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Pyrazolo-triazoles as Light Activable DNA Cleaving Agents

Stefano Manfredini,[a,*] Chiara Beatrice Vicentini,[a] Maurizio Manfrini,[a] Nicoletta Bianchi,[b] Cristina Rutigliano,[b] Carlo Mischiati[b] and Roberto Gambari[b]

[a]*Dipartimento di Scienze Farmaceutiche, Università di Ferrara, I-44100 Ferrara, Italy*
[b]*Dipartimento di Biochimica e Biologia Molecolare Via Fossato di Mortara 17-19, Università di Ferrara, I-44100 Ferrara, Italy*

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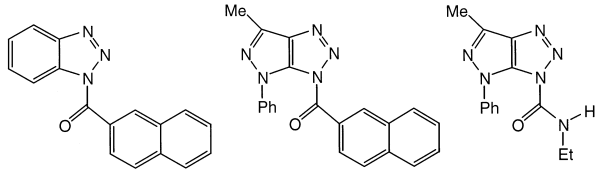
Abstract—In view of the continuous interest in new DNA cleaving compounds, both for the development of new therapeutic agents and for the possible use as reagents in nucleic acids research, a few pyrazolo[3,4-*d*][1,2,3]triazole derivatives have been obtained and investigated for their antiproliferative activity and capability to cleave DNA, after light-activation. A possible in situ activation, i.e. in neoplastic tissues, of less cytotoxic derivatives, may lead to potential antitumor compounds endowed with high therapeutic indexes.

Introduction

Bleomycin, Esperamicin, calicheamicin, dynemicin and neocarzinostatin are potent antitumor agents, based on a radical mechanism, which have attracted considerable interest in recent years due to their mode of action and potential use as reagents in nucleic acids research; in fact, these drugs exhibit potent DNA cleaving activity. Esperamicin, calicheamicin, dynemicin and neocarzinostatin activity is based on an en-diyne mechanism, due to an inducible cycloaromatization to an aryl or indenyl diradical, which abstracts hydrogens from proximate deoxyribosyl sites, leading to DNA scission.[1] Based on this mechanism of action, a few simple aryl (mono) radicals (i.e. **1**) have also been recently investigated as nucleic acid cleaving agents.[2] Indeed, benzotriazole (**4**) has been shown to be photochemically transformed into the intermediate **5** (Scheme 1).[2]

This reaction is proposed to proceed from the lowest excited singlet state of **4** (π,π^*), leading initially to an azoimine (**5**) stable only at low temperature, that converts thermally or photochemically to **6**. Singlet **6** can react directly or undergo intersystem crossing to the triplet that is capable of hydrogen abstraction and thereby of serving as an agent for DNA cleavage.[2] However, to the best of our knowledge, beside the above reported studies, the biological properties of these compounds were not further investigated.



1 2 3

As has been shown in the field of classical DNA-binders, both binding potency and specificity can be modulated by the substitution, on the reference molecule, of benzene ring with various heterocycles;[3] therefore, it was hypothesized that the introduction of a heterocyclic moiety would have been of importance to get further insight into structure–activity relationships. In these regards a pyrazole nucleus offers the advantages of stability and easy feasibility.[3] Taking advantage of our studies in the field of biologically active pyrazolo-related compounds, we have prepared two new representative pyrazolo-triazole derivatives (**2** and **3**), with the aim of identifying compounds able to modulate DNA-base specificity and photoinducible selective cytotoxicity. The 3-methyl-1-substituted-1-*H*-pyrazolo moiety was selected, besides for the facile preparation, because it has already been explored in the design of potential acridine analogues and DNA binders.[4,2] The synthesized compounds were tested for their ability to bind and cut DNA in comparison with the reported benzo-triazole **1**. Synthesis, photoinducible radical formation and preliminary biological studies will be briefly discussed here.

*Corresponding author. Tel.: +39-0532-291-292; fax: +39-532-29-1296; e-mail: mv9@dns.unife.it

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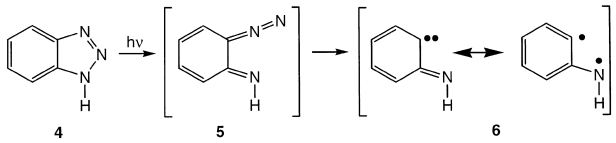
Results and Discussion

Chemistry

Compounds **2** and **3** were selected, at the beginning of the study, on the basis of their synthetic accessibility and prepared as shown in Scheme 1 from diazopyrazolylureas,[5]



Scheme 1.

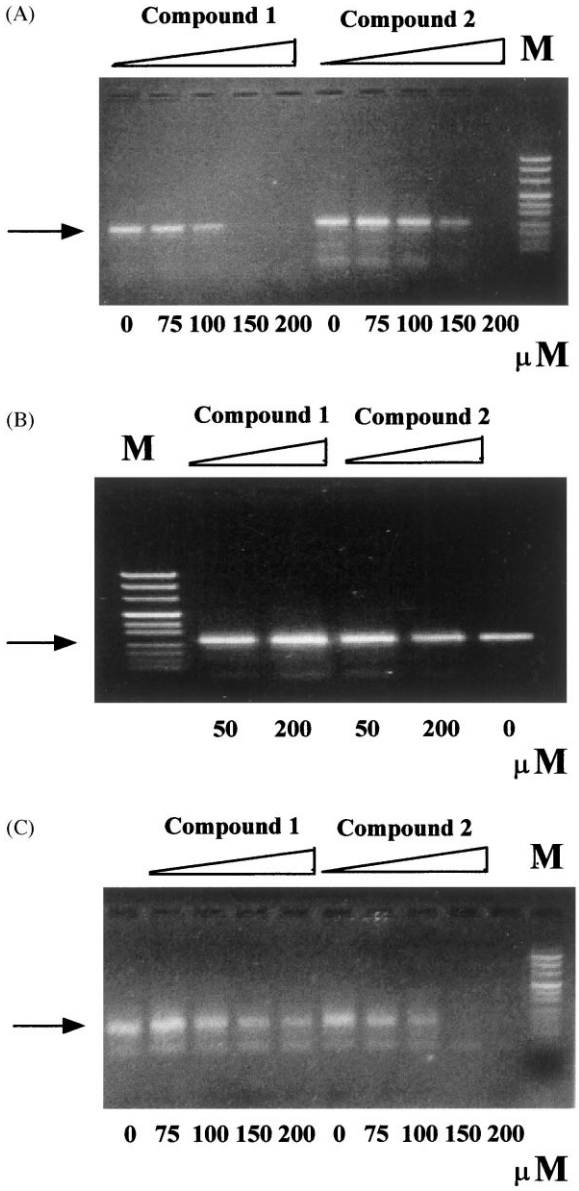


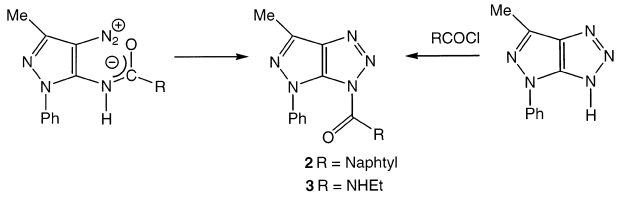
Figure 1. A–C. Effects of compounds **1** and **2** on PCR-mediated amplification of c-myc (A, B) and estrogen receptor (ER) (C) gene sequences. Target DNAs were 20 ng of purified c-myc PCR products and 20 ng of pBLCAT8ERCAT1 plasmid, as required. Before PCR, target DNA was incubated in the absence or in the presence of the indicated μM concentrations of DNA-binding drugs with (A, C) or without (B) light irradiation, as described in text. After PCR (20 cycles), 10 μl of each PCR mixture were analysed by agarose gel electrophoresis. M = molecular weight marker (pUC mix, MBI, Fermentas, Vilnius, Lithuania). ER and c-myc PCR products are arrowed.

or by treatment of pyrazolo-triazole with 2-naphthoyl chloride.

To verify the capability of title compounds to undergo radical generation after light irradiation, which is the basis of the expected mechanism of action, the triazole derivatives (**1–3**) were dissolved in ethanol (350 ml) (C = 2 mM) and photolysed for 6 h, giving rise to triazole ring opening along with solvolysis products (data not shown). These results are in agreement with other studies conducted on benzotriazole itself.[2,6]

Biology

The effects of the synthesized compounds were studied by arrested PCR.[7] The results are described in Fig. 1 and demonstrate that both **1** and **2** inhibit PCR-mediated amplification of the human c-myc oncogene promoter (A + T/G + C ratio = 0.607) after light-activation (IC_{50} 100 and 150 μM for **1** and **2**, respectively) (Fig. 1A). By contrast, no inhibitory activity was found even at 200 μM concentrations of **1** and **2** in the absence of light-activation (Fig. 1B). When the same experiment described in Fig. 1A was performed using PCR primers amplifying a 5′ portion of the human estrogen receptor (ER) gene (A + T/G + C ratio = 3.46), it was found that only compound **2** was able to block PCR-mediated amplification when added at a concentration of 150 μM (Fig. 1C). Compound **1** was found to retain very low activity in inhibiting PCR-mediated amplification of ER sequences (Fig. 1C). These findings, on one hand, indicate that compound **1** exhibits higher levels of sequence selectivity when compared to compound **2**; on the other hand,



Scheme 2.

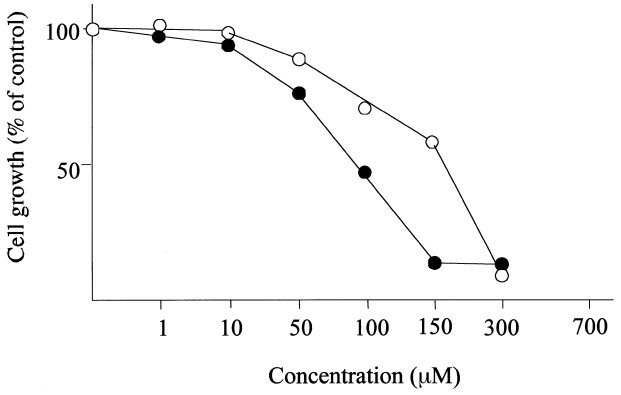


Figure 2. Effects of compounds **1** and **2** on in vitro growth of human T-lymphoid Jurkat cells. Cells were cultured in the absence or in the presence of different concentrations of compounds **1** (open symbols) and **2** (filled symbols). After 7 days, the values of cell number/mL were determined in each Jurkat cell population. The data values of cell number/mL in treated cells were compared to those of untreated cultures and expressed as % of control.

compound **2** is expected to target, unlike compound **1**, also A + T rich genomic DNA stretches. Figure 2 shows the *ex vivo* antiproliferative tests performed on the human T-lymphoid Jurkat cell line, demonstrating that **1** and **2** are both weak inhibitors of tumor cell growth when added at concentrations as high as 150 and 100 μM, respectively. However, compound **2** was significantly more active than compound **1**, thus confirming our initial hypothesis, also supported by susceptibility to fragmentation and PCR experiments, that the substitution of the benzene ring for an heterocyclic one, namely pyrazole, could be of importance for both cytoxicity and sequence selectivity. In our opinion these preliminary results validate the concept, advanced in this paper, that heterocyclic-annellated triazoles represent a new class of DNA-photocleaving agents worthy of investigation.

Conclusions

In conclusion, we have demonstrated that these compounds are active in inhibiting PCR-mediated amplification of human gene sequences only upon light-activation. Because this produces radical fragmentation of the triazole nucleus (see Chemistry), one among the possible hypotheses is that the biological activity described in the present paper is related to the formation of radical species interacting with DNA. Low levels of sequence selectivity were observed for compound **2**; therefore, this compound, despite being more active in inhibiting tumor cell growth than compound **1**, can be hardly envisaged as a possible tool for DNA study. However, it is remarkable that a possible in situ activation of these compounds, i.e. in neoplastic tissues, of low cytotoxic derivatives, may lead to potential antitumor compounds endowed with high therapeutic indexes. Taken together, the preliminary data presented here indicate the described compounds, as well as heterocyclic-annellated triazoles in general, as suitable candidate molecules for the design of new DNA-photocleaving agents and for the development of potential therapeutic agents. Further studies are currently ongoing to address these issues.

Experimental

Biology

The sequences of the primers used for polymerase chain reaction[8] were the following: ER-forward, 5′-G A C G C A T G A T A T A C T T C A C C-3′; ER-reverse, 5′-G C A G A A T C A A A T A T C C A G A T G-3′; c-myc-forward, 5′-C G T G G G G A A A G A A A A A A G T C C-3′; c-myc-reverse, 5′-T G C C T C T C G C T G G A A T T A C T A C A G-3′.

Taq DNA polymerase (Perkin–Elmer, Cetus, USA) was added at 2.5 U/25 μl final concentration. The nucleotide sequence of a 3.2 kb genomic region located upstream of the human estrogen receptor (ER) gene sequence originally designated exon 1 was investigated and described in our laboratory.[9] This region contains nucleotide sequences target of distamycin A and distamycin analogues.[10] The primers for PCR amplification of the human c-myc oncogene are able to amplify a promoter region containing P1 and P2 promoter elements.[11] For PCR-mediated amplification the target DNA was human genomic DNA. PCR buffer, Taq DNA polymerase and the four dNTPs were added as elsewhere described.[12] Conditions of PCRs were: denaturation, 92 °C, 1 min; annealing, 55 °C, 1 min; elongation, 72 °C, 1 min (35 cycles). The effects of DNA-binding drugs were analysed after incubating target DNA at room temperature, for 5 min, with increasing amounts of the compounds, as reported in the text, followed by polymerase chain reaction.[9,13] Amplified DNA was analysed by electrophoresis on 2.5% agarose, in TAE (0.04 M Tris-acetate, 0.001 EDTA), 0.5 μg/ml ethidium bromide. Light activation of the DNA-binding drugs used was obtained by performing PCR using the Hidaho PCR thermal cycler.

Chemistry

Melting points were obtained in open capillary tubes and are uncorrected. Infrared spectra were recorded on a Perkin–Elmer Paragon 500 FT-IR spectrophotometer. Nuclear magnetic resonance (^{1}H NMR) spectra were determined for solution in $CDCl_3$ on a Bruker AC-200 spectrometer and peak positions are given in parts per million (ppm) downfield from tetramethylsilane as internal standard. Mass spectra, Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF), were taken on the Hewlett-Packard HPG2025 A mass spectrometer operating in a positive linear mode. Ultraviolet spectra were recorded on a Jasco 510 spectrometer. Irradiation of the samples was performed with Pyrex-filtered light from a Helios Italquartz 125-W medium-pressure ($\lambda > 300$ nm) mercury lamp. Ambient temperature was 22–25 °C. All drying operations were performed over anhydrous magnesium sulphate. Compound **1**[2] was prepared as described for **2**.

Preparation of 6-methyl-4-phenyl-3-(2-naphtoyl)pyrazolo[3,4-*d*][1,2,3]triazole (2). A solution of naphthoylchloride (0.48 g, 2.5 mmol) in EtOAc (6 mL) was added dropwise to a vigorously stirred mixture of 6-methyl-4-phenylpyrazolo[3,4-*d*][1,2,3]triazole[14] (0.5 g, 2.5 mmol) and triethylamine (0.35 mL, 2.5 mmol) in EtOAc (10 mL). After 90 min stirring at room temperature, the precipitate was filtered. The resulting solid was dissolved in EtOAc and washed with water. After being dried the organic layer was evaporated to dryness. The residue was triturated with Et_2O and filtered to give **2** as a white powder (0.53 g, 60% yield), mp: 147–148 °C; IR (KBr) 1699 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.76 (s, 3H, CH_3), 7.25–8.16 (m, 11H, $C_6H_5 + C_{10}H_7$), 8.94 (s, 1H, $C_{10}H_7$); UV (EtOH) nm λ max 231 (ε = 14977). MS $(M+H)^+$ 354.2 (calcd 353.13).

Acknowledgements

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