Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 3385–3390

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Inhibition of Topoisomerase I by Naphthoquinone Derivatives

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Received 31 July 1998; accepted 9 October 1998

Abstract: Alkannin and shikonin are naturally occurring naphthoquinones. We have tested several derivatives of the title compounds and we have found that naphthoquinones bearing at least one phenolic hydroxyl group are potent inhibitors of topoisomerase I. The ability of the tested compounds to complex Zn^{++} parallels with a few exceptions their topoisomerase I inhibition properties while their intercalation and redox properties do not.

Introduction

Quinone derivatives may be toxic to cells by a number of mechanisms[1,2] including redox cycling, arylation, intercalation, induction of DNA strand breaks, generation of free radicals and alkylation via quinone methide formation.[3a] As a consequence, the molecular framework of a great number of pharmaceuticals and biologically important compounds contains a quinone moiety. Nowadays, their important pharmacological activity is also attributed to the inhibition of special proteins, such as: bacterial topoisomerase II–DNA gyrase–(antibacterial),[4] mammalian topoisomerases I and II (antitumor),[5,6] and HIV-1 integrase and proteinase (antiviral).[7,8] Representative examples of this class of compounds are the well known anticancer drugs of the anthracycline series—doxorubicin and mitoxanthrone—the action of which is believed to occur via topoisomerase II inhibition.[6] In addition, a number of naphthoquinone analogues like plumbagin, shikonin, naphthazarin as well as β-lapachone, have also been found to inhibit similar enzymes.[2,9,10] The number of reports concerning the biological evaluation of new natural products and synthetic analogues of this class of compounds, is constantly increasing.[7-12] Such a report, pertaining to the inhibition of topoisomerase I by naturally occurring and semisynthetic shikonin derivatives,[11] has prompted us to disclose related preliminary results from our laboratories.

Herein, we report on the strong inhibition of topoisomerase I by some naturally occurring and synthetic naphthoquinone derivatives. This activity is correlated with several physicochemical and biological properties of the tested compounds aiming to a better understanding of their mode of action.

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0960-894X/98/$ - see front matter
PII: S0960-894X(98)00600-3

Results and Discussion

Designed amino, alkylamino, and hydroxy derivatives of the naphthoquinone core (**2–4** and **7–10**; Figure 1) were synthesized and tested for topoisomerase I inhibition along with some commercially available homologues (**1** and **5–6**). In addition, naturally occurring shikonin **11**, alkannin **12**, acetylalkannin **13**, a mixture of alkannin isovalerate and angelate **14**, as well as the crude extract of all pigments of *Alkanna tinctoria* were also tested. Figure 2 illustrates the results of their interaction with DNA in the presence of topoisomerase I. The minimum concentration of each substance tested at which inhibition of this enzyme was observed (MIC) is reported in Figure 1.

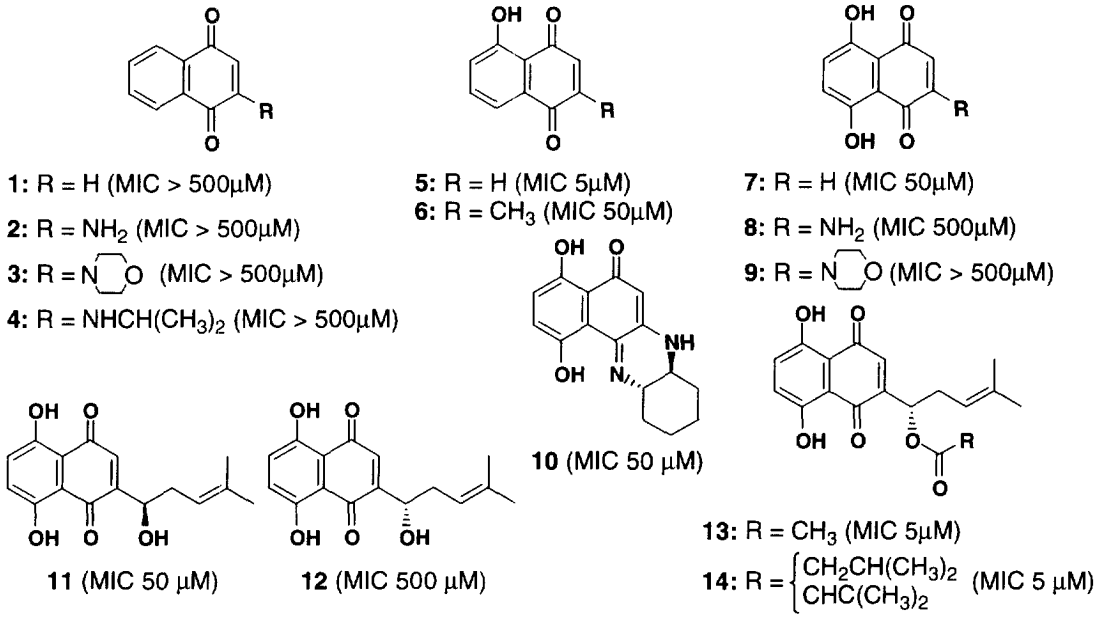

Figure 1. Chemical structure and minimum inhibition concentration (MIC) against topoisomerase I, of compounds **1–14**.

We have used naphthazarin **7**, as a reference for our comparisons, since it represents the main core of all compounds tested. Regarding the inhibition of topoisomerase I, our experimental results clearly suggest that naphthoquinones deprived of a phenolic hydroxyl are completely inactive (compare **1–4** with the rest of the compounds). However, the presence of both phenolic hydroxyls is not necessary (for example compare **7** with **5** or **6**), in contrast to previous findings regarding inhibition of HIV-1 integrase.[7]

The presence of a substituent at position 2 of the aromatic moiety, is generally affecting activity. Polar substituents like amines, are decreasing activity (**7** vs. **8**) making compound **9**, with a tertiary substituted amine, completely inactive. However, the rigid derivative **10** retains the activity of the parent framework **7**. On the other hand, alkyl substituents seem to have a less dramatic effect. Thus, the methyl substituted compound **6** (plumbagin) is less active than juglone (**5**). The effect of an α-hydroxylated side chain on the activity of the

parent framework depends on its stereochemistry (compare **7** with **11** and **12**). Finally, in accordance with previous findings,[11] we have also observed that acylated analogues like **13** or **14** are the most potent. This observation implies an activity through nucleophilic trapping of a quinone methide intermediate.[3] It should be noted that the biological assays were performed under mild alkaline (TRIS) and reductive (DTT) conditions. However, this mechanism of action cannot explain the important activity of compounds like **5**.

In order to better correlate the biological profile with the structural features of the tested compounds, intercalation as well as redox and complexation properties were also considered. The redox potentials of compounds **1** and **5–7** are available in the literature.[12] Naphthazarin (**7**) has a redox potential of –280 eV and plumbagin (**6**) –420 eV. Juglone (**5**), which is more active against topoisomerase I than **7** or **6**, has a redox potential in between (–390 eV), while naphthoquinone (**1**), which is completely inactive, –580 eV. From the above, it is concluded that the inhibition of topoisomerase I by naphthoquinone derivatives, is unrelated with their redox properties. Regarding intercalation abilities, it is known that plumbagin is a weak intercalator of DNA,[9] while shikonin[9] and naphthazarine[7] do not intercalate at all. Since all the above compounds show significant inhibition of topoisomerase I, their activity apparently does not correlate with their intercalation properties.

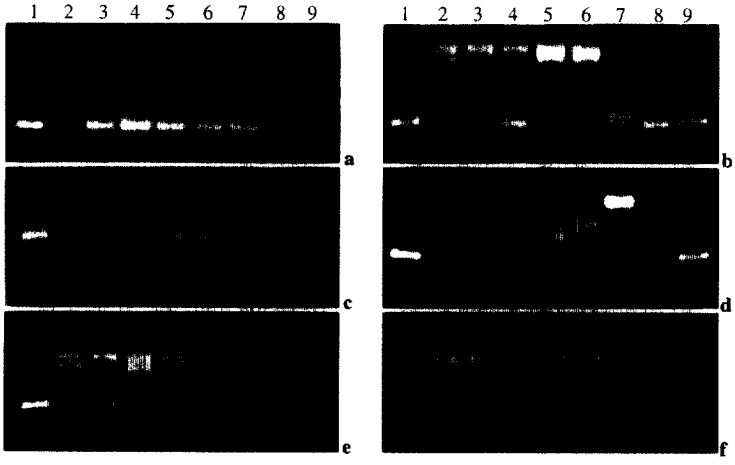

Figure 2. Effect of naphthoquinones on DNA topoisomerase I. In all gels, Lane 1: absence of topoisomerase I and inhibitors. Lane 2: absence of inhibitors. **a.** Lanes 3-9: presence of 500 μM of inhibitor **13**, **12**, **11**, dyes mixture, **14**, **7**, and **5** respectively. **b.** Lanes 3-9: presence of 500 μM of inhibitor **9**, **3**, **4**, **2**, **8**, **10**, and **6** respectively. **c.** Lanes 3-9: presence of 50 μM of inhibitor **13**, **12**, **11**, dyes mixture, **14**, **7**, and **5** respectively. **d.** Lanes 3-9: presence of 50 μM of inhibitor **9**, **3**, **4**, **2**, **8**, **10**, and **6** respectively. **e.** Lanes 3-9: presence of 5 μM of inhibitor **13**, **12**, **11**, dyes mixture, **14**, **7**, and **5** respectively. **f.** Lanes 3-9: presence of 5 μM of inhibitor **9**, **3**, **4**, **2**, **8**, **10**, and **6** respectively.

Hydroxynaphthoquinones, with their juxtaposed phenolic and keto groups, are effective chelators of divalent metal ions. For that reason, Pommier *et al.* suggested that they may inhibit topoisomerases by binding to a zinc finger domain of the protein.[7] If this is the case, their activity should be in close relation with their complexation properties. We have studied, by UV spectroscopy, the complexation properties of compounds **1–3** and **7–8** with Cu^{++}, Zn^{++} and Mg^{++}. Naphthoquinones **1–3**, deprived of a phenolic hydroxyl, showed negligible interaction with the metal ions, paralleling the results with the topoisomerase inhibition bioassays. On the other hand, phenolic derivatives **7–8**, chelate the metal ions studied except Mg^{++}. Figure 3 depicts the

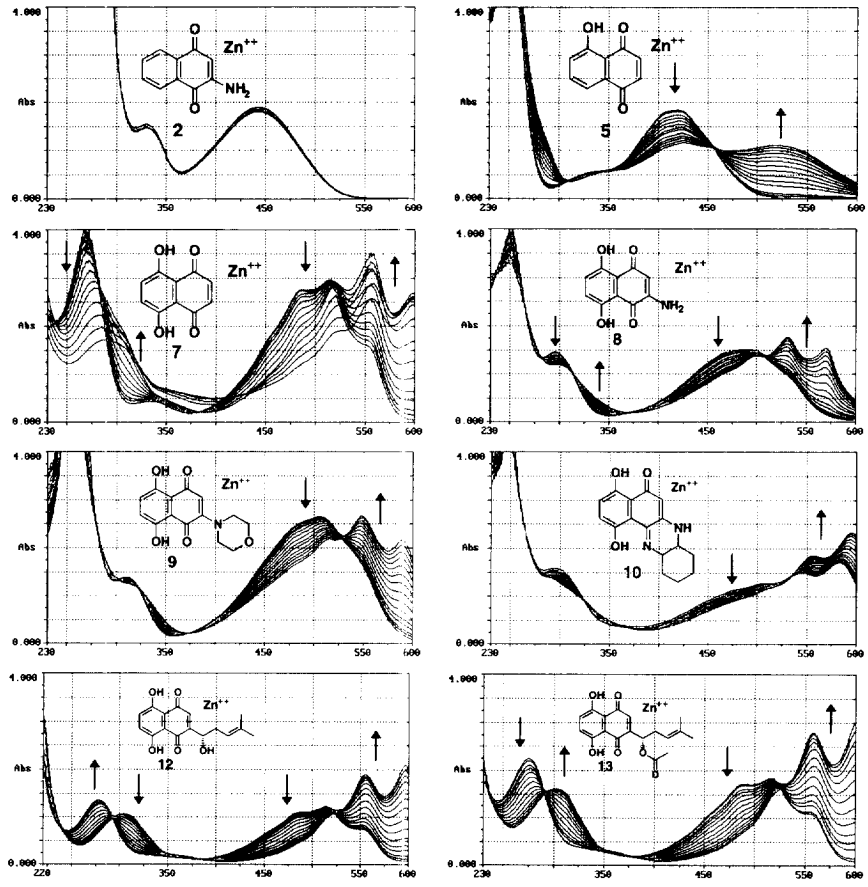

Figure 3. Recorded UV spectra of the titration of compounds **2, 5, 7–10, 12** and **13** with Zn^{++}.

UV spectra of compounds **2**, **5**, **7–10**, **12** and **13**, after titration with a methanolic solution of $Zn(OAc)_2$. Analogous experiments using $Mg(OAc)_2$ showed negligible change in the UV spectrum of the tested compounds, while titration with a methanolic solution of $Cu(OAc)_2$ resulted in the formation of precipitates (presumably Cu•quinone complexes). The above observations support Pommier's assumption, suggesting a binding of the phenolic naphthoquinones with a zinc finger domain of topoisomerase I. However, compound **10** although a good inhibitor of topoisomerase I does not seem to strongly interact with Zn^{++}. This discrepancy could perhaps be attributed to a different mode of interaction of this inhibitor with the enzyme. Furthermore, compound **9** is completely inactive, although it interacts well with Zn^{++}, and shikonin (**11**) and alkanin (**12**) differ greatly in their inhibitory potency although one would expect them to interact similarly with Zn^{++}. These observations suggest that metal chelation, although important, is not the only interaction in play and that stereo-electronic requirements imposed by the side chain are also important for efficient enzyme inhibition.

Finally, we have found that the crude extract of the pigments *of Alkanna tinctoria* (named as dyes mixture in Figure 2), shows equal activity with some of its isolated pure components **12**, **13**, and **14**. Therefore, a more detailed examination of its minor constituents is needed.

In conclusion, naphthoquinones bearing at least one phenolic hydroxyl group are potent inhibitors of topoisomerase I. The ability to complex Zn^{++} seems to be important and in close relation with their inhibition activity implying binding with a zinc finger domain of the enzyme. Enlightened by the above results, we are pursuing the synthesis and evaluation of more elaborate synthetic as well as naturally occurring derivatives of the title compounds.

Materials and methods.

General Experimental Procedures and Materials. Topoisomerase-I was purchased from Promega (source wheat germ). Juglone, Plumbagin and Naphthoquinone were purchased from Sigma and were used without further purification. Naphthazarin was prepared according to the literature.[13] The synthetic compounds were synthesized, purified and characterized according to the literature: compounds **3, 4** and **9** ref. 14, compounds **2** and **8** ref. 15, compound **10** ref. 16.

Isolation of Pigments from *Alkanna tinctoria*: Air-dried roots of *Alkanna tinctoria* were extracted thoroughly with hexane at room temperature. The solvent was removed at reduced pressure at temperature below 50 °C. The dark viscous residue was extracted with cold MeOH to separate the soluble pigments and fluorescent compounds from the insoluble waxes. The methanolic solution was then treated with cupric acetate, whereupon the pigments precipitated as insoluble Cu•chelates. These were washed exhaustively first with hexane and then with MeOH to ensure removal of fluorescing impurities. The mixture of free pigments, obtained by decomposition of the purified Cu-complexes with 10% HCl, was chromatographed on silica gel. The column was eluted successively with hexane and hexane/benzene mixtures of increasing polarity and finally with benzene. The hexane fraction was a mixture of five compounds: alkannin, deoxyalkannin, alkannin angelate, alkannin isovalerate and alkannin acetate, which were isolated and purified by repeated column chromatography.

Evaluation of the complexation properties: A solution of the tested compound in methanol (50 mL, 0.20 mM), was titrated with a solution of $Zn(OAc)_2.H_2O$ in the same solvent (2 mM) and the UV spectrum (200 to 600 nm) of the resulting solutions were recorded. Typical spectra obtained are presented in Figure 3.

Determination of DNA Topoisomerase-I Activity: Topoisomerase-I activity was determined by detection of the relaxation of superhelical pBR322. The assay mixture (20 μL) contain 50 mM TRIS (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, topoisomerase I (10 U) and varied amounts of naphthoquinone derivatives. The reactions were kept at 37 °C and terminated by the addition of 5 μL of solution of 2% SDS, 20% glycerol, and 0.05% bromophenol blue. Electrophoresis was carried out over 1% agarose gel

plate, equilibrated with 1xTBE buffer and stained with ethidium bromide after completion of the electrophoresis.

Acknowledgment: ZFP would like to thank IKY for a scholarship. P. N. Gerolymatos, Pharmaceutical Co., Ltd. (Athens, Greece), is acknowledged for purchasing the roots of *Alkanna tinctoria* TAUSCH, which were collected in Anatolia, Turkey.

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   (b) Alternatively, as one of the referees pointed out, acylation could potentate the inhibitory effect to a great extent through increase of the binding force. Although increase in enzyme affinity can not be ruled out, the binding site will have to be very tolerant to acyl groups of different steric requirements.
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