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Report

Quinone isomers of the WS-5995 antibiotics: synthetic antitumor agents that inhibit macromolecule synthesis, block nucleoside transport, induce DNA fragmentation, and decrease the growth and viability of L1210 leukemic cells more effectively than ellagic acid and genistein *in vitro*

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Antibiotic WS-5995A (code name J4) and two of its synthetic analogs, *o*-quinone J1 and model *p*-quinone J7, which show some structural similarity with both ellagic acid (EA) and genistein (GEN), were compared for their antileukemic activity in L1210 cells *in vitro*. Overall, J4 is more cytostatic and cytotoxic than J1 and J7, suggesting that methyl and methoxy substitutions, a *p*-quinone moiety, and a hydrogen bonding phenolic group may enhance the antitumor potential of these naphthoquinone lactones, which are all more potent than EA and GEN. For instance, the lead compound J4 inhibits tumor cell proliferation and viability at day 4 (IC_{50}: 0.24–0.65 μM) more effectively than EA (IC_{50}: 5–6 μM) and GEN (IC_{50}: 7 μM). Since J4 does not increase but rather decreases the mitotic index of L1210 cells at 24 h, it is not an antitubulin drug but might arrest early stages of cell cycle progression like EA and GEN. A 1.5- to 3-h pretreatment with J4 is sufficient to inhibit the rates of DNA, RNA and protein syntheses (IC_{50}: 2.0–2.5 μM) determined over 30- to 60-min periods of pulse-labeling in L1210 cells *in vitro*, whereas EA (IC_{50}: 20–130 μM) and GEN (IC_{50}: 40–115 μM) are less effective against macromolecule synthesis. In contrast to 156 μM EA, which is inactive, a 15-min pretreatment with 10–25 μM J4 has the advantage of also inhibiting the cellular transport of both purine and pyrimidine nucleosides over a 30 s period *in vitro*, an effect which can be mimicked by 156 μM GEN. Hence, the WS-5995 analogs and GEN may prevent the incorporation of [^{3}H]adenosine and [^{3}H]thymidine into DNA because they rapidly block the uptake of these nucleosides by the tumor cells. After 24 h, the concentration-dependent induction of DNA cleavage by J4 peaks at 10 μM and declines at 25 μM, whereas EA and GEN are ineffective at 10 μM but maximally stimulate DNA cleavage at 62.5 μM. Like EA and GEN, the mechanism by which J4 induces DNA fragmentation is inhibited by actinomycin D, cycloheximide, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, *N*-tosyl-L-phenylalanine chloromethyl ketone and $ZnSO_4$, suggesting that J4 triggers apoptosis by caspase and endonuclease activation. Because they are more potent than EA and GEN, and affect both nucleoside transport and DNA cleavage, the WS-5995 antitumor antibiotics might be valuable in polychemotherapy to potentiate the action of antimetabolites and sensitize multidrug-resistant tumor cells. [© 2001 Lippincott Williams & Wilkins.]

Key words: Apoptosis, DNA cleavage, ellagic acid, genistein, L1210 cells, macromolecule synthesis, mitotic index, nucleoside transport, tumor cell growth and viability, WS-5995 antibiotics.

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Introduction

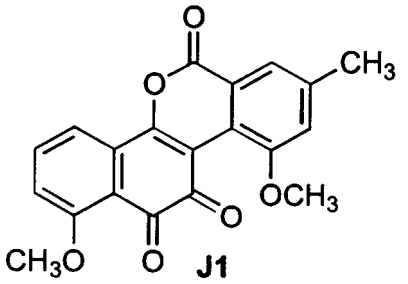
As precursors of reactive quinone methides, some natural and synthetic benzoquinones (BQs) and

naphthoquinones (NQs) function as bioreductive alkylating agents and have antitumor activity.[1–3] The cytotoxicity of quinones may be due to two competing mechanisms: soft electrophilic arylation and redox cycling oxidation.[4] Following bioreduction, conventional *p*-quinonoid-based cytotoxins generate semi-quinone free radicals (FRs) but the potential for *o*-quinone isomers to participate in similar chemistry also exists. However, the various quinone antitumor drugs used clinically, such as the anthracycline antibiotics doxorubicin (DOX) and daunorubicin (DAU), mitomycin C and BQ derivatives, have a complex chemical structure with a number of active functional groups and the exact contribution of the quinone group to their antitumor activity remains uncertain.[5–7] DOX and DAU covalently bind to and intercalate into DNA, inhibit DNA replication and RNA transcription, are DNA topoisomerase (Topo) II poisons, produce oxidative stress and damage biomembranes, induce DNA breakage and chromosomal aberrations, trigger apoptosis, and have a wide spectrum of anticancer activity.[5–9]

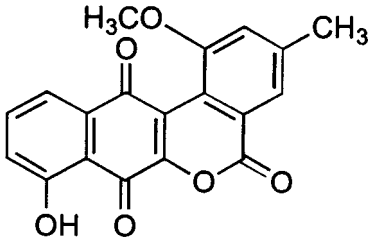
A limited number of quinoid structures with one (BQs), two (NQs) or three rings [anthraquinones (AQs)] and various substitutions may induce cytotoxic effects by a variety of mechanisms, which makes it difficult to assess structure–activity relationships (SARs).[1,10–14] Based on short-term photosynthetic inhibition, three-ring compounds are generally non-toxic to freshwater algae, but two-ring compounds are more toxic than related one-ring compounds.[11] At 1 μM, 2-methyl-1,4-NQ (vitamin K_3) is not or only marginally cytotoxic but sensitizes multidrug-resistant (MDR) P388 tumor cells to DOX.[15] The NQs include about 30 antitumor compounds, but the sodium salt of the monohydroxy NQ lapachol is the only compound active in the L1210 tumor system.[16] The synthetic geranyl and dibromoallyl derivatives of lapachol have also antitumor activity.[1] Among dihydroxy NQs, skikinin/alkannin esters show antibiotic and anticancer activity.[1] Most benzoisochromanquinones show antibiotic and antifungal activity, and fredericamycin A is a potent antitumor antibiotic.[1] Even though several groups of natural AQs and anthracyclinones contain interesting antitumor antibiotics, the number of bioactive 1,2-, 1,4- and 9,10-AQs appears quite limited, and only five of them elicit antitumor effects.[1,16,17] Among synthetic aminoalkylamino AQs, mitoxantrone is the only 9,10-AQ equipotent to DOX and DAU.[18] Mitoxantrone, a DNA-damaging agent which inhibits mammary tumor cells *in vitro* and *in vivo*, is somewhat effective in MDR tumor cells, synergistic with methotrexate (MTX) and 5-fluorouracil (5-FU), and less cardiotoxic than DOX.[18–27] However, we discovered recently that 1,4-AQ can also reduce tumor cell growth and viability in the same nM range as DAU *in vitro*, and may have a more versatile mechanism of action than DAU because it may interact with both membrane and nuclear targets to block nucleoside transport, inhibit nucleic acid and protein syntheses, and actively induce DNA fragmentation.[28]

Other types of biological interactions have been reported for a number of BQs, NQs and AQs, which are capable of inhibiting purified glutathione S-transferase activities *in vitro*,[10] inducing aquatic toxicity in the *Tetrahymena pyriformis*[14] and *Selenastrum capricornutum*[11] assay systems, blocking viral infectivity,[13] and quenching chlorophyll fluorescence in chloroplasts.[11] Interestingly, *o*-quinones have been reported to be the most toxic, whereas internal quinones with benzenoid substitution on both sides of the quinoid ring are not toxic at saturation.[14] Substitution of the 1,4-BQ ring by electron-donating methoxy or hydroxy groups decreases toxicity, 2,5-substitution being less toxic than 2,6-substitution.[14] Hence, fully substituted tetramethoxy- and tetrachloro-1,4-BQ derivatives, which cannot conjugate or arylate protein thiols, are significantly less toxic than 1,4-BQ.[14] In contrast, since the hydrophilicity or hydrophobicity of AQ substituents controls the fraction of chlorophyll accessible to quinone in chloroplasts, hydroxy substituents enhance the ability of 9,10-AQ to quench the singlet photoexcited state of light-harvesting chlorophyll (LHC), thereby reducing even more the population of LHC and its fluorescence intensity.[12]

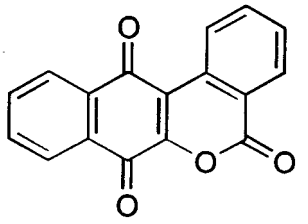
Though several pyranonaphthoquinone antibiotics have been identified, including nanaomycin A, kalafungin and eleutherin, relatively few members of the corresponding NQ lactones have been found. One member of this class is WS-5995A (code name J4 in Figure 1) and its structural analogs, including its open form WS-5995C, which are antibiotics structurally similar with the gilvocarcin antitumor antibiotics reported to function as DNA intercalators and Topo inhibitors (reviewed in Qabaja *et al.*[29]). Since the antitumor activity of J4 was unknown, substrate-dependent regioselective acid catalyzed quinolactonization procedures were recently developed and applied to the total synthesis of the WS-5995 antibiotics, allowing us to scrutinize the differential electrochemical reduction potentials and cytotoxicities of their quinone isomers.[29] Among the eight compounds synthesized and screened in our preliminary study, *p*-quinone J4, *o*-quinone J1 and unsubsti-



J1



J4 (WS-5995A)



J7

Figure 1. Structures of the *ortho* and *para* quinone compounds tested for their antileukemic activity *in vitro*.

tuted model *p*-quinone J7 (Figure 1) reduced the most L1210 tumor cell viability *in vitro*.[29] Because these lead antitumor compounds are similar in structure to both ellagic acid (EA) and genistein (GEN), the present study was undertaken to assess their molecular mechanism of action and compare their potency to those of EA and GEN. Interestingly, we found that these selected WS-5995 antibiotics are all more potent than EA and GEN, and may have an intriguing bifunctional mechanism of action, blocking nucleoside transport and actively inducing DNA fragmentation in order to prevent leukemic cells from synthesizing macromolecules, proliferating and remaining viable *in vitro*.

Materials and methods

Cell culture and drug treatments

All solutions of synthetic WS-5995 analogs, vincristine (VCR; from Lilly Research Laboratories, Indianapolis, IN), EA, GEN, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (all from Sigma, St Louis, MO) and benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk; from Calbiochem, La Jolla, CA) were dissolved and diluted in dimethyl sulfoxide (DMSO), whereas $ZnSO_4$ (JT Baker, Phillipsburg, NJ) and actinomycin D-mannitol (Act-D; Sigma) solutions were prepared in H_2O, and cycloheximide (CHX; Sigma) was dissolved in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS).[28,30,31] Suspension cultures of murine L1210 lymphocytic leukemia cells (ATCC, Rockville, MD) were maintained in continuous exponential growth by twice-a-week passage in RPMI 1640 medium supplemented with 8.25% fortified bovine calf serum (FCS; Hyclone, Logan, UT) and penicillin (100 IU/ml)-streptomycin (100 μg/ml), and incubated in the presence or absence of drugs at 37°C in a humidified atmosphere containing 5% CO_2. Since drugs were supplemented to the culture medium in 1 μl aliquots, the concentration of vehicle in the final incubation volume (0.5 ml) did not affect basal activity levels in control L1210 cells incubated in the absence of drugs.[28,30,31]

Cell proliferation and viability assays

For tumor cell growth, L1210 cells were resuspended in fresh FCS-containing RPMI 1640 medium, seeded in triplicate at an initial density of 7.5×10^3 cells/0.5 ml and incubated at 37°C in 48-well Costar cell culture plates (Costar, Cambridge, MA). Tumor cells were grown for 4 days in the presence or absence (control) of drugs and their density was monitored every 24 h using a Coulter counter (Beckman Coulter, Miami, FL).[28,30,31]

For tumor cell viability, L1210 cells suspended in FCS-containing RPMI 1640 medium were grown at 37°C in 48-well Costar cell culture plates for up to 4 days in the presence or absence (control) of drugs to evaluate their cytotoxicity. Decreasing concentrations of cells, such as 72×10^3 and 8×10^3 cells/0.5 ml/well, were initially plated in triplicate at time 0 in order to collect control samples with approximately equal cell densities after 2 and 4 days in culture, respectively. The viability of drug-treated cells was assessed from their ability to bioreduce the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega,

Madison, WI) in the presence of phenazine methosulfate (PMS; Sigma) into a water-soluble formazan product which absorbs at 490 nm.[32] At the appropriate time after drug treatment, cell samples (about 10^6/0.5 ml/well for controls) were further incubated at 37°C for 3 h in the dark in the presence of 0.1 ml of MTS : PMS (2 : 0.1) reagent and their relative cell viability was estimated by recording the absorbance at 490 nm, using a Cambridge model 750 automatic microplate reader (Packard, Downers Grove, IL).[28,30,31] Blank values for culture medium supplemented with MTS : PMS reagent in the absence of cells were substracted from the results.

Nucleoside transport and macromolecule synthesis

L1210 cells (1.24×10^6 cells/0.5 ml) were preincubated for 15 min at 37°C in the presence or absence (control) of drugs and then exposed to 1 μCi of [2,8-^{3}H]adenosine (30 Ci/mmol; American Radiolabeled Chemicals, St Louis, MO) or [methyl-^{3}H]thymidine (50 Ci/mmol; Amersham, Arlington Heights, IL) for only 30 s to, respectively, assess the cellular uptake of purine and pyrimidine nucleosides over such a very short period of time.[28,30,31,33] Reactions were diluted with 2 ml of ice-cold PBS and the unincorporated radiolabel was removed by centrifugation at 200 *g* for 10 min. After washing thrice with 2 ml of ice-cold PBS, intact cell pellets were harvested by centrifugation and incubated for 30 min in 1 ml of hypotonic lysis buffer (HLB) containing 10 mM Tris–HCl, pH 8.0, 1 mM EDTA and 0.2% Triton X-100. Cell lysates were mixed with 6 ml of Bio-Safe II (Research Products International, Mount Prospect, IL) and counted to estimate the cellular uptake of [^{3}H]adenosine or [^{3}H]thymidine. Drug inhibition was expressed as % of [^{3}H]adenosine or [^{3}H]thymidine transported into vehicle-treated control cells over similar 30-s periods.[28,30,31,33]

For nucleic acid and protein syntheses, L1210 cells were resuspended in fresh FCS-containing RPMI 1640 medium at a density of 8.28×10^5 cells/0.5 ml. To estimate the rate of DNA synthesis, cells were incubated at 37°C for 90 min in the presence or absence (control) of drugs and then pulse-labeled for an additional 30 min with 1 μCi of [^{3}H]thymidine. For RNA and protein syntheses, cells were incubated at 37°C for 3 h in the presence or absence (control) of drugs and then pulse-labeled for an additional 1 h with 2 μCi of [5,6-^{3}H]uridine (46 Ci/mmol; ICN Biomedicals, Irvine, CA) or 2.5 μCi of L-[3,4,5-^{3}H]leucine (120 Ci/mmol; American Radiolabeled Chemicals), respectively. The incubations were terminated by the addition of 0.5 ml of 10% trichloroacetic acid (TCA). After holding on ice for 15 min, the acid-insoluble material was recovered over Whatman GF/A glass microfiber filters and washed thrice with 2 ml of 5% TCA and twice with 2 ml of 100% EtOH. After drying the filters, the radioactivity bound to the acid-precipitable material was determined by liquid scintillation counting (LSC) in 6 ml of Bio-Safe NA (Research Products International).[28,30,31]

Mitotic index

L1210 cells (0.5×10^6/0.5 ml of FCS-containing RPMI 1640 medium) were incubated in triplicate for 24 h at 37°C in the presence or absence of drugs, including a known antimitotic, and collected by centrifugation at 200 *g* for 10 min to determine their mitotic index. For hypotonic treatment, cells were resuspended in 1 ml of 75 mM KCl for 20 min at 4°C. After fixation in 1 ml of MeOH : acetic acid (3 : 1), the final cell pellets were collected by centrifugation, resuspended in 75 μl of MeOH : acetic acid (3 : 1), dispensed onto glass slides, air dried and stained by spreading 40 μl of 0.1% crystal violet under a coverslip.[28,30,31] The percent of cells in mitosis was determined microscopically by counting 500 cells/slide. The mitotic index was calculated as the percent of mitotic cells in drug-treated cultures divided by the percent of mitotic cells in vehicle-treated controls.[28,30,31]

DNA cleavage and apoptosis

Drug-induced DNA cleavage was determined by intact chromatin precipitation, using L1210 cells which were prelabeled with 1 μCi of [^{3}H]thymidine for 2 h at 37°C, washed with 3×1 ml of ice-cold PBS, collected by centrifugation and resuspended in fresh FCS-containing RPMI 1640 medium at a density of 0.5×10^5 cells/0.5 ml.[9,28,30,31,34] Such cells containing prelabeled DNA were then incubated at 37°C for 24 h in the presence or absence (control) of drugs. After centrifugation at 200 *g* for 10 min to discard the drugs and wash the cells, the intact cell pellets were lysed for 30 min in 0.5 ml of HLB, centrifuged at 12 000 *g* for 30 min to collect the supernatants and resuspended in 0.5 ml of HLB. After another similar centrifugation, the radioactivities in the pooled supernatants (detergent-soluble low molecular weight DNA fragments) and the pellet (intact chromatin DNA) were determined by LSC: % DNA fragmentation = [c.p.m. in supernatant/c.p.m. in supernatant + pellet] $\times$ 100.[9,28,30,31,34]

For apoptosis, L1210 cells were incubated for various periods of time in the presence or absence (control) of drugs and DNA was extracted from samples with equal cell densities (10^6/0.5 ml), using a

salting out procedure.[30,35,36] After centrifugation at 200 *g* for 10 min, the cell pellets were washed twice with PBS and lysed overnight at 37°C in 0.34 ml of 10 mM Tris–HCl, pH 8.0, containing 2 mM EDTA, 400 mM NaCl, 1% SDS and proteinase K (0.5 mg/ml). Cell lysates were vortexed for 15 s with 0.1 ml of 6 M NaCl and centrifuged (3000 r.p.m. for 30 min). The supernatants (0.44 ml) were mixed with 0.88 ml of ice-cold 100% EtOH and kept at 4°C for 15 min for DNA precipitation. After centrifugation (13 000 r.p.m. for 15 min) at 4°C, the air-dried DNA pellets were dissolved in 0.34 ml of 10 mM Tris–HCl, pH 8.0, with 1 mM EDTA (TE buffer) and incubated for 2 h at 37°C in the presence of RNase (0.1 mg/ml). After another round of EtOH precipitation and centrifugation, the final air-dried pellets were dissolved in 50 μl of TE buffer and their DNA concentrations determined spectrophotometrically at 260 nm. Equal amounts of DNA samples (6 μg/7.5 μl of TE buffer) were mixed with 1.5 μl of 10 mM Tris–HCl, pH 7.5, containing 50 mM EDTA, 10% Ficoll 400 and 0.4% orange G, and loaded on each lane. About 0.5 μg of 100 bp DNA ladder and 0.75 μg of lambda DNA/*Eco*RI+*Hin*dIII (both from Promega) were similarly applied to each gel to provide size markers in the range 100–1500 and 125–21 226 bp, respectively. Horizontal electrophoresis of DNA samples was performed for 3.7 h at 60 V in 1.5% agarose gels containing ethidium bromide (1 μg/ml) with 90 mM Tris–HCl, pH 8.0, containing 90 mM boric acid and 2 mM EDTA as a running buffer. DNA fragments were visualized and photographed with Polaroid 667 film under UV transillumination.[30,36]

Results

Drugs

The chemical structures and code names of the three quinone compounds of the WS-5995 family selected to be tested for their antileukemic activity in the present study *in vitro* are shown in Figure 1. These lead antitumor compounds were synthesized by acid promoted quinolactonization of NQs, a published method providing direct access to either *ortho* or *para* isomers, and their correct nomenclatures are as follows:[29] J1, 1,10-dimethoxy-8-methyl-11,12-dihydro-6H-dibenzo[*c*,*h*]chromene-6,11,12-trione; J4, WS-5995A; J7, 7,12-dihydro-5H-dibenzo[*c*,*g*]chromene-5,7,12-trione. Importantly, J4 and J7 are both *p*-quinones, whereas J1 has the thermodynamically less stable *o*-quinone substructure. Our desire was to assess the contribution of the different quinone classes to overall antitumoral activity. The antitumor effects of J1, J4 and J7 were assessed, and compared to those of EA and GEN, two known antitumor agents which share some structural similarities with the WS-5995 framework. We selected EA and GEN as reference compounds since they both possess similar hydroxylated functionality to that found in the WS-5995 skeleton, and additionally, EA contains the lactone group as found in J1, J4 and J7.

Inhibition of tumor cell growth and viability

At concentrations of 25 μM or above, EA and GEN inhibit almost totally the proliferation and viability of L1210 cells at day 4 but these maximal cytostatic and cytotoxic activities of EA and GEN can be mimicked by smaller 1.6–10 μM concentrations of J4, J1 and J7. These relative potencies of J4, J1, J7, EA and GEN can easily be compared using the full concentration–response curves of Figures 2 and 3, where the striped areas at 100% represent the control levels of L1210 cell growth and viability after 4 days in culture. When ranked by decreasing effectiveness, the antiproliferative activities of J4, J1, J7, EA and GEN are, respectively, characterized by IC_{50} values of 0.24, 0.75, 1.1, 5 and 7 μM (Figure 2), whereas the cytotoxic activities of the same drugs are, respectively, characterized by IC_{50} values of 0.65, 2, 3.5, 6 and 7 μM (Figure 3). Hence, all three NQ lactones of the WS-5995 type tested are more cytostatic and cytotoxic than EA and GEN, and the lead compound J4, which is consistantly more effective than J1 and, especially, J7, is an antileukemic agent at least 9–20 times more potent than EA and GEN.

Inhibition of DNA synthesis and nucleoside transport

A 1.5-h treatment with J4, J1 or J7 is sufficient to inhibit, in a concentration-dependent manner, the rate of DNA synthesis determined over a 30-min period of pulse-labeling in L1210 cells *in vitro* (Figure 4). As compared to the three WS-5995 compounds, which become effective against DNA synthesis at 1.6–4 μM and maximally inhibit the rate of [^{3}H]thymidine incorporation into DNA at 10–25 μM, concentrations of 25 μM or above must be used to demonstrate the inhibitory effects of EA and GEN on DNA synthesis. When ranked by decreasing effectiveness, the concentration-dependent inhibitions of DNA synthesis by J4, J1, J7, EA and GEN are, respectively, characterized by IC_{50} values of 2, 4, 5, 20 and 50 μM, suggesting that, under these experimental conditions, J4 prevents L1210 cells from synthesizing DNA 10–25 times more effectively than EA and GEN (Figure 4).

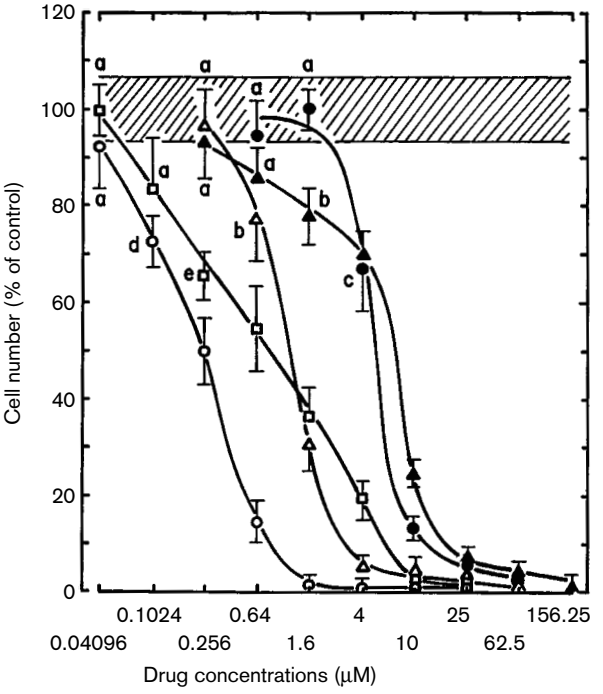


Figure 2. Comparison of the concentration-dependent inhibitions of L1210 cell proliferation by J1 (□), J4 (○), J7 (△), EA (●) and GEN (▲) at day 4 *in vitro*. Cell growth results are expressed as percent of the number of vehicle-treated control cells (100 ± 7%, striped area) after 4 days in culture (838 127 ± 55 568 cells/ml). Bars: means ± SD (n=3). [a]Not different from control; [b]$p<0.025$, [c]$p<0.01$ and [d]$p<0.005$, smaller than control; [e]$p<0.005$, smaller than control; and $p<0.05$, greater than 0.256 μM J4.

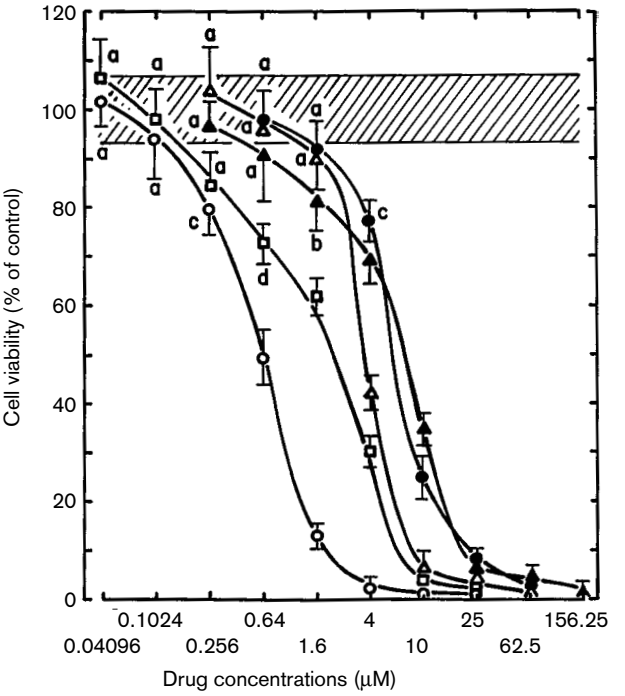


Figure 3. Comparison of the concentration-dependent inhibitions of L1210 cell viability by J1 (□), J4 (○), J7 (△), EA (●) and GEN (▲) at day 4 *in vitro*. Cell viability results are expressed as percent of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control cells (100 ± 7%, striped area) at day 4 (A_{490nm}=1.661 ± 0.112). The blank value (A_{490nm}=0.417 at day 4) for cell-free culture medium supplemented with MTS : PMS reagent has been substracted from the results. Bars: means ± SD (n=3). [a]Not different from control; [b]$p<0.025$ and [c]$p<0.01$, smaller than control; and [d]$p<0.005$, smaller than control and greater than 0.64 μM J4.

An interesting finding is that, in contrast to EA which serves as a negative control in the assays, a 15-min treatment with J4, J1, J7 or GEN is sufficient to block, in a concentration-dependent manner, the cellular transport of both [^{3}H]adenosine and [^{3}H]thymidine occuring over only 30 s *in vitro* (Figure 5). At 25 μM, J4, J1 and J7 inhibit the transport of these radiolabeled purine and pyrimidine nucleosides into L1210 cells by 58–71, 54–65 and 28–37%, respectively. The inhibitory effect of GEN is barely significant at 25 μM (data not shown) but, at 156.25 μM, this compound blocks nucleoside transport by 82–90% (Figure 5). However, 62.5 and 156.25 μM concentrations of EA are totally unable to significantly alter the cellular transport of [^{3}H]adenosine and [^{3}H]thymidine (Figure 5), even though such concentrations of EA, respectively, inhibit by 65 and 97% the incorporation of [^{3}H]thymidine into DNA used to assess the rate of DNA synthesis (Figure 4).

Inhibition of RNA and protein syntheses

Besides DNA synthesis, a 3-h treatment with J4, J1 or J7 can also inhibit, in a concentration-dependent manner, the rates of RNA and protein syntheses determined over 60-min periods of pulse-labeling in L1210 cells *in vitro* (Figures 6 and 7). The ranking of these WS-5995 compounds for their effectiveness against RNA and protein syntheses (Figures 6 and 7) is identical to that for their inhibition of DNA synthesis (Figure 4): J4 > J1 > J7, all three NQ lactones being more effective than EA and GEN. The concentration-dependent inhibitions of RNA synthesis by J4, J1, J7, EA and GEN are, respectively, characterized by IC_{50} values of 2, 5, 12, 30 and 40 μM, whereas the inhibitions of protein synthesis by the same drugs are, respectively, characterized by IC_{50} values of 2.5, 6.5, 18, 130 and 115 μM. Under these experimental conditions, therefore, J4 prevents L1210 cells from

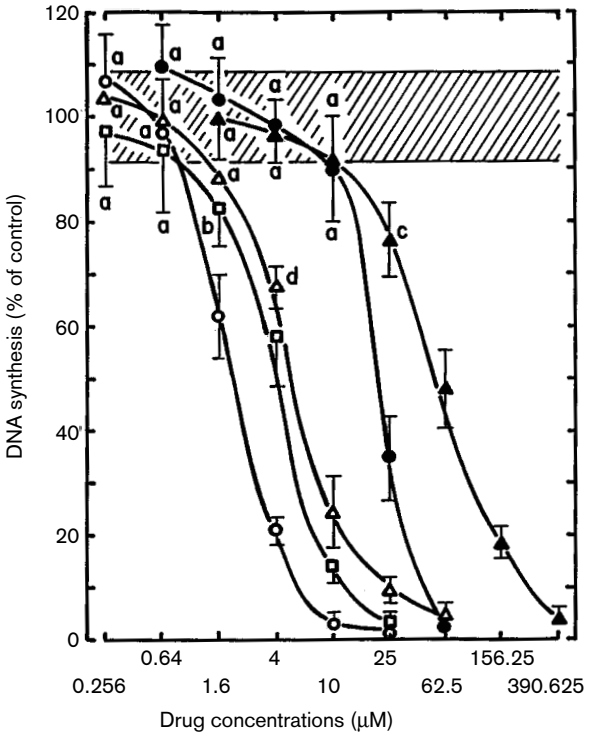


Figure 4. Comparison of the concentration-dependent inhibitions of DNA synthesis by J1 (□), J4 (○), J7 (△), EA (●) and GEN (▲) in L1210 cells *in vitro*. DNA synthesis in vehicle-treated control cells was 20536±1930 c.p.m. (100±9%, striped area). The blank value (759±49 c.p.m.) for control cells incubated and pulse-labeled at 2°C with 1 μCi of [^{3}H]thymidine has been substracted from the results. Bars: means±SD (n=3). [a]Not different from control; [b]$p<0.05$, smaller than control and greater than 1.6 μM J4; and [c]$p<0.025$ and [d]$p<0.01$, smaller than control.

synthesizing RNA and proteins, respectively, 15–20 and 46–52 times more effectively than EA and GEN (Figures 6 and 7).

Alteration of the mitotic index

Control populations of L1210 cells cultured for 24 h in the absence of drugs contain only 2.76% of mitotic cells. In relation with its ability to block tubulin polymerization and cell cycle progression in M phase, a 24-h treatment with 0.1 μM VCR produces a 5.4-fold increase in the mitotic index (Figure 8). Such known microtubule de-stabilizing anticancer drug, therefore, serves as a positive control in this antimitotic assay.[28,30,31] In contrast, none of the 10–62.5 μM concentrations of J1, J4 and J7 tested, which are already highly cytostatic and cytotoxic at 24 h, are able to raise the mitotic index of L1210 cells, demonstrating that the WS-5995 compounds are not antimitotic drugs that disrupt microtubule dynamics to trigger their anticancer activities. In relation with the known abilities of EA and GEN to, respectively, cause G_1 and G_2 arrest,[37–43] 25–62.5 μM EA and 156.25–390.625 μM GEN actually decrease the percent of mitotic cells by 62–91 and 97–99%, respectively (Figure 8). Since all concentrations of WS-5995 compounds tested significantly decrease the percent of tumor cells undergoing mitosis by 52–99% (Figure 8), J4, J1 and J7 are also likely to prevent cell cycle progression to mitosis.

Induction of DNA cleavage and apoptosis

L1210 cells containing [^{3}H]thymidine-prelabeled DNA were used to quantitatively determine whether WS-5995 compounds could induce DNA fragmentation over a 24-h period *in vitro* (Figure 9). High concentrations of EA, an inhibitor of both Topo I and II activities, and GEN, an inhibitor of Topo II activity, which are known to induce DNA breakage and apoptosis, are used as positive controls in this DNA fragmentation assay.[37,38,40–48] The concentration-dependent inductions of DNA cleavage caused by J4, J1 and J7 at 24 h are biphasic, all three WS-5995 compounds being more effective than EA and GEN (Figure 9). For instance, DNA breakage is initiated at 1.6 μM concentrations of J4 and J1 and 4 μM J7, and peak levels of 52, 43 and 35% DNA cleavage are respectively triggered by 10 μM concentrations of J4 and J1 and 25 μM J7 but higher concentrations of 25 μM J4 and J1 and 62.5 μM J7 lose their ability to induce DNA fragmentation. In contrast, 10 μM EA and 25 μM GEN fail to initiate DNA breakage, and 62.5 μM or higher concentrations of these compounds must be used to induce lower levels of 20–30% DNA cleavage after 24 h (Figure 9). Hence, J4 is capable of nearly doubling the peak induction of DNA cleavage caused by 6.25 times greater concentrations of EA and GEN (Figure 9).

Time- and concentration-dependent analyses of drug-induced internucleosomal DNA cleavage further indicate that J4 is a DNA-damaging agent substantially more potent than EA and GEN (Figure 10). No or very little DNA cleavage is visualized in controls, whereas DNA cleavage bands with a characteristic pattern of internucleosomal ladder suggestive of apoptosis become increasingly visible over a 3-day period in L1210 cells treated with 1.6–10 μM concentrations of J4 (Figure 10A). Interestingly, the peak of maximal DNA fragmentation observed at 24 h in response to 10 μM J4 shifts to lower concentrations with increasing times of drug exposure. In accord with the graph of the previous DNA cleavage assay (Figure 9), 10 μM J4 induces much more DNA fragmentation than 4 and

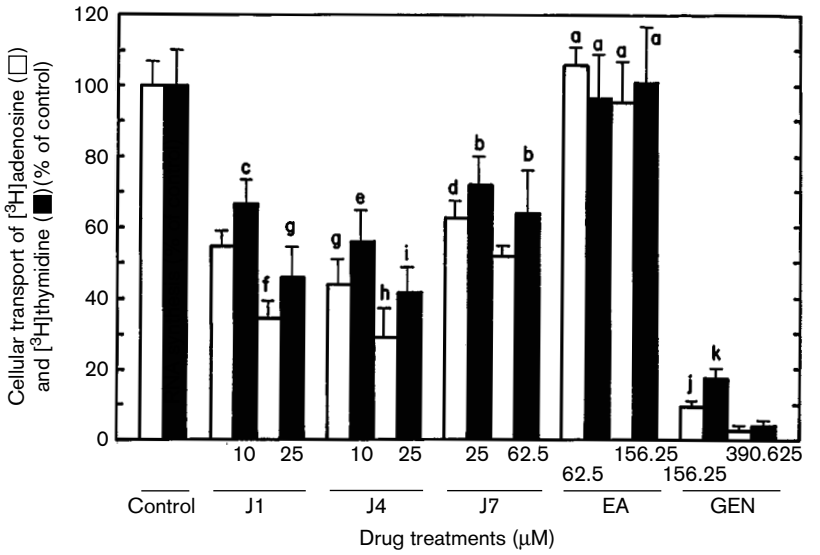


Figure 5. Comparison of the abilities of 10 and 25 μM J1 or J4, 25 and 62.5 μM J7 or EA, and 156.25 and 390.625 μM GEN to inhibit the cellular transport of purine (open) and pyrimidine (closed) nucleosides in L1210 cells *in vitro*. Results are ex-pressed as percent of [^{3}H]adenosine (18 637 $\pm$ 1269 c.p.m., 100 $\pm$ 7%, open control) or [^{3}H]thymidine (8631 $\pm$ 794 c.p.m., 100 $\pm$ 9%, closed control) transported into vehicle-treated control cells over 30 s. Bars: means $\pm$ SD (n=3). [a]Not different from controls; [b]$p<0.025$, [c]$p<0.01$ and [d]$p<0.005$, smaller than respective controls; [e]Not different from 25 μM J7; [f]$p<0.005$ and $p<0.01$, respectively smaller than 25 and 62.5 μM J7; [g]$p<0.025$, smaller than respective effects of 25 μM J7; [h]$p<0.025$ and [i]$p<0.05$, smaller than respective effects of 62.5 μM J7; and [j]$p<0.025$ and [k]$p<0.01$, smaller than respective effects of 25 μM J4.

1.6 μM at 24 h but 4 μM J4 becomes slightly more effective than 1.6 and 10 μM at 48 h and 1.6 μM J4 becomes even more effective than 4 and 10 μM at 72 h, suggesting that the events triggered by lower concentrations of J4 require substantially longer periods of time to fully induce DNA fragmentation (Figure 10A). When the full concentration–response for J4-induced DNA fragmentation is established at 24 h, no or only faint bands of DNA fragments are detected in controls and cells treated with 0.256 and 0.64 μM J4, whereas bands of lower molecular weight DNA become increasingly apparent at 1.6 and 4 μM, peak at 10 μM, but start disappearing at 25 μM (Figure 10B). As observed in Figure 9, the intensities of the DNA ladders caused by EA and GEN are less pronounced than that resulting from the 10 μM J4 treatment, EA being more effective at 62.5 than at 25 μM but 62.5 and 156.25 μM concentrations of GEN equally inducing the formation of DNA ladders indicative of apoptosis (Figure 10B).

Finally, non-toxic concentrations of Act-D, CHX, z-VAD-fmk, TPCK and $ZnSO_4$, which by themselves do not alter the basal level of DNA cleavage in L1210 cells after a 1 h preincubation period followed by 24 h of treatment (data not shown), were tested for their ability to affect the molecular mechanisms by which J4 induces DNA fragmentation and apoptosis (Figures 11 and 12). Such treatments with Act-D, CHX, z-VAD-fmk, TPCK and $ZnSO_4$, which are known to, respectively, inhibit RNA and protein syntheses, and caspase and endonuclease activities, either totally abolished or drastically reduced the ability of 10 μM J4 to maximally induce DNA cleavage and apoptosis at 24 h (Figures 11 and 12). For instance, considering that the control level of DNA cleavage is 4.8%, the level of DNA fragmentation induced by J4 (38.9%) at 24 h is inhibited by 71, 77, 54, 96 and 86% in the presence of Act-D, CHX, z-VAD-fmk, TPCK and $ZnSO_4$, respectively (Figure 11). The ladder of DNA fragments that characterizes apoptosis induction by the same J4 treatment is similarly reduced in the presence of Act-D, CHX, z-VAD-fmk, TPCK and $ZnSO_4$ (Figure 12). These preliminary data suggest that, like EA and GEN, the SW-5995 compounds may use, at least partially, an active process of apoptosis that requires gene expression, new RNA and protein syntheses, and caspase and endonuclease activations in order to trigger DNA fragmentation.

Discussion

To the best of our knowledge, this is the first report characterizing the antitumor effects of the WS-5995 antibiotics. On all markers tested, the effectiveness of these antitumor NQ lactones is consistently ranked as

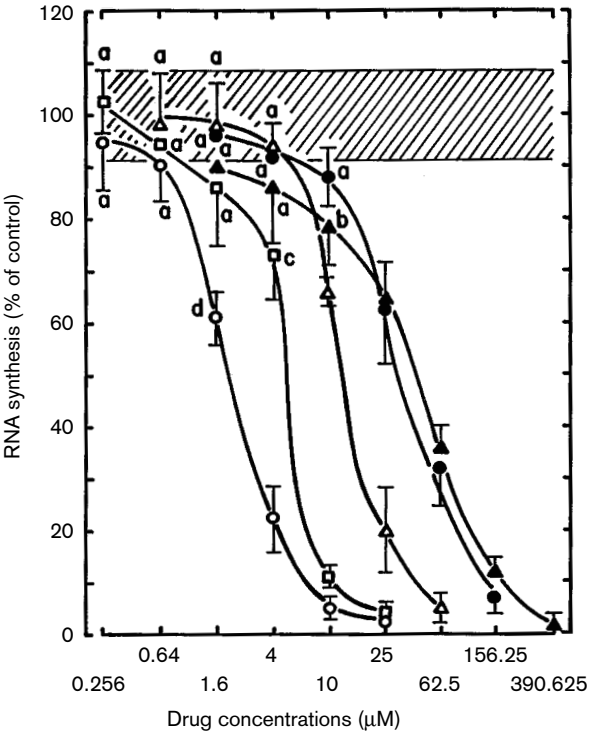


Figure 6. Comparison of the concentration-dependent inhibitions of RNA synthesis by J1 (□), J4 (○), J7 (△), EA (●) and GEN (▲) in L1210 cells *in vitro*. RNA synthesis in vehicle-treated control cells was 55 910 ± 4864 c.p.m. (100 ± 9%, striped area). The blank value (879 ± 112 c.p.m.) for control cells incubated and pulse-labeled at 2°C with 2 μCi of [^{3}H]uridine has been substracted from the results. Bars: means ± SD (n=3). [a]Not different from control; [b]$p<0.05$ and [c]$p<0.025$, smaller than control; and [d]$p<0.005$, smaller than control and $p<0.025$, smaller than 1.6 μM J1.

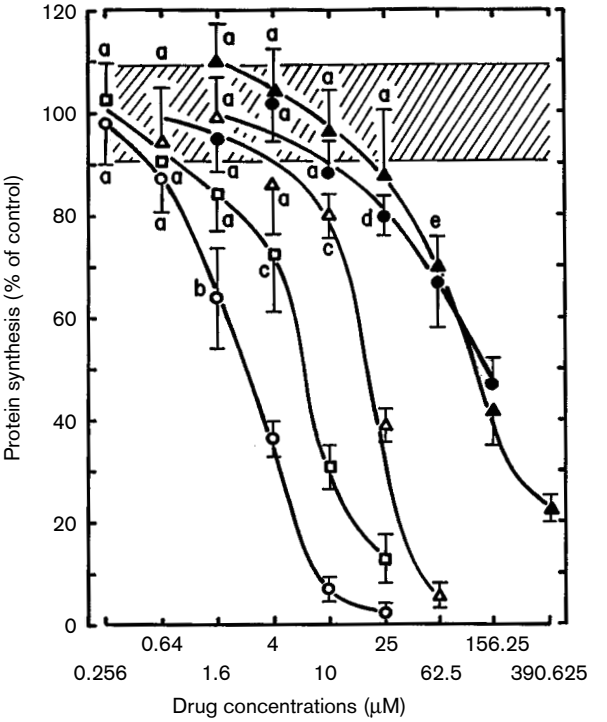


Figure 7. Comparison of the concentration-dependent inhibitions of protein synthesis by J1 (□), J4 (○), J7 (△), EA (●) and GEN (▲) in L1210 cells *in vitro*. Protein synthesis in vehicle-treated control cells was 12 726 ± 1171 c.p.m. (100 ± 9%, striped area). The blank value (1041 ± 125 c.p.m.) for control cells incubated and pulse-labeled at 2°C with 2.5 μCi of [^{3}H]leucine has been substracted from the results. Bars: means ± SD (n=3). [a]Not different from control; [b]$p<0.01$, smaller than control and $p<0.05$, smaller than 1.6 μM J1; and [c]$p<0.05$, [d]$p<0.025$ and [e]$p<0.01$, smaller than control.

J4>J1>J7, but it would be premature to speculate about SARs while the ability of various WS-5995 analogs to bind to nucleoside transporters, interact covalently with DNA, inhibit Topo activities and affect the production of reactive O_2 species and FRs is still unknown. So far, our preliminary data suggest that both *p*- and *o*-quinone isomers have antitumor potential and that, as compared to the J7 skeleton, the methyl, methoxy and/or hydroxy substitutions of J4 enhance its antitumor activity. What is intriguing is the antitumoral activity of J1, since relatively few *o*-quinone antitumor agents are known.

The magnitudes at which J4, J1 and J7 inhibit tumor cell proliferation (respective IC_{50} values: 0.24, 0.75 and 1.1 μM) match their cytotoxic activities (respective IC_{50} values: 0.65, 2 and 3.5 μM) at day 4 (Figures 2 and 3). Decreased tumor cell viability after J4 treatment may be a reliable predictor of anticancer activity.[49] The rates of DNA, RNA and protein syntheses (Figures 4, 6 and 7) are also inhibited to the same degrees by J4 (respective IC_{50} values: 2, 2 and 2.5 μM), J1 (respective IC_{50} values: 4, 5 and 6.5 μM) and J7 (respective IC_{50} values: 5, 12 and 18 μM). Moreover, J4, J1 and J7 inhibit DNA synthesis (Figure 4) in relation with their effectiveness against the cellular transport of purine and pyrimidine nucleosides (Figure 5), suggesting that the WS-5995 compounds may prevent DNA assembly because they rapidly block, at least partially, the cellular uptake of DNA precursors. For J4, concentrations in the 1.6–4 μM range are sufficient to totally inhibit tumor cell growth and viability (Figures 2 and 3), whereas higher concentrations of 10 μM must be used to maximally inhibit macromolecule syntheses (Figures 4, 6 and 7) and induce DNA cleavage (Figure 9). However, these apparent discrepancies may be due in part to different experimental conditions and cellular responses to various periods of drug exposure: the rates of nucleic acid and protein syntheses over 30–60 min are

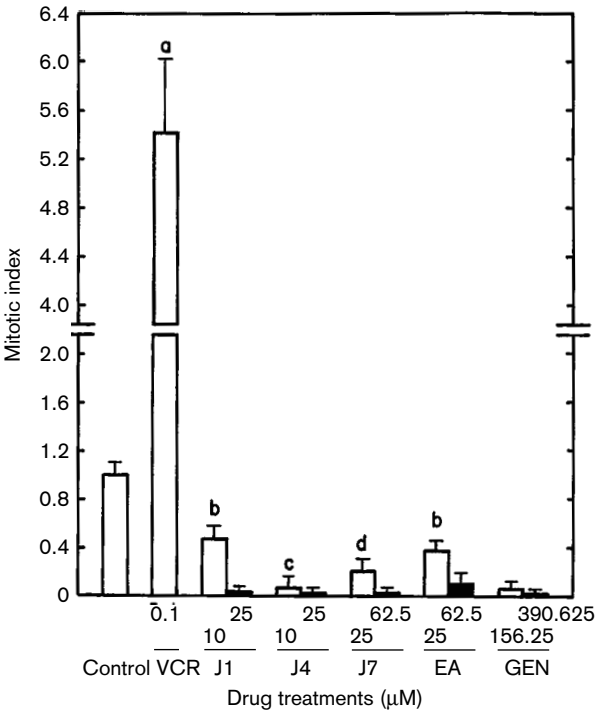


Figure 8. Comparison of the abilities of 10 and 25 μM J1 or J4, 25 and 62.5 μM J7 or EA, and 156.25 and 390.625 μM GEN to decrease the mitotic index of L1210 cells after 24 h *in vitro*. Results are expressed as the percent of mitotic cells in drug-treated cultures divided by the percent of mitotic cells in vehicle-treated controls. The mean percent of mitotic cells in control at 24 h was $2.76 \pm 0.35\%$. Bars: means $\pm$ SD (n=3). [a]$p < 0.0005$, greater than control; [b]$p < 0.005$, smaller than control; [c]$p < 0.01$, smaller than 10 μM J1; and [d]$p < 0.05$, smaller than 25 μM EA but greater than 25 μM J1 and J4.

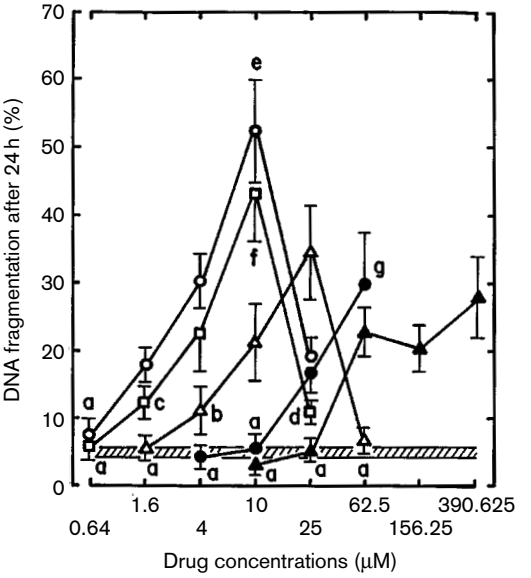


Figure 9. Comparison of the concentration-dependent effects of J1 (□), J4 (○), J7 (△), EA (●) and GEN (▲) on DNA cleavage in L1210 cells containing ^{3}H-prelabeled DNA *in vitro*. Results are expressed as [c.p.m. in supernatant/c.p.m. in supernatant + pellet] $\times$ 100 at 24 h. For untreated controls ($5.2 \pm 0.5\%$ DNA fragmentation, striped area), the supernatant (DNA fragments) is 2025 ± 203 c.p.m. and the pellet (intact DNA) is $37\,024 \pm 3998$ c.p.m.. Bars: means $\pm$ SD (n=3). [a]Not different from control; [b]$p < 0.05$, [c]$p < 0.025$ and [d]$p < 0.005$, greater than control; [e]$p < 0.05$, greater than 25 μM J7; [f]not different from 10 μM J4 and 25 μM J7; and [g]not different from 25 μM J7 and 62.5, 156.25 and 390.25 μM GEN.

inhibited in tumor cells treated for only 1.5–3 h with J4 (Figures 4, 6 and 7) and the peak of DNA cleavage is induced by a 24-h J4 treatment (Figure 9), whereas the more spectacular reductions of tumor cell growth and viability are the results of 4-day long J4 treatments (Figures 2 and 3). It should be noted that, after a short 15-min period of treatment, concentrations of J4 in the 10–25 μM range must be used to block only about 50% of the nucleoside transport occuring over 30 s (Figure 5), but that the level of DNA cleavage and the pattern of DNA laddering characteristic of apoptosis that are maximally induced by 10 μM J4 at 24 h (Figures 9 and 10) can be mimicked by 4 μM J4 at 48 h and 1.6 μM J4 at 72 h (Figure 10A). Hence, the antileukemic activity of J4 *in vitro* is likely to be due to a combination of drug concentration and duration of drug exposure.

An interesting finding is that the three WS-5995 compounds tested are all more effective than the known antitumor agents EA and GEN at inhibiting L1210 tumor cell growth/viability and macromolecule syntheses, and at inducing DNA fragmentation and apoptosis. The weaker antitumor activity of EA and GEN might be due to the fact that they lack the quinone functionality of the NQ lactones tested. The findings are of potential significance, since there is current interest in the development of new phenolic based agents (including members of the EA and GEN families) as chemopreventatives and antioxidants. In this context, the entire WS-5995 class may be worthy of further scrutiny.

As compared to EA and GEN, our lead compound J4 is 20–29 times more cytostatic (Figure 2), 9–11 times more cytotoxic (Figure 3), 10–25 times more effective at inhibiting DNA synthesis (Figure 4), 15–20 times better against RNA synthesis (Figure 6) and 46–52 times more potent against protein synthesis (Figure 7). Moreover, 10 μM J4 decreases the mitotic index to the same degrees than 62.5 μM EA and 156.25 μM GEN (Figure 8), and almost doubles the level of DNA cleavage induced by 62.5 μM EA and GEN (Figure 9),

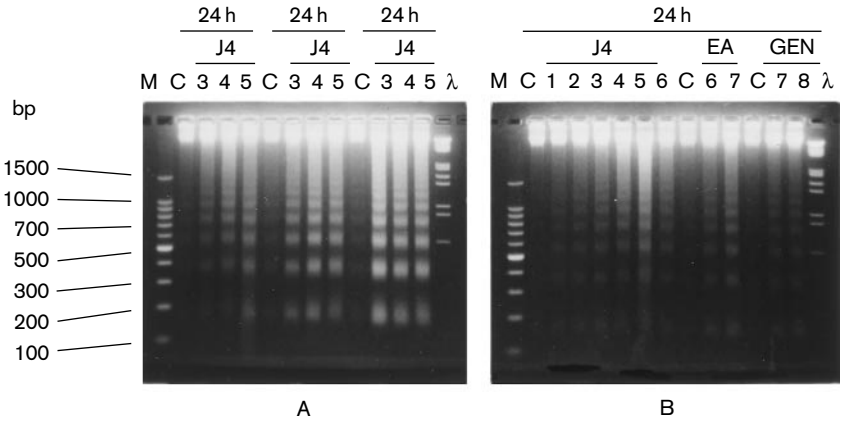


Figure 10. Agarose gel analysis of drug-induced internucleosomal DNA cleavage in L1210 cells *in vitro*. (A) Time course of DNA fragmentation in cells incubated at 37°C for 1, 2 and 3 days in the presence or absence (control: lanes C) of 1.6 (lanes 3), 4 (lanes 4) and 10 (lanes 5) μM J4. (B) Concentration-dependent level of DNA fragmentation in cells incubated at 37°C for 1 day in the presence or absence (control: lanes C) of 0.256, 0.64, 1.6, 4, 10 and 25 μM J4 (lanes 1–6), 25 and 62.5 (lanes 6 and 7) μM EA or 62.5 and 156.25 (lanes 7 and 8) μM GEN. Cellular DNA extracts (6 μg/well) were loaded onto a 1.5% agarose gel containing ethidium bromide (1 μg/ml), separated by electrophoresis for 3.7 h at 60 V, and photographed under UV light. A typical ladder pattern indicating the presence of DNA equivalent to the size of single and oligo nucleosomes is characteristic of apoptosis. Size markers are shown in lanes M (0.5 μg of 100-bp standard DNA ladder) and λ (0.75 μg of λ DNA/*Eco*RI+*Hin*dIII markers).

suggesting that the WS-5995 antitumor antibiotic is at least 6–15 times more potent than EA and GEN at arresting cell cycle progression and triggering DNA fragmentation. In contrast to EA, the treatments with 10 μM J4 and 156.25 μM GEN that inhibit DNA synthesis have the additional advantage of also blocking the cellular transport of purine and pyrimidine nucleosides (Figure 5), and J4 is likely to be a much more potent inhibitor of nucleoside transport than GEN since the 10 μM concentration of GEN which fails to inhibit [^{3}H]thymidine incorporation into DNA (Figure 4) is also unable to prevent the cellular uptake of [^{3}H]thymidine (data not shown). Finally, the percent of DNA cleavage and pattern of DNA laddering caused by J4 (Figures 9 and 10) are susceptible to Act-D, CHX, z-VAD-fmk, TPCK and $ZnSO_4$ inhibitions (Figures 11 and 12), suggesting that, like EA and GEN, the ability of WS-5995 compounds to trigger an active process of apoptosis that requires new RNA and protein syntheses and caspase and endonuclease activations in order to induce DNA fragmentation may play a significant role in their molecular mechanism of antitumor activity. Because of its ability to interact with both membrane and nuclear targets to block nucleoside transport, inhibit nucleic acid and protein syntheses, cleave DNA, and reduce tumor cell growth/viability more effectively than EA and GEN, J4 may represent a novel class of bifunctional quinone antitumor drugs perhaps valuable to develop new means of polychemotherapy. In any case, the antitumor potential of our lead NQ lactone, J4, appears superior to those of EA and GEN because it has either a greater potency or a more versatile mechanism of action that includes nucleoside transport inhibition. However, the eventual pro-oxidant and cardiotoxic effects of the WS-5995 compounds should be investigated and, even though J4 is cytostatic/cytotoxic in the low μM range and more potent than EA and GEN, one should remember that other clinical or experimental quinone antitumor drugs, such as DAU and 1,4-AQ, are effective in the low nM range in the same L1210 tumor system *in vitro*.[28] However, under similar conditions, the quinone antitumor drug DAU does not affect nucleoside transport.[28,31]

The antitumor effects of hydrolyzable tannins containing EA and gallic acid (GA) monomer units have been reviewed.[50] The antileukemic effects of EA (IC_{50} values: 5–130 μM) observed in the present study substantiate the reports that concentrations of EA, GA and their derivatives in the 1–750 μM range block Topo I activity, inhibit FR production, prevent cell transformation, decrease tumor cell growth/viability and induce apoptosis in different cell and cell-free systems *in vitro*.[51–58] However, higher concentrations of EA up to 20 mM have been used *in vitro* to demonstrate its protective effects against lipid peroxidation in liver microsomes and benzo[*a*]pyrene

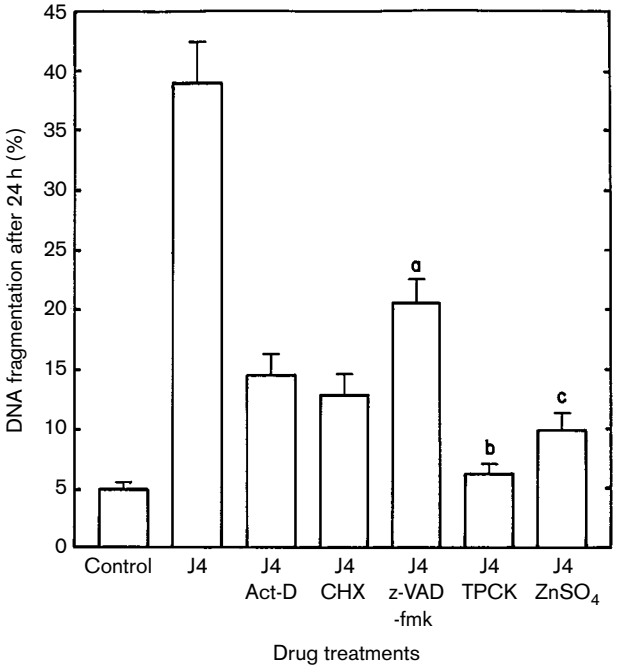


Figure 11. Inhibition of J4-induced DNA cleavage by Act-D, CHX, z-VAD-fmk, TPCK and $ZnSO_4$ in L1210 cells *in vitro*. Cells containing ^{3}H-prelabeled DNA were preincubated at 37°C for 1 h in the presence of vehicle, Act-D (1 μg/ml), CHX (25 μg/ml), 200 μM z-VAD-fmk, 50 μM TPCK or 100 μM $ZnSO_4$ and, after supplementing their culture medium with either vehicle (control) or 10 μM J4, these incubations were continued for an additional 24 h to determine the level of DNA cleavage as indicated in Figure 9. For drug-untreated controls preincubated and incubated in the presence of vehicle only (4.8 ± 0.5% DNA fragmentation), the supernatant is 1714 ± 183 c.p.m. and the pellet is $33\,658 \pm 3803$ c.p.m.. Bars: means $\pm$ SD (n=3). [a]$p < 0.005$, smaller than J4; [b]not different from control; and [c]$p < 0.005$, greater than control.

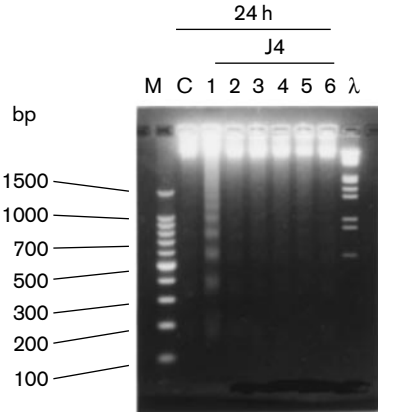


Figure 12. Agarose gel analysis of J4-induced internucleosomal DNA fragmentation after 24 h in L1210 cells treated *in vitro* with Act-D, CHX, z-VAD-fmk, TPCK or $ZnSO_4$. The conditions of the experiment were identical with those of Figures 10 and 11. Size markers are shown in lanes M and λ. Lane C: vehicle-treated controls. Lane 1: 24 h treatment with 10 μM J4. Preincubation for 1 h with Act-D (lane 2), CHX (lane 3), z-VAD-fmk (lane 4), TPCK (lane 5) or $ZnSO_4$ (lane 6) followed by 24 h treatment with J4.

metabolism in cultured keratinocytes.[59,60] EA binds to DNA, affects p53 and p21 expression, can inhibit various DNA-modifying enzymes, including Topo I and II, gyrase, DNA polymerase, reverse transcriptase and integrase, and induces G_1 arrest and apoptosis in cancer cells *in vitro* (reviewed in Thulstrup *et al.*[61]).[37,54] However, the formation of insoluble EA–metal ion complexes that activate intrinsic blood coagulation may preclude the utilization of systemic EA treatments *in vivo*.[62]

The antileukemic effects of GEN (IC_{50} values: 7–115 μM) observed in the present study match those already described for similar concentrations of GEN in the literature but, to the best of our knowledge, we are the first to report that 156.25 μM GEN totally blocks nucleoside transport. The polyhydroxylated isoflavone GEN is a phytoestrogen which neither intercalates into DNA nor alters Topo I activity, but is known to inhibit tyrosine protein kinase (TPK) activity and interact with the ATP binding domain of Topo II to inactivate this enzyme (IC_{50}: 111 μM), thereby producing protein-linked DNA strand breaks, decreasing tumor cell proliferation and stimulating tumor cell differentiation.[44–46,63–67] GEN does not exert its antiproliferative activity through the estrogen or androgen receptors and, as observed in Figure 2, 10–150 μM concentrations have been used in the literature to demonstrate that GEN inhibits the growth of various tumor cells more effectively at day 4 than at day 1, induces the differentiation of malignant tumor cells and decreases *in vitro* angiogenesis (reviewed in Wei *et al.*[68]).[42,44,65–67,69–72] As compared to Figure 3, GEN, which is reportedly more cytotoxic toward neoplastic than toward normal cells (reviewed in Wei *et al.*[68]), decreases the viability of HL-60 and MOLT-4 leukemic cells at 24 h with IC_{50} values of 31 and 48 μM, respectively.[38] Besides TPK, GEN may affect cell proliferation by interacting with phospholipase C, phosphatidylinositol kinases, ribosomal S6 kinase or MAP kinases downstream from growth factor receptor.[73] The antioxidant and antiinflammatory activities of GEN have been demonstrated at 15–45 μM *in vitro*. GEN inhibits the cellular formation of $O_2^{-\cdot}$ and H_2O_2, microsomal lipid peroxidation and oxidative DNA damage (reviewed in Wei *et al.*[74]).[75] GEN suppresses NADH oxidase and respiratory chain activities in mitochondria (reviewed in Wei *et al.*[74]) and prevents the cytochrome P-450-mediated oxidative metabolism

of benzo[*a*]pyrene.[76] Moreover, GEN decreases the transcriptional activations of inducible cyclooxygenase and inducible nitric oxide synthase, thereby inhibiting the production of prostagladins and NO (reviewed in Wei *et al.*[68]).[77] GEN can scavenge exogenously added H_2O_2,[74] and its $RO_2^{\cdot}$ and $^{\cdot}OH$ FR-absorbing activities are, respectively, more or less potent than those of Trolox, a water-soluble α-tocopherol analog.[78,79] Interestingly, GEN may sensitize MDR cells to anticancer drugs by inhibiting glutathione reductase activity, depleting reduced glutathione and thus preventing these drugs from being detoxified by glutathione S-transferase-mediated conjugation.[80]

In contrast to VCR, which is a microtubule destabilizing anticancer drug known to interact with the Vinca binding site on tubulin to block microtubule assembly and the progression of tumor cells undergoing mitosis,[30] the inability of J4 to raise the mitotic index of L1210 cells after 24 h suggests that this WS-5995 antibiotic is not a mitotic spindle poison and neither interacts with tubulin nor alters the polymerization/depolymerization of microtubules in order to induce its cytostatic/cytotoxic effects. In fact, anticancer quinones have been reported to cause G_2 arrest,[6] and the ability of 10–62.5 μM concentrations of J4, J1 and J7 to dramatically reduce the percent of mitotic cells at 24 h (Figure 8) like the increasing concentrations of DAU known to first block tumor cells in G_2 and then prevent cell cycle traverse all together (reviewed in Perchellet *et al.*[31]) suggests that the WS-5995 compounds might also block early stages of cell cycle progresion to prevent tumor cells from reaching the M phase. This hypothesis is substantiated by the fact that 10 μM J4 decreases the mitotic index of L1210 cells at 24 h as much as 62.5 μM EA and 156.25 μM GEN (Figure 8), our two reference compounds which are, respectively, known to arrest tumor cells in G_1 and G_2.[37–43] In contrast to low concentrations of GEN of 20 μM or below, which upregulate the cyclin-dependent kinase inhibitors $p27^{KIP1}$ and $p21^{WAF1}$, produce a G_1 block but do not stimulate apoptosis,[81] high concentrations of GEN in the 60–150 μM range are reported to decrease the mitotic index,[39] maximally induce a partially reversible arrest in G_2,[38,40,41] and trigger DNA fragmentation and apoptosis in various tumor cells lines at 24 h (reviewed in Constantinou *et al.*[41]).[38,39,42,81] At 50–150 μM, GEN has been shown to rapidly cause nuclear fragmentation characteristic of apoptosis[38] by decreasing the mitochondrial membrane potential and stimulating the release of cytochrome *c*,[48] upregulating p53,[41] inhibiting the antiapoptotic role of NF-κB activity,[47] and downregulating *bcl*-2, which is inactivated by phosphorylation and loses its antiapoptotic potential because it can no longer bind to *bax*.[41]

The fact that, within 24 h, J4 can produce as much, or even more, internucleosomal DNA fragmentation in L1210 cells as several known Topo I and II inhibitors, such as camptothecin and DAU in previous studies[28,31] and EA and GEN in Figures 9 and 10, suggests that the ability of WS-5995 compounds to produce DNA strand breaks may play a major role in their mechanism of antitumor activity. Since the shape of the concentration–response curves for the effects of J4, J1 and J7 on DNA cleavage (Figure 9) resembles the biphasic response to DAU,[9] which has already been reported and discussed before (reviewed in Perchellet *et al.*[31]), it is tempting to speculate that the mechanisms by which the NQ lactones and DAU induce DNA fragmentation share some similarity. The Topo II-associated DNA lesions occuring in cells exposed to anthracycline quinone antibiotics may facilitate subsequent internucleosomal DNA fragmentation by endogenous nucleases and trigger apoptosis.[9]

Since apoptosis is an active and cell cycle phase-specific process, which requires the expression of specific genes, the synthesis of new mRNA and proteins and the activation of caspase and endonuclease enzymes, inhibitors of such mechanisms can prevent DNA fragmentation in anthracycline-treated cells (reviewed in Ling *et al.*[9]). Hence, the abilities of the inhibitors of RNA (Act-D) and protein (CHX) syntheses, and caspase (z-VAD-fmk), non-caspase proteases (TPCK) and endonuclease ($ZnSO_4$) activities to similarly block or reduce the mechanisms of DNA fragmentation by DAU and 1,4-AQ in a previous study[28] and J4 (Figures 11 and 12), EA and GEN (data not shown) in the present study suggest that, just like 0.256–1.6 μM DAU, 0.256–4 μM 1,4-AQ[28] and 62.5 μM EA or GEN, 1.6–10 μM concentrations of J4 might also activate caspases, non-caspase proteases and nucleases in order to trigger apoptosis, break DNA and induce tumor cell killing. In spite of their increasing cytotoxicity, the highest concentrations of 25 μM J4 and J1 or 62.5 μM J7 tested in our study might inhibit RNA and protein syntheses (Figures 6 and 7), reduce the level of Topo targets, inactivate caspases and endonucleases, and/or arrest cell cycle traverse to such degrees that they might actually block their own ability to sustain the active molecular mechanisms required for internucleosomal DNA fragmentation and apoptosis, and produce the paradoxical biphasic curve of DNA cleavage shown in Figure 9. An alternate explanation might be that, in response to increasingly cytotoxic concentrations or durations of J4 treatments (Figures 9 and 10), increasing fractions of DNA-damaged tumor cells become necrotic, lose their membrane integrity and are eliminated from the

pellet of intact cells from which cellular DNA is extracted to assess drug-induced DNA fragmentation by intact chromatin precipitation or agarose gel electrophoresis.

J4, which inhibits nucleoside transport and induces DNA cleavage, might disrupt a wider spectrum of molecular targets in populations of unsynchronized tumor cells than another drug affecting a single of these events. For nucleotide synthesis, cells use purine and pyrimidine nucleosides generated either through *de novo* synthesis or through the utilization of salvage pathways. MDR is sometimes associated with increases in the number of nucleoside transporters and their rate of transport, resulting in the increased uptake of adenosine.[82] By blocking the rescue effect of exogenous nucleosides, nucleoside transport inhibitors may potentiate or prolong the antitumor activity of antimetabolites which already inhibit the *de novo* pathway for nucleoside synthesis (reviewed in Perchellet *et al.*[28]). Moreover, nucleoside transport inhibitors may also circumvent DOX resistance by interfering with both P-glycoprotein and nucleoside transport in MDR cells.[83,84] As a bifunctional inhibitor of nucleoside transport and inducer of DNA fragmentation, J4 might be valuable in polychemotherapy to potentiate the antitumor actions of MTX and 5-FU, and sensitize MDR tumor cells that have become unresponsive to the cytotoxicities of other conventional DNA-damaging anticancer agents.

Conclusion

The WS-5995 antibiotics may represent a new class of quinone antitumor drugs, which block the cellular transport of purine and pyrimidine nucleosides and induce DNA fragmentation by an active apoptotic process in order to inhibit nucleic acid and protein syntheses, and decrease the proliferation and viability of leukemic cells more effectively then EA and GEN *in vitro*. Further SAR studies are warranted to elucidate in detail the molecular mechanism of action and demonstrate the anticancer potential *in vivo* of these NQ lactones, which might be valuable to develop new means of polychemotherapy.

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