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Preclinical report

Synthesis and cytogenetic effects of aminoquinone derivatives with a di- and a tripeptide

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Quinones are of significant interest due to their important role in specific cellular functions. Quinoproteins are a big class of oxyreductative agents occurring in bacteria and other organisms. In this investigation derivatives of 2-amino-1,4-benzoquinone, 2-amino-1,4-naphthoquinone and 2-amino-5,8-dihydroxy-1,4-naphthoquinone with a di- and a tripeptide were prepared for first time. The effect of the synthesized compounds on sister chomatid exchange (SCE) rates and human lymphocyte proliferation kinetics on a molar basis was studied. Among these coupled products the most effective in inducing SCEs and depressing proliferation rate indices is the coupling product of 2-amino-1,4-naphthoquinone with the tripeptide GHK (10). Next in order of magnitude in inducing cytogenetic effects is 2-amino-1,4-naphthoquinone (2) and its coupling products with glycine and serine (4 and 5), while the rest displayed marginal activity. [© 2002 Lippincott Williams & Wilkins.]

Key words: Biological activity, cytostatic, cytotoxic, derivatives of aminoquinones with peptides, enzymic effect.

Introduction

Proteins containing quinonoid factors have been found in many mammalian and bacterial cells and are part of several enzymes.[1] Many metalloproteins, all ferritins and bacterioferritins contain pyrroloquinolinoquinone (PPQ), topaquinone (TPQ) and/or tryptophanyl-tryptophane-quinone (TTQ).[2,3] It has been also shown that many drugs used in cancer chemotherapy contain quinones.[4] Synthesis of various alkylamino derivatives of naphthoquinones and coupling products with free or protected amino acids have been reported.[5,7]

We have previously reported the synthesis of derivatives of aminoquinones with N-protected amino acids.[8] An attempt to expand this study to derivatives with oligopeptides and examine their antineoplastic activity is the aim of the present work. The peptides were synthesized either in solution or solid phase technique. Their N-protected derivatives were further coupled to aminoquinones including 2-amino-1,4-benzoquinone, 2-amino-1,4-naphthoquinone and 2-amino-5,8-dihydroxy-1,4-naphthoquinone. The stability of these products towards a number of proteases was investigated.

Sister chomatid exchanges (SCEs) have been proposed as a very sensitive method for detecting DNA damage and/or subsequent repair. Lately, SCE and proliferation rate index (PRI) values have been suggested as one of the indices of evaluating chemotherapeutic efficiency *in vitro*[9,10] and *in vivo*.[11,12] The ability to excise and repair various types of DNA damage is probably a general property of living cells.[13] Unrepaired damage expressed as SCEs in normal cells caused by certain chemicals may indicate inability for repair of damage induced by the same chemicals in cancer cells.[9–12,14,15]

Materials and methods

All quinones, diisopropylcarbodiimide (DIC), *N*-hydroxy-benzotriazole (HOBt) and *N*-Boc amino acid derivatives were purchased from Sigma (Steinheim, Germany). Fmoc amino acid derivatives and acid-labile 2-chlorotrityl chloride resin were

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purchased from Biopharmaceutical Labs (Patras, Greece). Reagents and solvents used were analytical grade.

The purity of the compounds was established by thin-layer chromatography (TLC) and analytical high-performance liquid chromatography (HPLC). The elution in HPLC chromatography was performed under isocratic conditions using CH_3OH:H_2O 70:30 and a Separon SGX C_{18} column (7 μm, 200 mm × 4.6 mm i.d.). Melting points were determined on a Buchi capillary apparatus and are uncorrected. All commercial products were used without further purification.

Enzymes

Aminopeptidase I from *Streptomyces griseus* (EC 3.4.11.10) and carboxypeptidase A Type II-DFP from bovine pancreas (EC 3.4.17.1) were purchased from Sigma. Pepsin from porcine stomach mucosa (EC 3.4.23.1), trypsin from bovine pancreas (EC 3.4.21.4) and α-chymotrypsin from bovine pancreas (EC 3.4.21.1) were obtained from BDH (Poole, UK).

Hydrolysis

Compound **5** at a concentration of 50 mM (100 μl) was used as substrate for the hydrolysis with the mentioned enzymes. Hydrolysis with aminopeptidase I (5 U) was done at 25°C, pH 8.0 adjusted with 50 mM Tris–HCl for 1 h. Experiments with carboxypeptidase A, trypsin and α-chymotrypsin were performed at their optimum pH (7.0, 7.6 and 7.0, respectively) in the same buffer using 2.5 U of enzyme. Pepsin was also used at its optimum pH 3.0 (adjusted with 50 mM CH_3COONH_4) at 37°C for 1 h. A similar procedure was applied for the hydrolysis of compound **7**.

Synthesis

Aminoquinones including 2-amino-1,4-benzoquinone (**1**), 2-amino-1,4-naphthoquinone (**2**) and 2-amino-5, 8-dihydroxy-1,4-naphtho-quinone (**3**) were prepared as reported.[5,8]

Gly-His-Lys (GHK, **8**) was synthesized by the solid-phase technique as previously reported[16] and obtained as Fmoc-Gly-His(Mtt)-Lys(Mtt)-O-resin. It was then treated with 5% TFA for 40 min, filtered and washed with methanol. The filtrate containing the product **9** that was over 90% homogeneous, as judged by TLC in different systems, was used for coupling with 2-amino-1,4-naphthoquinone without other purification.

Boc-Asn-Gly-OH (**7**) was synthesized in solution from Boc-Asn-OH and HCl.Gly-OMe using dicyclohexylcarbodiimide (DCC) as coupling reagent in DMF. Boc-Asn-Gly-OMe was then saponified the with 0.1 N KOH for 1 h to give compound **7** with a final yield 0f 0.18 g (41%).

Coupling of **9** with 2-amino-1,4-naphthoquinone

Fmoc-Gly-His-Lys-OH (**9**) (50 mg, 0.09 mM) was dissolved in 5 ml DMF and equimolar amounts of HOBt and DIC (0.05 g and 0.1 ml, respectively) were added and the mixture was stirred for 20 min at 0°C and for another 15 min at room temperature. The mixture was filtered and to the filtrate was added 0.09 g (0.5 mM) 2-amino-1,4-naphthoquinone. The mixture was stirred for 2 h at room temperature. Then it was evaporated to dryness, and the residual was diluted with 15 ml ethyl acetate and extracted with 10 ml sodium bicarbonate 1%. The organic layer was dried with $MgSO_4$ and evaporated to give an oil residue which was purified on a silica gel column with chloroform:methanol (70:30) to give 28 mg of product (**11**), yield 0.08 g (44%), MS $(M+H)^+$ calculated: 719; found: 719. Analysis: $C_{39}H_{39}N_7O_7$ (C: 65.18, H: 5.43, N: 13.64).

Coupling of **9** with 2-amino-5,8-dihydroxy-1,4-naphthoquinone

It was prepared as previously described. Yield 15%. MS $(M+H)^+$ found: 751; calculated: 751. Analysis: $C_{39}H_{39}N_7O_9$ (C: 62.4, H: 5.2, N: 13.06).

Coupling of **7** with 2-amino-1,4-naphthoquinone

It was prepared similarly to the above procedure using HOBt and DIC as coupling reagents. The product was obtained after purification on a silica gel column, eluted with chloroform:methanol 70:30 as in the previous case. Yield 15%. MS $(M+H)^+$ found: 459; calculated: 459.

Biological experiments

Lymphocyte cultures were prepared by adding 10 drops of whole blood (containing about 3×10^6

lymphocytes) from normal subjects to 5 ml of chromosome medium (Gibco, Middlesex, UK; including phytohemagglutinin). Cultures were treated with chemicals for 72 h at the beginning of culture life. The chemicals were dissolved in dimethylsulfoxide (DMSO) and then further diluted in culture medium. The final concentration of DMSO in culture medium was always less than 0.2%, which does not induce SCEs.

For the demonstration of SCE,[14] 5-bromodeoxyuridine (5-BrdUrd) at 4 μg/ml was added 24 h after initiation of culture. During the entire period, all cultures were maintained in the dark to minimize photolysis of BrdUrd. Metaphases were collected during the last 2 h with colchicine at 0.3 μg/ml. Air-dried preparations were made and stained with the fluorescence plus Giemsa procedure.[14] The preparations were scored for cells in their first, second, third and subsequent divisions, and second-division cells that were suitably spread were scored blindly for SCEs and lymphocyte proliferation kinetics. A minimum of 30 cells was scored for each culture in order to establish mean SCE values. For PRIs, at least 100 cells were scored. The PRI was calculated according to the formula PRI=(M1+M2+M3+$\cdots$)/N, where M1, M2 and M3 indicate those metaphases corresponding to first, second and third or subsequent division, and N is the total number of metaphases scored.[17] For the statistical evaluation of the experimental data the χ^2-test was used for the cell kinetics comparisons, whereas for the SCE frequencies Student's t-test was performed to determine whether any values deviated significantly from the controls ($p<0.05$).

We also calculated the correlation between SCEs and PRI values. The formula for the Pearson product moment correlation coefficient r was applied. Then a criterion for testing whether r differs significantly from zero was applied whose sampling distribution is Student's t-test with $n-2$ d.f.

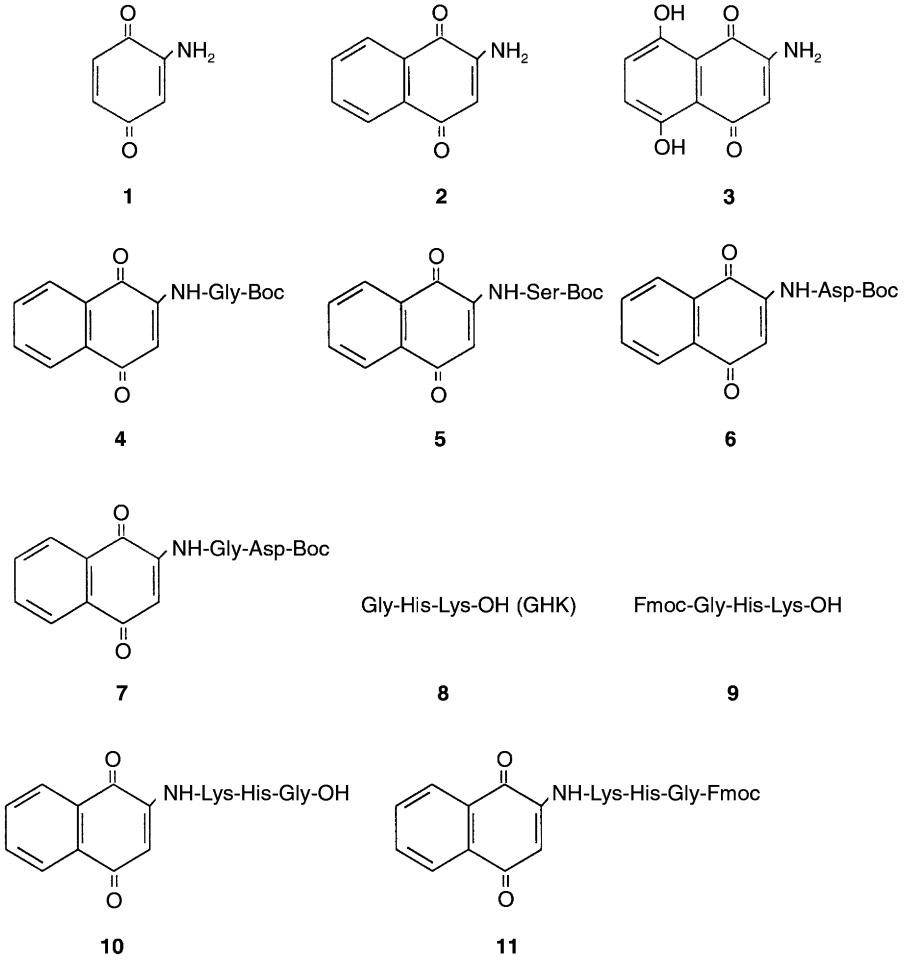

Figure 1. Structures of compounds listed in this paper.

Table 1. The effect of compounds **1**–**11** on SCE and PRI values in normal human lymphocyte cultures

Compound	Concentration (μm)	SCEs/cell $\pm$ SE[a]	PRI[b]
Control	–	8.44 $\pm$ 0.48	2.21
1	62.5	8.26 $\pm$ 0.48	2.08
	125.0	10.28 $\pm$ 0.72	1.84**
2	62.5	19.72 $\pm$ 1.59*	1.20**
	125.0	–	–
3	62.5	11.25 $\pm$ 0.89*	1.93**
	125.0	11.29 $\pm$ 0.61*	1.80**
4	62.5	16.38 $\pm$ 0.98*	1.66**
	125.0	–	–
5	62.5	13.21 $\pm$ 0.61*	1.79**
	125.0	15.07 $\pm$ 0.98*	1.40**
6	62.5	9.00 $\pm$ 0.43	2.13
	125.0	9.18 $\pm$ 0.54	2.27
7	62.5	11.55 $\pm$ 0.53*	1.88**
	125.0	13.70 $\pm$ 0.83*	1.82**
8	62.5	7.98 $\pm$ 0.58	2.21
	125.0	8.01 $\pm$ 0.72	2.05
9	62.5	–	–
	125.0	–	–
10	62.5	32.51 $\pm$ 2.53*	1.74**
	125.0	31.79 $\pm$ 2.46*	1.29**
11	62.5	16.18 $\pm$ 0.91*	1.28**
	125.0	–	–

[a]Mean SCE values/cell $\pm$ SE after counting 30 second-division metaphases.
[b]For definition see in the text.
*Significant increase ($p < 0.01$) over the control by *t*-test.
**Significant decrease ($p < 0.01$) over the control by χ^2-test.
SCEs have been correlated with corresponding PRI values (r=–0.66, t=3.52 and for 16 d.f. $p < 0.01$).

Results and discussion

In a previous work,[8] we reported the synthesis of derivatives of aminoquinones with N-protected amino acids. The same aminoquinones were used in the present study for coupling with a di- and a tripeptide in an attempt to investigate whether oligopeptides can also be linked to these aminoquinones (Figure 1). It was found that the derivatives of the di- and the tripeptide with 2-amino-1,4-naphthoquinone were obtained in higher yields (30 and 45% for the di- and tripeptide, respectively) than the derivatives of the same peptides with the other two aminoquinones.

In order to investigate the stability of the amide bond between the aminoquinone and the amino acid and/or peptide towards a series of common proteases, the coupling products of Boc-Ser, Boc-Gly and Boc-Asn-Gly-OH with 2-amino-1,4-naphthoquinone were used. The results showed that all enzymes used, with the exception of aminopeptidase I, hydrolyze the amide bond between aminoquinone and the amino acid and/or the peptide. Aminopeptidase I did not have any effect on these substrates as was expected. These data further indicate that quinonyl amino acids or peptides and proteins with quinonoid factors that exist in mammals could be hydrolyzed by the common proteases. Table 1 gives the SCEs and PRI values for 2-amino-1,4-benzoquinone (**1**), 2-amino-1,4-naphthoquinone (**2**) and 2-amino-5,8-dihydroxy-naphthoquinone (**3**). As shown, the higher SCE value was obtained with compound **2**. The PRI value for this compound was 1.20 compare to 2.08 and 1.93 for compounds **1** and **3**, respectively. SCEs values are an indication of DNA damage and/or subsequent repair (cytogenetic action), whereas PRI values are an indication of cytotoxicity.[15] According to the above statements, compound **2** gave the best results in terms of SCE and PRI values.

Compound **2** was then coupled with the tripeptide GHK. Table 1 shows their cytogenetic and cytotoxic effect in the same experimental material. It should be emphasized here that the tripeptide GHK was selected as a small and biological active peptide (growth factor) for coupling with the mentioned aminoquinones. In a previous work the antibacterial properties of the synthetic GHK have been shown. It was found that the SCE values of the GHK (or compound **8**) remain in the control level, whereas compound **11** (GHK coupled with 2-amino-1,4-naphthoquinone) has a statistically significant

cytogenetic and cytotoxic synergistic effect (PRI=1.74 and SCE=32.51). Smaller activities in terms of the SCE and PRI values were shown by the products of coupling **2** with Boc-Gly and Boc-Ser (**4** and **5**). All other quinines and the coupling products presented marginal activity.

Chemically induced cytotoxicity, in that it delays cell turnover times, is clearly manifested as a change in the relative proportions of cells in their first, second and subsequent divisions.[14,15] Studies in search for a relationship between SCE induction and other expressions of genotoxicity have shown a positive relationship between SCEs and reduced cell survival and alteration in cell cycle kinetics.[15] The findings indicate that the effectiveness in SCE induction by potential antitumor agents in cancer cells *in vitro*[10] and *in vivo*[11] is positively correlated with *in vivo* tumor response to these agents, and suggest that the SCE assay could be used to predict both the sensitivity of human tumor cells to chemotherapeutics and heterogeneity of drug sensitivity of individual tumors.[10,11] The SCE assay has predictive value as a clinical assay for drugs for which a strong correlation between cell killing and induction of SCEs has been established.[10] A correlation between potency for SCE induction and effectiveness in cell cycle delay by potential chemotherapeutics has been recently identified *in vivo*.[12] In the present study our results show a high correlation between the potency for SCE induction and effectiveness in cell division delay in normal human lymphocytes ($p<0.01$). Chemotherapeutics in many instances are expected to induce severe damage to the neoplastic cell. However, if an agent penetrates a cell and induces damage its biological effect may be minimized by the cell's capacity for repair. The ability to excise and repair DNA damage is a property of cancer as well as of normal cells, since both cell types have similar DNA repair mechanisms.[9–12,14,15] Therefore, DNA damage induced by potential chemotherapeutics to normal cells reflects the level of desirable damage which might be induced by the same chemotherapeutics upon cancer cells.[15]

The high SCE values in combination with the low PRI values might be considered as an indication of the antineoplastic activity of compounds. The results, even though are encouraging, need further investigation. The mechanism of molecular action of the most effective compounds (**10**, **4** and **5**) is not known. Possibly their action resembles:

(i) That of mitomycin, which is postulated to form an essential semiquinone intermediate, because recent evidence implicates such a species in preliminary non-covalent binding to DNA, quite possibly by intercalation as a prerequisite for cross-link formation.

(ii) That of streptonigrin, which is an antibiotic that contains the same α aminoquinone moiety present in mitomycin but it is not cross-linked. *In vitro* it will attack DNA to produce single-strand breaks, provided O_2 and an electron source are present, and the reaction is inhibited by the enzymes catalase and superoxide dismutase whose effect appears to be synergistic. The hydroxyl radical OH is the ultimate reactive species that imitates attack on DNA, most likely via a series of reactions commencing with the abstraction of a hydrogen ion from position 4 of the deoxyribose ring.

In closing, aminoquinones can serve as substrates for anchoring not only amino acids but peptides of different length as well. In addition, the biological results showed that by anchoring selected oligopeptides, the potent 2-amino-1,4-naphthoquinone may lead to derivatives with enhanced synergistic activity. It is evident that this study must be pursued with more derivatives of aminoquinones with oligopeptides.

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