Inhibition of human topoisomerase I and activation of caspase-3 by aza-angucyclinones and arylaminopyrimido[4,5-c]isoquinoline-7,10-quinones

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Abstract. Cancer is the second cause of death in the world after cardiovascular diseases. Cancer cells acquire capacities not present in normal cells, such as self-sufficiency, resistance to antiproliferative stimuli, evasion of apoptosis, unlimited replication, invasiveness and metastasis. Consequently, it is of major interest to explore and develop molecules with anticancer activity directed to specific targets. In this study, we aimed to evaluate two series of polycyclic quinones: aza-angucyclinone and arylaminopyrimido[4,5-c]isoquinoline-7,10-quinones, in their capacity to inhibit human topoisomerase I (TOP1) and to trigger apoptosis through activation of caspase-3. We evaluated the capacity of the two series of polycyclic quinones to inhibit TOP1, using a DNA supercoiled relaxation assay and their capacity to induce apoptosis through the activation of caspase-3 in HL60 cells. Both series of quinones inhibited TOP1 activity over 50%. When we evaluated the pro-apoptotic capacity of both series of quinones, at therapeutically relevant concentrations, the arylaminquinones ADPA-ICC (methyl 7-(4-methoxyphenyl)amino-1,3-dimethyl-5,8-dioxo-5,8-dihydroisoquinoline-4-carboxylate), P4 (9-phenylamino-3,4-dihydrophenanthridine-1,7,10(2H)-trione) and the aza-angucyclinone OH-6H (8-hydroxy-2,4-dimethyl-2H,4H-benzo[g]pyrimido[4,5-c]isoquinoline-1,3,7,12-tetraone) increased the caspase-3 activity by approximately 2-fold over the control. The series of the arylaminquinones and aza-angucyclinones showed differential antiproliferative capacity. We further identified a group of them that showed antiproliferative capacity possibly through inhibition of TOP1 and by activation of caspase-3. This group of molecules may represent a potential pharmacological tool in the treatment against cancer.

Introduction

Cancer is the second cause of death worldwide, after cardiovascular diseases (1). Cancer cells acquire capacities not present in normal cells, such as self-sufficiency, resistance to antiproliferative stimuli, evasion of apoptosis, unlimited replication, invasiveness and metastasis. These characteristics are common for most types of cancer (2). The loss of cell cycle regulation and the autonomous growth of cancer cells shows a correlation with the upregulation of biomarkers, such as the enzyme human topoisomerase I (TOP1) in several types of cancers, such as that of the digestive tract and lung (3,4). This enzyme is present in all eukaryotic cells and is key for cell survival due to its key function: to relax supercoiled DNA, through a specific cleavage of the DNA (5). On the other hand, evasion of programmed cell death (apoptosis) is a process in which the normal cell becomes malignant, with the ability to form a tumor (6).

Several studies have shown that some compounds induce apoptosis in cancer cell lines, through an increase in the activity of caspases, both of the extrinsic (caspase-8) and intrinsic (caspase-9 and -3) pathway, positioning these compounds as potential agents for the treatment of cancer (7-9).

Chemotherapeutic agents, such as the inhibitors of TOP1 act by stabilizing the complex formed between the enzyme and the DNA, causing the death of the cancer cell. Camptothecin was the first TOP1 inhibitor to be isolated, showing strong antitumor activity. Unfortunately, its use had to be discontinued, due to its adverse effects. Water-soluble derivates of camptothecin, such as topotecan and irinotecan, were developed (10), resulting in stronger activity and less toxicity. Quinones are among the several groups of antitumor drugs, due to their lower toxicity (11). They exert anticancer activity through several mechanisms that include DNA intercalation, alkylation of biomolecules generation of oxygen reactive species and probably inhibition of TOP1 (12).

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Our group has recently synthesized and characterized the in vitro antitumor capacity of several polycyclic quinones (13-18). These molecules may represent an interesting therapeutic tool. We aimed to study the mechanism of action of two series of polycyclic quinones: named aza-angucyclinones and arylaminopyrimido[4,5-c]isoquinoline-7,10-quinones. For this, we evaluated their capacity to inhibit human TOP1 and to induce apoptosis.

Materials and methods

Chemicals. Polycyclic quinones were synthesized as previously described (15,17). The chemical structures of the aza-angucyclinones used in this study appear in Fig. 1. P1, 8-hydroxy-3,4-dihydro-2H-benzo[j] phenanthridine-1,7,12-trione; P3, 8-hydroxy-6-methyl-3,4-dihydro-2H-benzo[j] phenanthridine-1,7,12-trione; FG-2, 3,3,6-trimethyl-3,4-dihydro-2H-benzo[j]phenanthridine-1,7,12-trione; FG-5, 3,3-dimethyl-8-hydroxy-1,2,3,4-tetrahydro-1H-benzo[j] phenanthridine-1,7,12-trione; OH-6H, 8-hydroxy-2,4-dimethyl-2H4AH-benzo[g]pyrimido[4,5-c]isoquinoline-3,7,12-tetraene.

The chemical structures of the arylaminopyrimido[4,5-c] isoquinoline-7,10-quinones used in the study are shown in Fig. 1. AD, methyl 7-phenylamino-1,3-dimethyl-5,8-dioxo-5,8-dihydroisoquinoline-4-carboxylate; ADPA-1CC, methyl-7-(4-methoxyphenyl)amino-1,3-dimethyl-5,8-dioxo-5,8-dihydroisoquinoline-4-carboxylate; P4, 9-phenylamino-3,4-dihydrophenanthridine-1,7,10(2H)-trione; A, 2,4-dimethyl-8-phenylamino- 4H-pyrimido[4,5-c]isoquinoline-1,3,7,10-tetraene; B, 8-(4-methoxyphenyl)amino-2,4-dimethyl-4H-pyrimido[4,5-c] isoquinoline-1,3,7,10-tetraene; C, 2,4,6-trimethyl-8-phenylamo- no-4H-pyrimido[4,5-c]isoquinoline-1,3,7,10-tetraene; D, 8-(4-methoxyphenylamino)-2,4,6-trimethyl-4H-pyrimido[4,5-c] isoquinoline-1,3,7,10-tetraene.

Plasmid DNA pGEM®-T extraction. Plasmid DNA pGEM®-T supercoiled was extracted from colonies of Escherichia coli TOP10 (TOP10 Electrocomp™ kit, Invitrogen) grown in agar/ LB/ampicillin media for 24 h at 37°C. Then, bacteria were inoculated with 50 ml of Terrific Broth™ (Gibco, USA), for 24 h with agitation (300 rpm) at 37°C. After this period, the plasmid DNA pGEM® was extracted using the E.Z.N.A.t Fasfilter® Plasmid Midiprep kit (Omega Biotek, USA) with ethidium bromide (EtBr).

TOP1 activity assay. The assay was based on the principle of increasing the mobility of supercoiled DNA in an agarose gel after treatment with TOP1, according to Liu et al (19). Briefly, supercoiled DNA pGEM®-T (63 ng/µl) was incubated with 6 units of human TOP1 (1 ml, Invitrogen) in 1.0 µl of relaxation buffer (stock 10X; 50 mM Tris-HCl, pH 7.5; 0.1 mM EDTA; 50 mM KCl; 10 mM MgCl2; 0.5 mM DTT; 30 µg/ml BSA) and the volume was adjusted to 10 µl with ultrapure H2O. All the chemical compounds were dissolved in DMSO and pre-incubated at 37°C for 1 min at a final concentration of 750 µmol/l. The reaction was started adding the plasmid pGEM®-T and incubating the mixture at 37°C x 30 min. At the end of this period, the mixture was submitted to electrophoresis on 0.8% agarose gels in TAE buffer, containing EtBr (1 mg/ml), and then photographed under UV light. Then the gels were photographed, digitalized and analyzed using the Gene Scope v.1.73 software. We estimated the percentage of DNA that remained supercoiled, or alternatively, quantified the appearance of relaxed DNA.

Apoptosis assay in HL-60 cells. The pro-apoptotic potential of the polycyclic compounds was assessed using the non-adherent cell line HL-60 (derived from human promyelocytic leukemia cells, ATCC CCL-240). A cell stock was prepared as follows: HL-60 cells were grown in 30 ml RPMI-1640 medium supplemented with 10% heat-inactivated BSA, 2 g/l NaHCO3, 1 mM sodium pyruvate, 100 UI/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. Cells were incubated at 37°C in a humidified atmosphere, with 5% CO2. The RPMI-1640 medium was changed every 48 h. From the stock cells, 3 ml were separated to analyze each quinone and 3 ml for each control. Each Petri dish contained approximately 2x10⁶ cells. This assay was performed in triplicate. For each compound, we used a quarter of their IC50, according to previous reports (15,17). Each Petri dish was incubated at 37°C for 12 h in the presence of the quinones. As positive control we treated HL-60 cells with 50 µM etoposide, and as negative control, cells only with the corresponding vehicle (DMSO). After this treatment, we measured the activity of caspase-3 using a colorimetric assay (caspase-3 Colorimetric Assay, R&D Systems, Inc.). Briefly, HL-60 cells were collected in Eppendorf tubes (1.5 ml) and centrifuged at 2,800 rpm x 10 min at 4°C. Immediately after this period, the supernatant was discarded and 50 µl of lysis buffer were added to each tube, resuspending the pellet and incubating on ice for 10 min. Then, the tubes were centrifuged at 10,000 rpm x 1 min at 4°C. From each tube, we collected 50 µl of supernatant (cell lysate), and transferred to the wells of ELISA microplates. Each well was loaded with 50 µl of reaction buffer 2X plus 5 µl of colorimetric substrate. The microplates were incubated at 37°C x 120 min. Finally, the microplates were read at 405 nm.

Statistical analysis. The results of the caspase-3 activity are presented as average ± standard deviation (SD). SPSS v.12.0 software was used to evaluate normality of the data (Kolmogorov-Smirnov) and variance homogeneity (Bartlett). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by the Dunnett's post-hoc test. A P-value <0.005 was considered significant.

Results

Purification of plasmid DNA and standardization of the relaxation assay. First, we extracted plasmid DNA pGEM®-T from TOP10 E. coli and analyzed its quality and purity. Using electrophoresis in agarose gels, plasmid DNA appeared supercoiled DNA and of about 2,500 bp, with purity over 90% (Fig. 2A).

Next, we standardized the supercoiled DNA relaxation assay as follows. Briefly, we first evaluated whether DMSO, the vehicle for the polycyclic quinones studied, had any effect on DNA relaxation (Fig. 2B). We analyzed the effect of DMSO in the presence or absence of human TOP1. DMSO did not affect the migration on agarose gels neither on DNA treated with TOP1 nor in DNA without treatment (supercoiled). Then, we validated the DNA relaxation, with increasing concentrations...
of EtBr, a DNA intercalating agent (Fig. 2C). EtBr effectively caused changes in DNA topology, causing relaxation that changed DNA migration. Next, we proceeded to determine the optimal concentration of TOP1 (Fig. 2D), enzymatic kinetics (Fig. 2E) and plasmid DNA concentration (Fig. 2F). As shown in the figures, incubation of plasmid DNA with different concentrations of TOP1, at different incubation times and at different DNA concentrations, caused mobility that shifted the bands to the level of 5,000 bp according to the DNA ladder. With these results we established the optimal conditions for the assay at 37°C, DNA concentration of 60 ng/ml, with 6 UI of human TOP1, with 30 min of incubation time.
Inhibition of TOP1 activity assays. The ability of polycyclic quinones to inhibit TOP1 activity was evaluated on relaxation assays, as indicated above. All the quinones were used at 750 µmol/l final concentration. As a control of TOP1 inhibition, we used camptothecin. At the end of the incubation period, the mixture was submitted to electrophoresis on agarose gels.

First, we explored the range of concentrations of the polycyclic quinones to be used in the DNA relaxation assay. For this, we chose one compound of each class: one angucyclinone (FG5) and one arylaminoquinone (C). We assayed these compounds in a range of concentrations from 0 to 1,000 µg/ml (Fig. 3A). Both compounds presented activity from 250 µg/ml, with a relative maximal at 750 µg/ml. For this reason we decided to use this concentration for assays with the rest of the compounds. Next, we assessed both series of quinones (Fig. 3B). Both series inhibited TOP1-induced DNA relaxation over 50% except the arylaminoquinones C and D, which basically showed no effect.
equivalent to a quarter of their previously reported IC\textsubscript{50} values for cytotoxicity (15,17). The aim was to assay their capacity to induce apoptosis at therapeutically relevant concentrations (~1 µmol/l). After this period, we determined the level of activation of caspase-3 using a colorimetric method. As a positive control, we used etoposide, an agent that inhibits TOP2, but which is known to induce apoptosis. We observed (Fig. 4) that the compounds OH-6H (3.9 µmol/l), P4 (1.45 µmol/l) and ADPA-1CC (2.35 µmol/l) induced caspase-3 activity significantly over control levels: 1.8±0.4, 2.1±0.23 and 1.48±0.04 (P<0.05 vs. control) respectively, while etoposide (50 µmol/l) increased caspase-3 activity by 3.7±0.2-fold over control.

Discussion

The usefulness of camptothecin analogs as pharmacological tools against cancer has led to the search of other classes of compounds that target human TOP1 activity (20). In the present study, we confirmed the hypothesis that aza-angucycliones and arylaminoquinones have potential anticancer activity through inhibition of TOP1 catalytic activity and induction of apoptosis. Most compounds of both families inhibited the catalytic activity of human TOP1, particularly FG-2 (75.3% inhibition) and the compound B (92.7% inhibition). Previously, both series of quinones showed important antiproliferative effects, in the range 4-53 µM, in other cancer lines (15,17), but the potential mechanisms were not studied.

Several studies show that the activation of apoptosis in cell cultures after the administration of antitumor agents that promote the release of cytchrome C, activation of caspases-3, -8, -9, DNA fragmentation dependent on caspase-2 (11), or through activation of death receptors (21). The series of quinones used in this study previously showed antitumor activity (15,17). We observed that the compounds OH-6H and P4, showed the highest capacity to induce activation of caspase-3 in HL-60 cells, at 4 and 1.5 µmol/l, respectively. Both were compared to etoposide, that increased 3.7-fold over control caspase-3 activity (at 50 µmol/l).

Apoptosis induced by quinones has been documented. Geldanamycin, a benzoquinone found in a strain of Streptomyces hygroscopicus, that is able to induce arrest of the cell cycle and apoptosis in tumor cells (22). Illudins, a class of quinone antibiotics, have been shown to induce apoptosis in animal tumor models (23); and hypocrellins, polycyclic quinones derived from Hypocrella bambusae, fungi used in traditional medicine, are able to induce caspase-3 activity and apoptosis (24). The molecular details of how angucyclinone 5-aza-analogues and arylaminopyrroimido[4,5-c]isoquinoline-7,10-quinones, exert the inhibition of TOP1 remain to be determined.

The effect of these compounds on TOP2 activity also has to be determined. HL-60 cells present TOP2 (25) and its contribution to the process of apoptosis is unknown. For instance, pyranonaphthoquinines such as eleuterine, ventiloquinone and thysanone and are able to inhibit TOP2 (26).

In conclusion, we present evidence that angucyclinone 5-aza-analogues and arylaminopyrroimido[4,5-c]isoquinoline-7,10-quinones have the potential to inhibit TOP1 and induce apoptosis by activation of caspase-3 in cancer cells. This group of molecules may represent a potential pharmacological tool in the treatment against cancer.

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