaz-1-en-1-yl)-1*H*-pyrido[2,3-*c*]pyrroles **5** and 2-(3,3-dialkyltriaz-1-en-1-yl)-1*H*-pyrido[3,2-*b*]pyrroles **6**. *Reagents and conditions*: a) AcOH, $NaNO_2$, 0 °C, RT, N_2 atmosphere, 1 h, then Na_2CO_3/H_2O, 0 °C, RT, N_2 atmosphere; b) CH_2Cl_2, RT, N_2 atmosphere, 22 h.

Results and Discussion

Synthesis of triazenopyridopyrroles 5 and 6

2-Triazenopyridopyrroles of type **5** and **6** were prepared in two steps starting from the 2-amino-3-ethoxycarbonylpyrrolopyridines **7** or **8**. The 2-aminopyridopyrroles **7** and **8** were converted, in nearly quantitative yields (96–98%), into the corresponding 2-diazo-3-ethoxycarbonylpyridopyrroles **9** and **10**, by diazotization in acetic acid with a stoichiometric amount of sodium nitrite under nitrogen atmosphere in the dark followed by neutralization with sodium carbonate. The strict control of the temperature at 0 °C both during diazotization and neutralization is crucial to obtain a high yield.[16] The 2-(3,3-dialkyltriaz-1-en-1-yl)-1*H*-pyrido[2,3-*c*]pyrroles **5a–g** and 2-(3,3-dialkyltriaz-1-en-1-yl)-1*H*-pyrido[3,2-*b*]pyrroles **6a–c** were obtained in acceptable yields (43–59%; see Table 1) through coupling reaction, with a large excess (ratio 1:10), of the suitable secondary amines **11a–g** in anhydrous dichloromethane in the dark at room temperature under nitrogen atmosphere or with bubbling anhydrous dimethylamine (Scheme 1). The reaction was carried out until the diazo stretch band (~2100 cm^{-1}) disappeared (22 h).

Biology

Antiproliferative activity

Cytotoxicity was determined by the MTT test after 72 h of treatment with compounds in five human tumor cell lines:[17] Jurkat (T-cell leukemia); K-562 (chronic myeloid leukemia); CEM (T-cell leukemia); LoVo (colon carcinoma), and A-431 (vulvar squamous cell carcinoma). Table 2 shows the extent of cell survival expressed as GI_{50}, the concentration which induces 50% of inhibition of cell growth after 72 h of cell incubation in the presence of the test compounds. Dacarbazine was used as reference compound because of its structural similarity with triazenopyridopyrrolo derivatives.

Compounds **5e** and **5f** showed good cytotoxicity in all tumor cells and they presented GI_{50} values lower than 10 μM in all test cell lines. These last two compounds are characterized by highly hydrophobic substituents at the nitrogen at position 3 of the triazene group such as benzyl and butyl groups. Other compounds showed cytotoxic activity at the concentrations used only toward the EGFR-overexpressing cell line (A-431),[18] and this fact could be linked to an inhibitory action toward EGFR. Dacarbazine was not cytotoxic in vitro, as it requires in vivo hepatic enzymatic activation.[1c]

Table 2. Growth inhibitory activity of triazenopyridopyrroles **5** and **6** against a panel of human tumor cell lines.

Compd	GI_{50} [μM] Jurkat[a]	K-562[a]	CEM[a]	LoVo[a]	A-431[a]
5a	>20	>20	>20	>20	12.6±2.7
5b	>20	>20	>20	>20	14.8±2.5
5c	>20	>20	>20	>20	11.5±1.4
5d	>20	>20	>20	>20	5.1±1.4
5e	2.2±0.4	5.5±0.4	4.9±0.8	3.3±0.6	4.8±0.6
5f	2.5±0.3	8.2±0.8	5.1±0.7	4.5±0.5	4.4±0.9
5g	>20	>20	>20	>20	>20
6a	>20	>20	>20	>20	7.6±0.9
6b	>20	>20	>20	>20	>20
6c	12.6±1.1	>20	>20	>20	10.9±1.5
DITC[b]	>20	>20	>20	>20	>20

[a] Concentration causing 50% growth inhibition of tumor cells after 72 h incubation; values are the mean ±SEM of at least three independent experiments. [b] DITC = dacarbazine.

Cytotoxicity in multidrug-resistant cell lines

One of the main problems of cancer chemotherapy is the onset of tumor cell resistance, which can derive from different mechanisms. Among these mechanisms, one is the overexpression of P-glycoprotein, which increases cell efflux of antitumor drugs. The cytotoxicity of the two most cytotoxic triazenopyridopyrroles was also evaluated in two resistant cell lines, because of the importance of resistance phenomena in the efficacy of cancer therapy: CEM^{Vbl100}, a T-cell leukemia line selected under continuous treatment with vinblastine which shows a classical multidrug-resistance phenotype and overexpresses the *mdr1* gene,[19] and $LoVo^{Doxo}$, a colon adenocarcinoma cell line resistant to doxorubicin and to a number of intercalating agents such as various anthracyclines, mitoxantrone, and ametantrone.[20] As shown in Table 3, the examined compounds are almost equally potent toward cells resistant to vinblastine and doxorubicin relative to the parent cell lines.

Table 3. Effect of triazenopyridopyrroles against various drug-resistant cell lines.

Compd	GI_{50} [μM] CEM	CEM^{Vbl100}	LoVo	$LoVo^{Doxo}$
5e	4.9±0.8[a]	8.6±0.9[a] (1.7)[b]	3.3±0.6[a]	6.1±0.9[a] (1.8)[b]
5f	5.1±0.7[a]	9.5±1.5[a] (1.9)[b]	4.5±0.5[a]	7.1±1.4[a] (1.6)[b]
Vbl[c]	0.004±0.0002[a]	0.21±0.03[a] (525)[b]	ND[d]	ND[d]
Doxo[e]	ND[d]	ND[d]	0.12±0.03[a]	13.5±0.12[a] (112.5)[b]

[a] Concentration causing 50% growth inhibition of tumor cells after 72 h of incubation; values are the mean ±SEM of at least three independent experiments. [b] Values in brackets are fold resistance, indicating the decrease potency of the compound in the resistant cell lines. [c] Vbl = vinblastine. [d] ND = not determined. [e] Doxo = doxorubicin.

Colony formation assay

Treating cells with the most active compounds for 24 h decreased the growth of colonies (7–14 days) in the clonogenic survival assay in a concentration-dependent manner. Exposure of cells to compounds **5e** and **5f** at 50 and 20 μM almost completely eliminated the formation of colonies (Figure 1). These results indicate that the compounds inhibited long-term survival of A-431 cells at micromolar concentrations.

Evaluation of cell death

The evaluation of the mode of cellular death was carried out with the two most cytotoxic compounds: **5e** and **5f**. We performed a flow cytometry test that allows us to investigate the kind of cellular death (necrosis or apoptosis) induced by 2-triazenopyridopyrroles. Apoptosis is characterized by a variety of morphological changes, for example, in the plasma membrane. In apoptotic cells, the phospholipid phosphatidylserine (PS) is early translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a Ca^{2+}-dependent phospholipid binding protein with high affinity for PS and it easily binds to cells with exposed PS. We performed biparametric cytofluorimetric analysis using propidium iodide (PI) and Annexin V–FITC conjugates, which stain DNA and PS residues, respectively.[21]

Experiments were performed in Jurkat cells after 6 and 24 h of incubation with the chosen compounds. After 6 h of incubation, no significant changes in Annexin-positive cells and cellular survival were detected with respect to control, whereas after 24 h, a concentration-dependent increase of late apoptotic cells was observed (Figure 2).

Evaluation of cell cycle

Cells were fixed and labeled with PI. This analysis was performed after 24 h of treatment of Jurkat cells with **5e** and **5f**. The results are presented in Table 4 and are expressed as percentage of cells in each cell-cycle phase for every sample, examples of cell-cycle analysis are shown in Figure 3. The most important change in the cell-cycle profile following incubation with triazenopyridopyrroles is the onset of a novel peak with lower DNA content than the G_1 peak. This new peak, often referred as sub-G_1, is found in cells undergoing apoptosis. Moreover, the percentages of the sub-G_1 peak in treated cells are consistent with the amount of dead cells found in the previous experiment. Thus, triazeno derivatives were able to induce cell death by apoptosis.

Involvement of organelles in cellular death

Some cytofluorimetric experiments were performed to check the involvement of some organelles such as mitochondria and lysosomes in cellular death induced by triazenopyridopyrroles. One typical feature of mitochondrial dysfunction during cell death is the loss of mitochondrial membrane potential. This loss was checked by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanine (JC-1) cell staining after 24 h of Jurkat

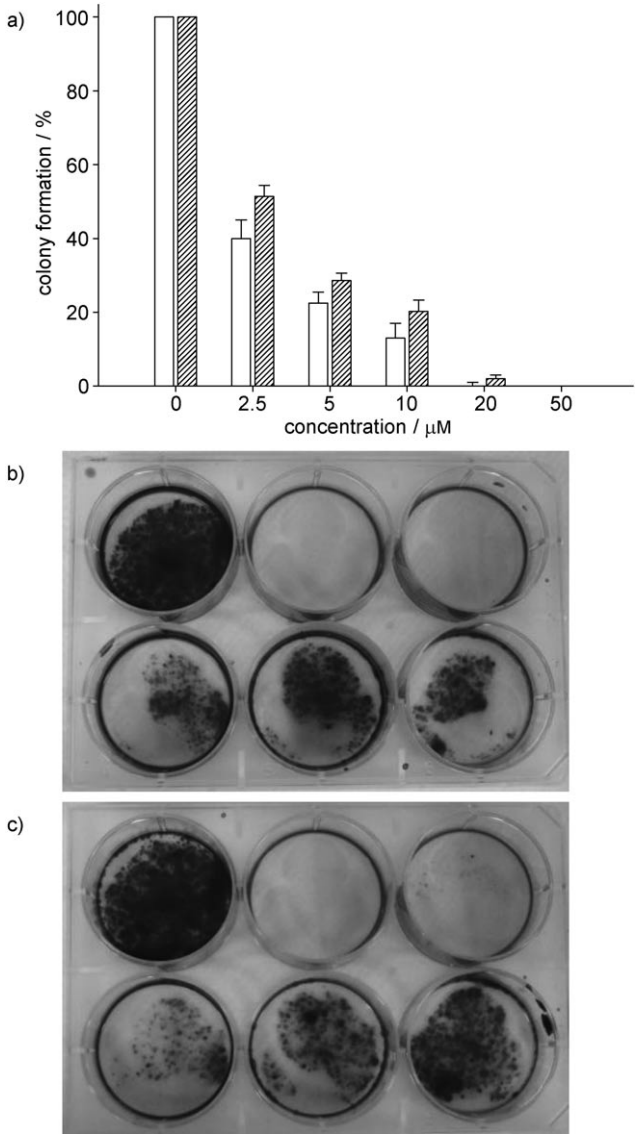


Figure 1. Colony formation assays were performed as described in the Experimental Section. a) Results are represented as percent colony formation relative to untreated cells (0 μM = 100%; **5e**: white bars, **5f**: hashed bars); error bars represent the mean ± SEM of three independent experiments. The images below show representative wells of colonies stained with crystal violet after treatment with the indicated concentrations of b) **5e** or c) **5f**.

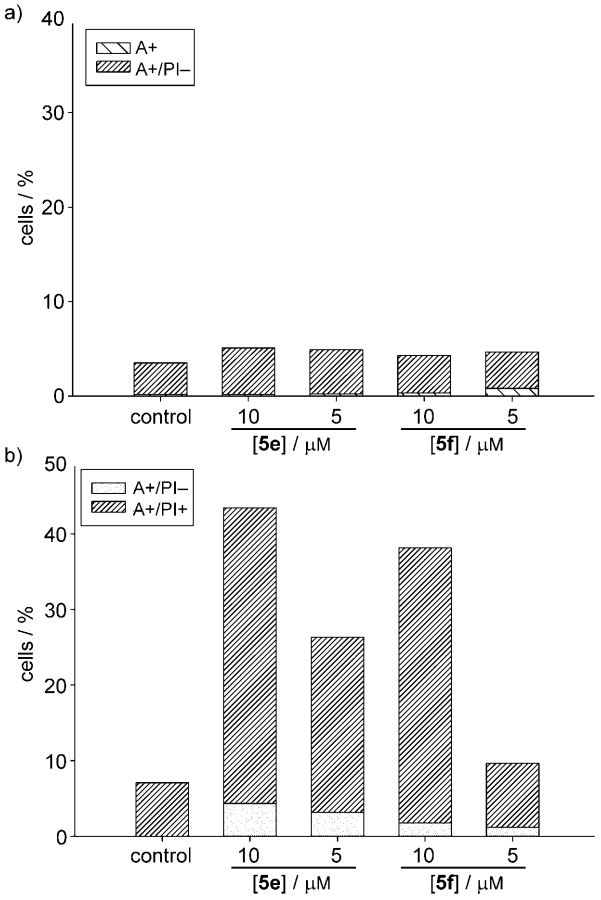


Figure 2. Exposure of phosphatidylserine and analysis of PI staining after a) 6 and b) 24 h of Jurkat cell treatment with **5e** and **5f** at 5 or 10 μM. Cells were stained with Annexin V–FITC (FL1) and of PI (FL3) and analyzed by flow cytometry; the percentages are expressed as the mean of three independent experiments. A+/PI−: fluorescence in FL-1, no fluorescence in FL-3; A+/PI+: fluorescence in FL-1 and FL-3.

Table 4. Cell-cycle analysis after 24 h of treatment with triazenopyridopyrroles.[a]

Compd	G_1 [%]	S [%]	G_2/M [%]	sub-G_1 [%]
Control	63.0	14.5	20.9	1.6
5e (10μM)	34.5	9.8	20.6	35.1
5e (5μM)	43.7	11.4	15.1	29.8
5f (10μM)	44.0	12.0	14.0	30.0
5f (5μM)	49.1	12.5	15.4	23.0

[a] Data indicate the percentage of cells in each phase.

cell treatment with compounds as described by Salvioli et al.[22] Lysosome involvement in cell death was also examined by staining Jurkat cells with acridine orange (AO), following the uptake method described by Zhao et al.[23] Although mitochondria seemed to be only marginally involved in apoptosis propagation, lysosomes played a major role in inducing cell death as can be observed by the concentration-dependent increase of lysosomal rupture in Figure 4.

EGFR inhibition

The human vulvar cancer cell line A-431, which overexpresses EGFR, was used to investigate whether these compounds could block EGF receptor signaling as many triazenopyridopyrroles were cytotoxic only toward this kind of cell line. After treating these cells with compounds for three hours, the blockage in EGF receptor signaling was detected by Western blotting using phospho-specific antibodies against the EGF receptor and its downstream signaling molecule ERK1/2. As a positive control, some cell samples were treated with a known EGFR inhibitor (AG1478). In Figure 5, a clear decrease in pEGFR and pERK1/2 signals was observed after treatment with AG1478, as a consequence of the inhibition of the activity of EGFR. In contrast, no band intensity decrease was detected in

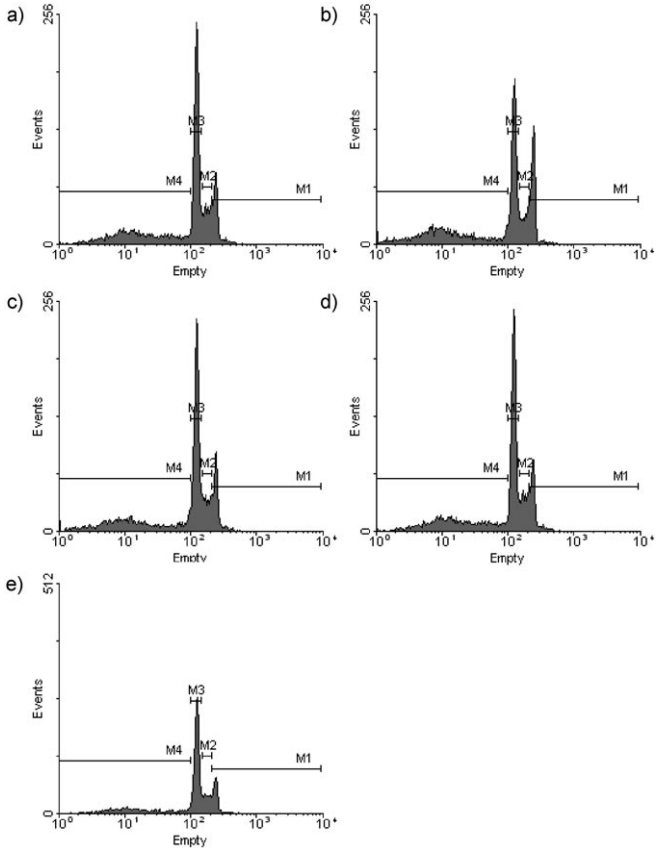


Figure 3. Representative cell-cycle profile of Jurkat cells incubated for 24 h with **5e** and **5f** at 5 and 10 μM. Cells were fixed and labeled with PI and analyzed by flow cytometry as described in the Experimental Section. a) control, b) 10 μM **5e**, c) 5 μM **5e**, d) 10 μM **5f**, e) 5 μM **5f**.

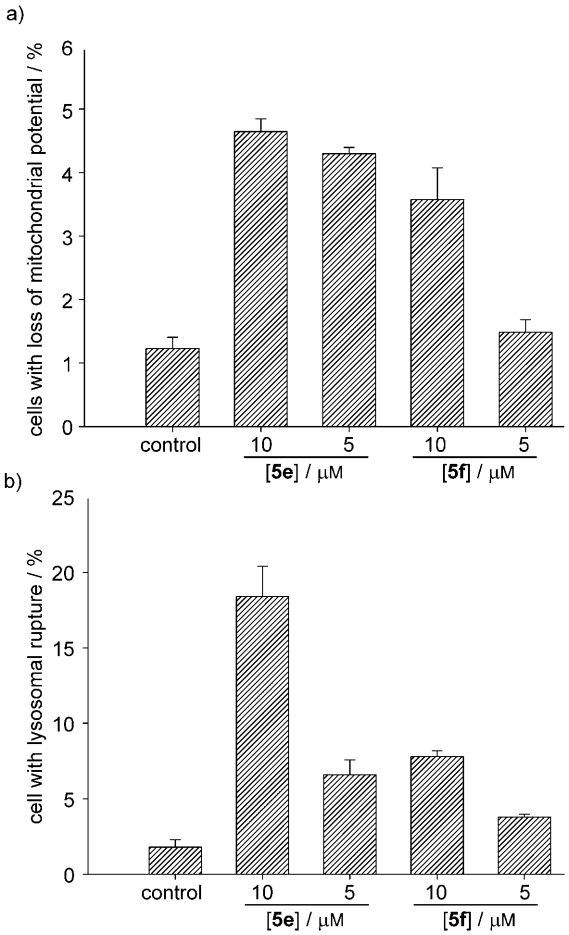


Figure 4. Involvement of organelles in cell death induced by triazenopyridopyrroles. a) Percent of cells with loss of mitochondrial membrane potential as measured by JC-1 staining after 24 h treatment of Jurkat cells with **5e** or **5f** at 5 and 10 μM. b) Percent of cells with lysosomal rupture as measured by flow cytometry AO fluorescence after 24 h treatment of Jurkat cells with **5e** or **5f**. Percentages are expressed as the mean ± SEM of at least three experiments.

triazeno derivatives samples relative to the EGF-stimulated control. Thus, the hypothesis is that EGFR is not the target of triazenopyridopyrrole compounds.

Evaluation of alkylating activity

As a dacarbazine mechanism is linked to covalent binding to DNA, the alkylation activity of some triazeno derivatives (**5d**–**5f**) was evaluated using a colorimetric assay, in which 4-(4′-nitrobenzyl)pyridine (NBP) was used as an alkylation substrate.[24] Alkylating compounds react with the pyridine nucleophilic nitrogen in a basic environment and the reaction can be observed by an absorbance increase. Triazenopyridopyrroles were not able to induce an absorbance increase at 545 nm; moreover, spectrophotometric measurements to check the stability in aqueous medium did not reveal any chemical decomposition of the compound (see, for example, the behavior of **5e** in Figure 6).

Conclusions

Herein we describe the synthesis and the antiproliferative properties of new derivatives having a triazenopyridopyrrole structure. Their antiproliferative activity was evaluated through

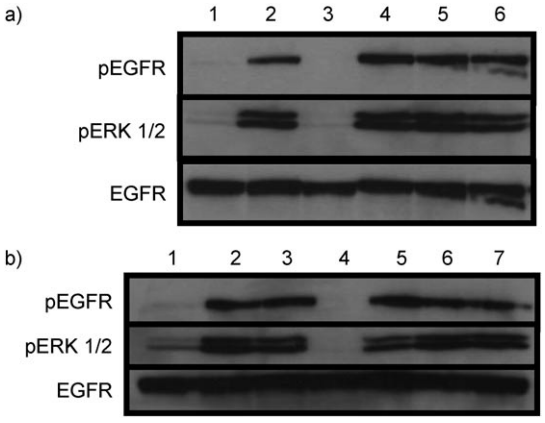


Figure 5. Cells were starved overnight and treated for 10 min with EGF (100 ng mL^{-1}) after 3 h of compound incubation. Cell lysates (100 μg) were separated by SDS-PAGE and the membrane probed with phospho-specific antibodies against the EGF receptor (P-Tyr1173) or ERK1/2 (P-Thr202/P-Tyr204). In addition, the membranes were probed with antibodies against total EGFR. a) 1: control without EGF, 2: control + EGF, 3: 5 μM AG1478 + EGF, 4: 20 μM **5a** + EGF, 5: 20 μM **5b** + EGF, 6: 20 μM **5c** + EGF; b) 1: control without EGF, 2: control + EGF, 3: 20 μM **5e** + EGF, 4: 5 μM AG1478 + EGF, 5: 20 μM **5f** + EGF, 6: 20 μM **6a** + EGF, 7: 20 μM **6c** + EGF.

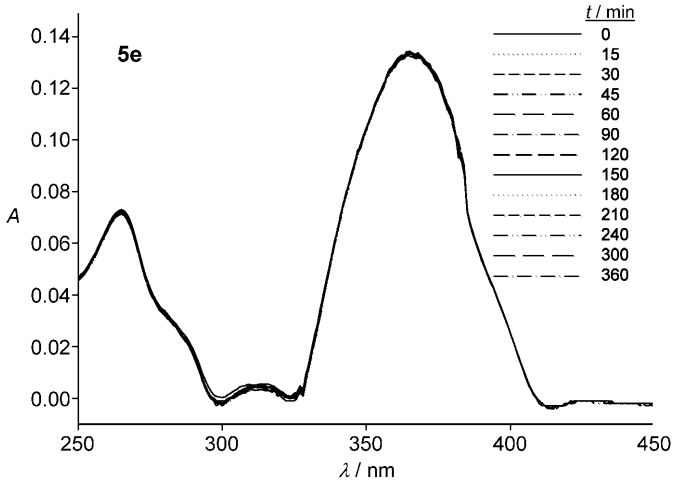


Figure 6. Representative example of compound stability in aqueous solution. The aqueous stability of **5e** (5 μM in PBS) at 37 °C at various time points is shown. The stability was verified by UV/Vis spectroscopy.

the classical MTT test in different human tumor cell lines and two of them, **5e** and **5f**, were cytotoxic in all cell lines investigated (GI_{50} range: 2.2–8.2 μM). These two compounds are characterized by the presence of two identical hydrophobic substituents with great steric hindrance at position 3 of the triazene group. As the MTT test only measures the cellular metabolic state, the antiproliferative effect of the two most active compounds was also checked using the colony formation assay, confirming their cytotoxicity.

One of the main problems of tumor treatment with chemotherapeutic drugs, together with the severe side effects, is the initiation of resistance, which is often mediated by the overexpression of the P-glycoprotein drug efflux pump. The most active compounds **5e** and **5f** also exhibited cytotoxic activity against drug-resistant cell lines (CEM^{Vbl100} and $Lovo^{Doxo}$), which overexpress that drug efflux pump. These results are promising as they suggest that these compounds might be useful in the treatment of drug refractory tumors.

The treatment of Jurkat cells with these derivatives caused cell death by apoptosis, as determined by the loss of membrane asymmetry (Annexin V positivity). Moreover, the cell-cycle effects of triazenopyridopyrroles were investigated to confirm the previous test results and to gain insight into their potential mechanism of action. Although the onset of the sub-G_1 peak was detected as a consequence of apoptotic machinery activation, there were no other clear changes in cell-cycle profiles of treated samples. Lysosomes seemed to be involved in apoptosis induction, whereas mitochondria had only a marginal role.

A possible alkylating activity was evaluated through a colorimetric method but triazenopyridopyrroles were found to be devoid of alkylating properties in vitro. Thus, the target of triazeno derivatives was not DNA, and this result seemed to be confirmed by the absence of accumulation of cells in the G_2 phase in the cell-cycle analysis. Moreover, **5e** and **5f** certainly have a different mechanism of action than dacarbazine, as they are cytotoxic in vitro without activation by liver microsomes.

As many triazeno derivatives were cytotoxic only toward the EGFR-overexpressing cell line and some triazene derivatives were found to be ATP competitive EGFR inhibitors, we also checked their possible EGFR inhibition. However, EGFR is not the target of triazeno derivatives. Further investigations are needed to identify the molecular target and are in progress.

Experimental Section

Chemistry

General: All melting points were taken on a Büchi-Tottoli capillary apparatus and are uncorrected; IR spectra were determined in bromoform with a Jasco FT/IR 5300 spectrophotometer; ^1H and ^{13}C NMR spectra were measured at 200 and 50.3 MHz, in $CDCl_3$ solution, using a Bruker Avance II series 200 MHz spectrometer [$(CH_3)_4Si$ as internal reference]. Column chromatography was performed with Merck silica gel 230–400 mesh ASTM or with Büchi Sepacore chromatography module (pre-packed cartridge system). Elemental analyses (C, H, N) were within ±0.4% of the theoretical values.

2-Diazo-3-ethoxycarbonylpyrrolo[2,3-*c*]pyridine (9) and 2-diazo-3-ethoxycarbonylpyrrolo[3,2-*b*]pyridine (10) were prepared as previously described[17] by reacting a solution of 2-amino-3-ethoxycarbonylpyrrolopyridine **7** and **8** (0.62 g, 3 mmol) in glacial acetic acid (6 mL) with a solution of sodium nitrite (0.21 g, 3 mmol) in a small amount of water (1 mL). The reaction was carried out at 0 °C under a nitrogen atmosphere. The mixture was neutralized at 0 °C with saturated Na_2CO_3 and the yellow solid precipitate was filtered off and dried under vacuum in the dark. The crude products, quickly shaken in cyclohexane and filtered off, gave 2-diazopyrrolopyridine **9** and **10**.

General method for the synthesis of 2-triazen-1-yl-1*H*-pyrido[2,3-*c*]pyrrolo-3-carboxylates (5a–g) and 2-triazen-1-yl-1*H*-pyrido[3,2-*b*]pyrrolo-3-carboxylates (6a–c). A solution of secondary amine (30 mmol) in anhydrous CH_2Cl_2 (20 mL) or bubbling anhydrous dimethylamine was added to a stirred solution of 2-diazopyrrolopyridine **9** or **10** (0.65 g, 3 mmol) in anhydrous CH_2Cl_2 (30 mL). The reaction mixture was kept in the dark at RT and under nitrogen atmosphere until the diazo stretch band at ~2100 cm^{-1} disappeared (22 h). Removal of the solvent and the excess of amine, under reduced pressure, gave the 2-triazenopyrrolopyridines **5** or **6**, respectively. The crude products were purified by column chromatography on silica gel using CH_2Cl_2/EtOAc (95:5) as eluent.

Ethyl 2-(3,3-diethyl-1-triazenyl)-1*H*-pyrido[2,3-*c*]pyrrolo-3-carboxylate (5a): yellow powder (0.46 g, 53%): R_f = 0.30 (CH_2Cl_2/MeOH 9:1); mp: 173–174 °C; ^1H NMR (200 MHz, $CDCl_3$): δ = 1.32–1.43 (m, 9H, 3×CH_3), 3.89–3.94 (m, 4H, 2×CH_2), 4.36–4.43 (m, 2H, CH_2), 8.02 (d, J = 5.5 Hz, 1H, H-4), 8.25 (d, J = 5.5 Hz, 1H, H-5), 8.73 (s, 1H, H-7), 9.50 ppm (s, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 10.9 (q), 14.2 (q), 14.6 (q), 43.0 (t), 50.3 (t), 59.8 (t), 97.3 (s), 115.8 (d), 130.7 (s), 131.4 (d), 134.5 (s), 138.4 (d), 153.9 (s), 164.7 ppm (s); IR (KBr): $\tilde{\nu}$ = 1681 cm^{-1} (CO); Anal. calcd (%) for $C_{14}H_{19}N_5O_2$: C 58.12, H 6.62, N, 24.21%; found: C 58.41, H 6.80, N 24.08.

Ethyl 2-(3,3-tetramethylelene-1-triazenyl)-1*H*-pyrido[2,3-*c*]pyrrolo-3-carboxylate (5b): yellow powder (0.46 g, 53%): R_f = 0.26 (CH_2Cl_2/MeOH 9:1); mp: 191–192 °C; ^1H NMR (200 MHz, $CDCl_3$): δ =

1.46 (t, $J=7.1$ Hz, 3H, CH_3), 2.05 (t, $J=3.3$ Hz, 4H, $2\times CH_2$), 3.82 (t, $J=3.3$ Hz, 2H, CH_2), 3.92 (t, $J=3.3$ Hz, 2H, CH_2), 4.43 (q, $J=7.1$ Hz, 2H, CH_2), 7.98 (d, $J=5.4$ Hz, 1H, H-4), 8.31 (d, $J=5.4$ Hz, 1H, H-5), 8.68 (s, 1H, H-7), 9.48 ppm (s, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta=14.6$ (q), 23.3 (t), 23.9 (t), 47.9 (t), 52.0 (t), 59.8 (t), 97.0 (s), 115.9 (d), 130.5 (s), 132.5 (d), 133.5 (s), 140.2 (d), 153.4 (s), 164.8 ppm (s); IR (KBr): $\tilde{\nu}=1980$ (NH), 1671 cm^{-1} (CO); Anal. calcd (%) for $C_{14}H_{17}N_5O_2$: C 58.52, H 5.96, N 24.37%; found: C 58.34, H 5.59, N 25.54.

Ethyl 2-(3,3-diisopropyl-1-triazenyl)-1*H*-pyrido[2,3-*c*]pyrrolo-3-carboxylate (5c): yellow powder (0.46 g, 48%): $R_f=0.23$ (CH_2Cl_2/MeOH 9:1); mp: 254–255 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta=1.35$–1.45 (m, 15H, $5\times CH_3$), 4.17 (sept, $J=6.5$ Hz, 1H, CH), 4.41 (q, $J=7.1$ Hz, 2H, CH_2), 5.38 (sept, $J=6.7$ Hz, 1H, CH), 7.97 (d, $J=5.4$ Hz, 1H, H-4), 8.30 (d, $J=5.4$ Hz, 1H, H-5), 8.63 (s, 1H, H-7), 9.39 ppm (s, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta=14.6$ (q), 19.0 (q×2), 23.5 (q×2), 49.0 (d), 51.2 (d), 59.6 (t), 96.8 (s), 115.7 (d), 130.4 (s), 132.8 (d), 133.5 (s), 140.7 (d), 152.9 (s), 164.8 ppm (s); IR (KBr): $\tilde{\nu}=2975$ (NH), 1708 cm^{-1} (CO); Anal. calcd (%) for $C_{16}H_{23}N_5O_2$: C 60.55, H 7.30, N 22.07; found: C 60.84, H 7.08, N 22.21.

Ethyl 2-(3,3-dimethyl-1-triazenyl)-1*H*-pyrido[2,3-*c*]pyrrolo-3-carboxylate (5d): yellow powder (0.36 g, 46%): $R_f=0.69$ (CH_2Cl_2/MeOH 9:1); mp: 186–187 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta=1.45$ (t, $J=7.1$ Hz, 3H, CH_3), 3.39 (s, 3H, CH_3), 3.58 (s, 3H, CH_3), 4.42 (q, $J=7.1$ Hz, 2H, CH_2), 7.98 (d, $J=5.5$ Hz, 1H, H-4), 8.31 (d, $J=5.5$ Hz, 1H, H-5), 8.67 (s, 1H, H-7), 9.56 ppm (s, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta=14.6$ (q), 37.1 (q), 43.9 (q), 59.8 (t), 97.4 (s), 115.9 (d), 130.4 (s), 132.9 (d), 133.2 (s), 140.5 (d), 152.4 (s), 164.7 ppm (s); IR (KBr): $\tilde{\nu}=3444$ (NH), 1685 cm^{-1} (CO); Anal. calcd (%) for $C_{12}H_{15}N_5O_2$: C 55.16, H 5.79, N 26.80; found: C 55.48, H 5.44, N 26.96.

Ethyl 2-(3,3-dibuthyl-1-triazenyl)-1*H*-pyrido[2,3-*c*]pyrrolo-3-carboxylate (5e): yellow powder (0.55 g, 52%): $R_f=0.45$ (CH_2Cl_2/MeOH 9:1); mp:147–148 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta=0.88$–1.00 (m, 7H), 1.34–1.47 (m, 7H), 1.65–1.78 (m, 4H), 2.84–2.92 (m, 1H), 3.72–3.91 (m, 3H), 4.41 (q, $J=7.1$ Hz, 2H, CH_2), 8.01 (d, $J=5.5$ Hz, 1H, H-4), 8.26 (d, $J=5.5$ Hz, 1H, H-5), 8.69 (s, 1H, H-7), 9.60 ppm (s, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta=13.7$ (q), 13.8 (q), 14.6 (q), 19.9 (t), 20.6 (t), 27.7 (t), 30.9 (t), 48.2 (t), 55.5 (t), 59.7 (t), 97.3 (s), 115.8 (d), 130.2 (s), 132.7 (d), 133.6 (s), 140.8 (d), 152.2 (s), 164.7 ppm (s); IR (KBr): $\tilde{\nu}=3438$ (NH), 1675 cm^{-1} (CO); Anal. calcd (%) for $C_{18}H_{27}N_5O_2$: C 62.58, H 7.88, N 20.27; found: C 62.47, H 8.12, N, 19.97.

Ethyl 2-(3,3-dibenzyl1-triazenyl)-1*H*-pyrido[2,3-*c*]pyrrolo-3-carboxylate (5f): yellow powder (0.61 g, 49%): $R_f=0.34$ (CH_2Cl_2/MeOH 9:1); mp: 133–135 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta=1.37$ (t, $J=7.1$ Hz, 3H, CH_3), 4.39 (q, $J=7.1$ Hz, 2H, CH_2), 4.88 (s, 2H, CH_2), 5.04 (s, 2H, CH_2), 7.15–7.34 (m, 10H, 10×CH), 8.00 (dd, $J=0.9$, 5.5 Hz, 1H, H-4), 8.30 (d, $J=5.5$ Hz, 1H, H-5), 8.68 (d, $J=0.9$ Hz, 1H, H-7), 9.46 ppm (s, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta=14.6$ (q), 49.6 (t), 57.8 (t), 59.8 (t), 98.26 (s), 116.0 (d), 127.9 (d), 128.0 (d×2), 128.4 (d), 128.7 (d×2), 129.0 (d×2), 129.3 (d×2), 130.8 (s), 132.9 (d), 133.5 (s), 134.4 (s), 135.0 (s), 140.3 (d), 151.9 (s), 164.6 ppm (s); IR (KBr): $\tilde{\nu}=3438$ (NH), 1683 cm^{-1} (CO); Anal. calcd (%) for $C_{24}H_{23}N_5O_2$: C 69.72, H 5.61, N 16.94; found: C 69.93, H 5.33, N 17.04.

Ethyl 2-(3,3-pentamethylelene-1-triazenyl)-1*H*-pyrido[2,3-*c*] pyrrolo-3-carboxylate (5g): yellow powder (0.53 g, 59%): $R_f=0.15$ (CH_2Cl_2/MeOH 9:1); mp: 198–199 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta=1.45$ (t, $J=7.1$ Hz, 3H, CH_3), 1.72 (s, 6H, $3\times CH_2$), 3.80 (s, 2H, CH_2), 4.07 (s, 2H, CH_2), 4.42 (q, $J=7.1$ Hz, 2H, CH_2), 7.97 (d, $J=5.5$ Hz, 1H, H-4), 8.29 (d, $J=5.5$ Hz, 1H, H-5), 8.68 (s, 1H, H-7), 9.58 ppm (s, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta=14.6$ (q), 23.9 (t), 24.6 (t), 26.4 (t), 44.6 (t), 53.9 (t), 59.8 (t), 97.2 (s), 115.8 (d), 130.6 (s), 132.9 (d), 133.3 (s), 140.5 (d), 152.9 (s), 164.7 ppm (s); IR (KBr): $\tilde{\nu}=2937$ (NH), 1675 cm^{-1} (CO); Anal. calcd (%) for $C_{15}H_{19}N_5O_2$: C 59.79, H 6.36, N 23.24; found: C 60.15, H 6.57, N 22.93.

Ethyl 2-(3,3-diethyl-1-triazenyl)-1*H*-pyrido[3,2-*b*]pyrrolo-3-carboxylate (6a): yellow powder (0.37 g, 45%): $R_f=0.20$ (CH_2Cl_2/MeOH 9:1); mp: 167–168 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta=1.28$–1.42 (m, 9H, $3\times CH_3$), 3.76–3.97 (m, 4H, $2\times CH_2$), 4.43 (q, $J=7.1$ Hz, 2H, CH_2), 7.04 (dd, $J=4.8$, 8.1 Hz, 1H, H-6), 7.52 (dd, $J=1.4$, 8.1 Hz, 1H, H-7), 8.48 (dd, $J=1.4$, 4.8 Hz, 1H, H-5), 9.45 ppm (s, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta=11.0$ (q), 14.3 (q), 14.7 (q), 42.8 (t), 49.9 (t), 59.8 (t), 98.3 (s), 117.2 (d), 117.4 (d), 127.0 (s), 144.1 (d), 145.5 (s), 152.0 (s), 164.2 ppm (s); IR (KBr): $\tilde{\nu}=2973$ (NH), 1683 cm^{-1} (CO); Anal. calcd (%) for $C_{14}H_{19}N_5O_2$: C 58.12, H 6.62, N 24.21; found: C 58.37, H 6.48, N 24.40.

Ethyl 2-(3,3-tetramethylelene 1-triazenyl)-1*H*-pyrido[3,2-*b*]pyrrolo-3-carboxylate (6b): yellow powder (0.37 g, 43%): $R_f=0.18$ (CH_2Cl_2/MeOH 9:1); mp: 164–165 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta=1.41$ (t, $J=7.1$ Hz, 3H, CH_3), 2.05–2.10 (m, 4H, $2\times CH_2$), 3.84 (t, $J=6.4$ Hz, 2H, CH_2), 3.94 (t, $J=6.2$ Hz, 2H, CH_2), 4.45 (q, $J=7.1$ Hz, 2H, CH_2), 7.06 (dd, $J=4.8$, 8.1 Hz, 1H, H-6), 7.53 (dd, $J=1.3$, 8.1 Hz, 1H, H-7), 8.49 (dd, $J=1.3$, 4.8 Hz, 1H, H-5), 9.49 ppm (s, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta=14.7$ (q), 23.4 (t), 23.9 (t), 47.8 (t), 51.8 (t), 59.9 (t), 98.2 (s), 117.3 (d), 117.5 (d), 127.0 (s), 144.0 (d), 145.3 (s), 152.5 (s), 164.3 ppm (s); IR (KBr): $\tilde{\nu}=1685$ cm^{-1} (CO); Anal. calcd (%) for $C_{14}H_{17}N_5O_2$: C 58.52, H 5.96, N 24.37; found: C 58.69, H 5.72, N 24.21.

Ethyl 2-(3,3-diisopropyl-1-triazenyl)-1*H*-pyrido[3,2-*b*]pyrrolo-3-carboxylate (6c): yellow powder (0.41 g, 43%): $R_f=0.15$ (CH_2Cl_2/MeOH 9:1); mp: 154–156.0 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta=1.32$–1.40 (m, 15H, $5\times CH_3$), 4.12 (sept, $J=6.6$ Hz, 1H, CH), 4.41 (q, $J=7.1$ Hz, 2H, CH_2), 5.39 (sept, $J=6.8$ Hz, 1H, CH), 7.03 (dd, $J=4.8$, 8.0 Hz, 1H, H-6), 7.55 (dd, $J=1.3$, 8.0 Hz, 1H, H-7), 8.46 (dd, $J=1.3$, 4.8 Hz, 1H, H-5), 9.53 ppm (s, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta=14.7$ (q), 19.1 (q×2), 23.5 (q×2), 48.8 (d), 50.9 (d), 59.8 (t), 97.7 (s), 117.1 (d), 117.6 (d), 127.1 (s), 143.7 (d), 145.3 (s), 152.6 (s), 164.3 ppm (s), IR (KBr): $\tilde{\nu}=3315$ (NH), 1691 cm^{-1} (CO); Anal. calcd (%) for $C_{16}H_{23}N_5O_2$: C 60.55, H 7.30, N 22.07; found: C 60.85, H 7.15, N 22.00.

Biology

Chemicals: If not otherwise indicated, all the chemicals were purchased from Sigma–Aldrich (Milan, Italy).

Cell lines: Jurkat cells (human T-cell leukemia), K-562 cells (human chronic myeloid leukemia), and CEM cells (human T-cell leukemia) were grown in RPMI-1640 medium; LoVo cells (human intestinal adenocarcinoma) were grown in Ham's F12 medium, and A-431 cells (vulvar squamous cell carcinoma EGFR-overexpressing) were grown in Dulbecco's modified Eagle's medium (DMEM). All media were supplemented with 115 U mL^{-1} penicillin G, 115 μg mL^{-1} streptomycin, and 10% fetal bovine serum (Invitrogen, Milan, Italy). $CEM^{Vbl-100}$ is a multidrug-resistant line selected against vinblastine and were grown in complete RPMI-1640 medium supplemented with vinblastine (0.1 μg mL^{-1}).[19] $LoVo^{Doxo}$ is a doxorubicin resistant subclone of LoVo cells and the cells were grown in complete Ham's F12 medium supplemented with doxorubicin (0.1 μg mL^{-1}).[20]

Cellular toxicity: Individual wells of a 96-well tissue culture microtiter plate (Falcon, Becton-Dickinson, Italy) were inoculated with

100 mL of complete medium containing 5×10^3 of the above cited cells. Plates were incubated at 37 C° in a humidified 5% incubator for 24 h prior to the experiments. After medium removal, 100 mL of the drug solution, dissolved in DMSO and diluted with the suitable complete medium, were added to each well and incubated at 37 °C for 72 h. DMSO concentration was always lower than 1%. Cell viability was assayed by the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide)] test as described previously.[17] Analogous experiments were performed with drug-resistant cell lines.

Colony formation assay: A-431 cells were plated at 1000 per well in six-well plates to provide an optimal counting density. Cells were treated with compounds at different concentrations for 24 h. After 24 h, medium was replaced with fresh medium and cells were cultured for one to two weeks until well-defined colonies had formed (replacing culture medium every two to three days). Cells were briefly permeabilized with 0.9% saline solution and stained with 0.5% crystal violet in 20% methanol. Colonies of $\geq$50 cells were then counted visually. Data points represent the average of three values and error bars represent the SD.

Externalization of phosphatidylserine: Surface exposure of phosphatidylserine (PS) by apoptotic cells was measured by flow cytometry by adding Annexin V–FITC to cells according to the manufacturer's instructions (Annexin V Fluos, Roche Diagnostic). Simultaneously, cells were stained with propidium iodide (PI). Samples were incubated in the dark for 15 min and then analyzed using a BD FACS Calibur (Becton Dickinson, New York, USA) flow cytometer. The fluorescence of FITC and PI were collected in FL1 and FL3, respectively.[21] At least 10000 events for each sample were acquired.

Cell-cycle analysis: Jurkat cells were incubated for 24 h with or without the test compounds, then fixed with ice-cooled ethanol (70%), treated overnight with RNAse (100 $\mu g\,mL^{-1}$) in phosphate saline buffer and finally stained with PI (10 $\mu g\,mL^{-1}$). Samples were analyzed on a BD FACS Calibur flow cytometer. Results of cell-cycle analysis were examined using WinMDI 2.9 (Windows Multiple Document Interface for Flow Cytometry).

Mitochondrial dysfunction: Jurkat cells were incubated with or without different concentrations of test compounds for 24 h. Cells were collected by centrifugation (5 min, RT, 300 *g*) and resuspended in Hank's Balanced Salt Solution (HBSS) containing JC-1 at a concentration of 1 μM. The cytofluorimetric analysis (BD FACS Calibur flow cytometer) was performed collecting green (FL1) and orange (FL2) fluorescence in at least 10000 events for each sample.[22]

Lysosome dysfunction: After 24 h of treatment with different concentrations of test compounds, Jurkat cells were stained with AO at a concentration of 1 μM in RPMI-1640 at 37 °C for 15 min. The fluorescence was directly recorded with a flow cytometer (BD FACS Calibur) using 488 nm wavelength as excitation and emission in the FL3 channel.[23]

Protein extraction and Western blotting: A-431 cells (1×10^6) were seeded in 6 cm Petri dishes; after 24 h, they were washed with PBS and starved overnight. Then, cells were incubated with or without 20 μM of compounds for 3 h and stimulated with EGF 100 $ng\,mL^{-1}$ for 10 min. Cell extracts were prepared after scraping in lysis buffer [50 mM Tris-HCl pH 8.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM DTT, 30 mM NaPPi, 10 mM NaF, 1 mM Na_3VO_4, 100 nM okadaic acid, complete protease inhibitors (Roche)], incubating on ice for 15 min and clearing by centrifugation (20 min, 4 °C, 16000 *g*); 100 μg of protein was loaded in 12.5% SDS-PAGE and then blotted to a PVDF membrane (Bio-Rad). Membranes were blocked with 0.2% casein, 0.1% Tween-20 in PBS (blocking buffer) for 1 h and incubated with polyclonal anti-phospho-EGFR (Tyr1173) (Santa Cruz), anti-phospho-ERK1/2 (P-p42/p44) (Thr202/Tyr204) (Cell Signaling) overnight or with anti-EGFR (Santa Cruz) for 2 h. After washing in blocking buffer, the membranes were incubated with the proper secondary antibody coupled to alkaline phosphatase (Jackson Immunoresearch Laboratories) for 1 h. Visualization was performed with CDP-star (Tropix) according to the manufacturer's instructions.[25]

Alkylation of 4-(4′-nitrobenzyl)pyridine (NBP): 5 μL of NBP in DMSO (80 $mg\,mL^{-1}$) and 5 μL of compounds in DMSO (final concentrations: 10–100 μM) were added to 500 μL of 100 mM phosphate buffer (pH 7.4). Mixtures were incubated at 37 °C for 30 min; then, 1 mL of 1-octanol and 0.1 mL 10 M NaOH were added. Solutions were shaken and centrifuged at 10000 *g* for 2 min. The extend of alkylation was checked spectrophotometrically by recording the absorbance of the upper layer at 545 nm.[24]

Evaluation of aqueous stability: Solutions of compounds were prepared in PBS and were incubated at 37 °C. Their stability was checked by looking for changes in UV/Vis spectra on a PerkinElmer Lambda 15 spectrophotometer after precise incubation times.

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Keywords: antiproliferative activity · antitumor agents · EGF receptors · triazeno derivatives

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