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Original Paper

Augmentation of 1-β-D-Arabinofuranosylcytosine (Ara-C) cytotoxicity in leukaemia cells by co-administration with antisignalling drugs

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The ribonucleotide reductase inhibitors hydroxyurea (HU), arabinosyl-2-fluoroadenine (F-Ara-A) and 2-chlorodeoxyadenosine (2-CdA) and the antisignalling drugs all-*trans* retinoic acid (ATRA), staurosporine and quercetin have been reported to enhance the cytotoxicity of 1-β-D-arabinofuranosylcytosine (ara-C). We tested the hypothesis that the ara-C-sensitising potency of the antisignalling agents is equipotent with that of the ribonucleotide inhibitors. The cytotoxicity, determined by the 3-(4,5 dimethylthiazol-2-y1-)5 diphenyltetrazolium bromide (MTT) assay, of combinations of ara-C with the agents named above was compared in the leukaemia cell lines HL-60, ara-C-resistant HL-60 (HL-60/ara-C) and U937. Furthermore, a range of protein tyrosine kinase inhibitors, genistein, CGP 52411, tyrphostin A48 and nordihydroguaiaretic acid (NDGA), for which ara-C-sensitisation has hitherto not been described, were included in the study. All three cell types acquired increased sensitivity to ara-C when co-incubated with HU or ATRA, but their ara-C sensitivity was not affected by quercetin or genistein. 2-CdA, CGP 52411, tyrphostin A48, staurosporine and NDGA were active as sensitisers against ara-C in HL-60 cells, CGP 52411 and tyrphostin A48 also in HL-60/ara-C cells, and 2-CdA, staurosporine and NDGA also in U937 cells. F-Ara-A increased ara-C toxicity in HL-60/ara-C and U937 cells. To address the mechanism of the observed sensitisation, the influence of agents with ara-C-sensitising properties on ara-C-induced apoptosis was investigated in HL-60 cells as measured by cell shrinkage, DNA loss and DNA fragmentation. HU, ATRA, tyrphostin A48 and NDGA augmented apoptosis induced by ara-C as assessed by all three indicators. CGP 52411 decreased the effect of ara-C on apoptotic indicators after incubation for 4 h, but not after 12 h. The results suggest that ATRA, CGP 52411, tyrphostin A48, staurosporine and NDGA may be suitable alternatives to the clinically applied ribonucleotide reductase inhibitors as modifiers of ara-C cytotoxicity in the treatment of acute myeloid leukaemia.

Key words: apoptosis, antisignalling drugs, ara-C, cytotoxicity, HL-60 cells, ribonucleotide reductase inhibitors

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INTRODUCTION

1-β-D-ARABINOFURANOSYLCYTOSINE (ara-C, cytarabine) plays a central role in the treatment of leukaemias, particularly acute myeloid leukaemias (AML). In the cell, ara-C is phosphorylated to its cytotoxic metabolite ara-cytosinetriphosphate (ara-CTP) via the action of a sequence of kinases. Incorporation of ara-CTP into DNA and subsequent induction of DNA strand breaks is considered to be the major mechanism by which ara-C exerts cytotoxicity. In clinical studies, ara-CTP levels have been correlated with manifestations of cytotoxicity or clinical outcome [1–3]. Resistance against ara-C is a major cause of failure of AML treatment. Mechanistically, acquisition of ara-C resistance can involve

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several steps in the metabolic toxification pathway of ara-C, and resistance can be moderated by co-administration with agents such as hydroxyurea (HU), 2-chlorodeoxyadenosine (2-CdA) and arabinosyl-2-fluoroadenine (F-Ara-A) [4]. Augmentation of cellular ara-CTP levels by these agents is one feasible mechanism to explain the increase in cellular sensitivity against ara-C caused by these agents. Combinations of ara-C with F-Ara-A or 2-CdA are currently under clinical phase I and II evaluation [5, 6]. Other drugs which have been reported to sensitise cells to ara-C are the kinase modulators quercetin [7], staurosporine [8] and bryostatin 1 [9, 10] and the differentiation-inducing drug all-*trans* retinoic acid (ATRA) [11, 12], agents which are thought to exert antineoplastic effects *per se* via perturbation of cellular signal transduction pathways and not via alteration of ara-C metabolism. From a mechanistic standpoint, the ara-C-sensitising activity of these agents is pertinent in the light of the fact that ara-C itself has been implicated as a modulator of signal transduction pathways. It has been shown to modulate the activity of a number of kinases, including mitogen-activated protein (MAP) kinase [13], protein kinase C [14] and cdc 2 kinase [15], to upregulate cyclin E [16], to decrease phosphorylation of retinoblastoma protein [17], to activate the transcription factors NFkappa B [18] and AP-1 [19] and to increase the rate of transcription of the c-*fos* and c-*jun* proto-oncogenes [20]. The role which these effects have as mechanistic arbiters of ara-C cytotoxicity is unclear. Induction of programmed cell death (apoptosis) has been proposed as a mechanistic component of the cell killing properties of ara-C [21], and perturbation of signal transduction is germane to induction of apoptosis. Sensitisation of cells to ara-C cytotoxicity by staurosporine was not accompanied by augmented ara-CTP levels [8], which suggests that this, and perhaps other, antisignalling drugs increase ara-C cytotoxicity independent of modulation of ara-C metabolism.

The ara-C-sensitising potencies of ribonucleotide reductase inhibitors and antisignalling drugs have hitherto not been rigorously compared. Therefore, we investigated the abilities of CdA, F-Ara-A and HU to sensitise cells to ara-C and juxtaposed their sensitising efficacy with that of quercetin, staurosporine and ATRA. Furthermore we wanted to test the hypothesis that kinase-inhibitory antisignalling drugs other than quercetin and staurosporine, for which sensitisation has been demonstrated (see above), augment ara-C cytotoxicity. Apart from quercetin and staurosporine we chose genistein, CGP 52411, tyrphostin A48 and nordihydroguaiaretic acid (NDGA). Quercetin, genistein and staurosporine are relatively non-specific kinase inhibitors [22, 23], CGP 52411 and tyrphostin A48 inhibit the epidermal growth factor (EGF) receptor-linked protein tyrosine kinase (PTK) with relative specificity [24, 25], and NDGA is a selective inhibitor of platelet-derived growth factor (PDGF) receptor-linked PTK [26]. As leukaemia cell models for these investigations, we chose human-derived HL-60 promyelocytic cells and U937 monocytic cells and a newly derived ara-C resistant variant, HL-60/ara-C cells. Furthermore we investigated whether sensitisation of HL-60 cells against ara-C by such agents may be accompanied by an increase in susceptibility towards ara-C induced apoptosis. Overall, the work was aimed at the comparative characterisation of some known and novel agents for their ara-C-sensitising activity with the ultimate rationale to help enlarge the oncologist's therapeutic armamentarium in the treatment of childhood leukaemias.

MATERIALS AND METHODS

Drugs and cell lines

Ara-C, HU, 2-CdA, F-Ara-A, ATRA, staurosporine, genistein and quercetin were purchased from Sigma Chemical Co. (Dorset, U.K.), NDGA from Fluka Chemie AG (Buch, Switzerland) and tyrphostin A48 from Calbiochem (Nottingham, U.K.). CGP 52411 was a gift from Dr E. Buchdunger (Ciba-Geigy, Basel, Switzerland). Ara-C, HU, 2-CdA and F-Ara-A were dissolved in phosphate-buffered saline (PBS), the other drugs in dimethylsulphoxide (DMSO) or ethanol. Stock solutions were stored at -20°C. Concentrations of DMSO and ethanol did not affect cell growth and viability. Incubations with 2-CdA, ATRA, tyrphostin A48, genistein and quercetin were protected from light. Strictly speaking ATRA is a 'signalling', not an 'antisignalling', drug, but for reasons of simplicity we incorporate ATRA under the heading 'antisignalling' agents.

HL-60 promyelocytic and U937 monocytic leukaemia cells were obtained from the European Collection of Cell Cultures (Salisbury, U.K.) and tested negative for mycoplasmal infection. An ara-C-resistant HL-60 cell line (HL-60/ara-C) was isolated by culturing cells for 6 months in the presence of ara-C at gradually increasing concentrations, as described previously [27]. Cells were cultured in RPMI-1640 medium (with phenol-red) (Gibco, U.K.) supplemented with 10% heat-inactivated fetal calf serum, glutamine (2 nmol/l), and incubations for cytotoxicity assays included penicillin (100 U/ml) and streptomycin (50 μg/ml). Cultures were grown under 5% CO_2 in a humidified atmosphere at 37°C.

Cytotoxicity assay

The cytotoxicity of ara-C without or with modulator was determined using the 3-(4, 5 dimethylthiazol-2-yl-)5 diphenyltetrazolium bromide (MTT) assay [28, 29]. Cells (10^6/ml HL-60 and HL-60/ara-C; 4×10^5/ml U937) were seeded in dishes with 96 round bottom wells (Nunc, Naperville, U.S.A.) and incubated with drugs for 24 h, then centrifuged (800 *g*, 5 min), washed with PBS and resuspended in a solution of MTT (5 mg/ml RPMI). After incubation for 4 h, cells were washed in PBS and the formazan crystals were dissolved in DMSO. Optical density was measured at 540 nm using a plate reader (Labsystems iEMS Reader MF). Experiments were conducted in octuplets and repeated at least twice. Potential chemical interactions between agents and MTT were ruled out by including tests with non-viable cells [30]. Erroneously high MTT values have been reported for cells with compounds which cause G2 arrest [31]. This problem was unlikely to confound the analysis described here, since ara-C cytotoxicity is mainly S phase specific [32], and the modulators were used at concentrations which were without any, or with only marginal, cytotoxicity when used on their own. Ara-C was used at two concentrations, 0.1 and 10 μmol/l in HL-60 cells, 100 and 2500 μmol/l in HL-60/ara-C cells, and 0.5 and 5 μmol/l in U937 cells. The ara-C concentrations which caused 50% cytotoxicity (IC_{50}) were 8.7 ± 0.39, 6399 ± 211 and 3.2 ± 0.5 μmol/l in HL-60, HL-60/ara-C and U937 cells, respectively. Modulators were employed at the following concentrations in HL-60, HL-60/ara-C and U937 cells, respectively: 2-CdA 0.05, 100 and 0.05 μmol/l; F-Ara-A 1, 100 and 1 μmol/l; quercetin 50, 50 and 10 μmol/l; genistein 10, 50 and 50 μmol/l; and for all three cell lines, HU 100 μmol/l; ATRA 1 μmol/l; staurosporine 0.01 μmol/l; CGP 52411 10 μmol/l; tyrphostin A48 50 μmol/l; and NDGA

10 μmol/l. Modulators were co-incubated with ara-C, without a pre-incubation period. Such a protocol has been shown to furnish significant, albeit not maximal, augmentation of levels of the active ara-C metabolite ara-CTP when incubated together with F-Ara-A, 2-CdA and HU [33]. In preliminary experiments we established that, using this protocol, F-Ara-A, 2-CdA and HU significantly exacerbated ara-C-induced cytotoxicity in the cells used here. It cannot be ruled out that scheduling plays a role in determining the interaction between ara-C and antisignalling agents, therefore the co-incubation protocol used here constitutes a simplification. The term 'sensitisation' in the text describes supra-additive cytotoxicity of the combination compared with the sum of cytotoxicities of ara-C and modulator alone.

Measurement of deoxycytidine kinase activity

Deoxycytidine kinase activity in HL-60 and HL-60/ara-C cells was determined according to Ruiz van Haperen and associates [34]. Enzyme activity was measured at low (10 μmol/l) and saturating (230 μmol/l) deoxycytidine concentrations, in the absence and presence of thymidine (1 mM) which inhibits mitochondrial thymidine kinase 2 activity [35].

Measurement of apoptosis

HL-60 cells (1×10^6/lane) were exposed for 4 h to tyrphostin A48 (50 μmol/l), ATRA (50 μmol/l), NDGA (10 μmol/l), HU 100 (μmol/l), CGP 52411 (10 μmol/l) or staurosporine (0.05 μmol/l) alone or in combination with ara-C (2–10 μmol/l) and analysed for markers of apoptosis. The modulators on their own at these concentrations failed to induce apoptosis, or caused only minimal apoptosis. Oligonucleosomal DNA fragmentation was determined on 1.8% agarose gels as previously described [36]. Electrophoresis was conducted at 20 V for 1 h in the presence of 2% sodium dodecylsulphate (SDS) and proteinase K (1.25 mg/ml) to ensure cell lysis, then for 3–4 h at 100 V. Hypodiploid cells (sub-G1 peak) were quantitated flow-cytometrically as described by Ormerod [37]. Following drug exposure, cells were fixed by exposure to 70% ethanol overnight at 4°C. Subsequently, cells were washed and resuspended in a solution containing RNaseA (0.05 mg/ml) and propidium iodide (PI, 25 μg/ml). The DNA histogram of the PI-stained nuclei was measured on a FACScan instrument (Becton Dickinson) (486 nm, flow rate 200 cells/sec). Clumped cells were excluded by bivariate histogram of the peak and the integrated area of the red fluorescent signal. Changes in cell size were detected with a Casy 1 counting and sizing instrument (Schaerfe System, Reutlingen, Germany) using a 150 μm aperture.

Differences in cytotoxicity and apoptosis indicators were analysed for significance by Student's *t*-test.

RESULTS

Modulation of ara-C cytotoxicity

Effects of the ribonucleotide reductase inhibitors HU, 2-CdA and F-Ara-A, or the antisignalling drugs genistein, quercetin, staurosporine, CGP 52411, tyrphostin A48, NDGA and ATRA on the cytotoxicity of ara-C in HL-60, newly derived HL-60/ara-C and U937 cells were investigated using the MTT assay. HL-60/ara-C cells were characterised by acquisition of 1000-fold resistance against ara-C and 333-fold cross-resistance against gemcitabine. The resistance is probably the corollary of the substantial decrease in deoxy-

Table 1. *Effect of drugs on* 1-β-D-*arabinofuranosylcytosine (ara-C)-induced cytotoxicity in three leukaemia cell lines (percentage decrease from number of untreated control cells)*

	Cells		
	HL-60	HL-60/ara-C	U937
HU	14 ± 7	24 ± 5	—†
Ara-C low conc.‡	7 ± 10	12 ± 7	21 ± 2
Ara-C high conc.§	58 ± 8	24 ± 4	69 ± 1
HU + Ara-C low conc.	39 ± 13*	36 ± 7	38 ± 15*
HU + Ara-C high conc.	77 ± 9	65 ± 9**	72 ± 2
F-Ara-A	9 ± 8	17 ± 7	—
Ara-C low conc.	1 ± 2	10 ± 9	21 ± 2
Ara-C high conc.	56 ± 2	21 ± 1	69 ± 1
F-Ara-A + Ara-C low conc.	24 ± 20	23 ± 17	31 ± 2*
F-Ara-A + Ara-C high conc.	58 ± 7	56 ± 16*	73 ± 2
2-CdA	15 ± 4	23 ± 9	—
Ara-C low conc.	12 ± 4	10 ± 9	21 ± 2
Ara-C high conc.	51 ± 6	21 ± 9	69 ± 1
2-CdA + Ara-C low conc.	49 ± 7**	14 ± 7	44 ± 4**
2-CdA + Ara-C high conc.	61 ± 4	43 ± 3	78 ± 2
ATRA	—	—	—
Ara-C low conc.	6 ± 6	4 ± 3	21 ± 2
Ara-C high conc.	52 ± 3	21 ± 5	69 ± 1
ATRA + Ara-C low conc.	12 ± 5	16 ± 5*	17 ± 6
ATRA + Ara-C high conc.	61 ± 4**	44 ± 5*	76 ± 1**
Staurosporine	—	12 ± 15	—
Ara-C low conc.	9 ± 10	11 ± 7	27 ± 4
Ara-C high conc.	51 ± 8	23 ± 11	73 ± 2
Staurosporine + Ara-C low conc.	39 ± 5*	30 ± 13	69 ± 4*
Staurosporine + Ara-C high conc.	81 ± 4*	58 ± 9	94 ± 1
Tyrphostin A48	—	—	—
Ara-C low conc.	9 ± 4	6 ± 3	21 ± 2
Ara-C high conc.	56 ± 5	19 ± 4	69 ± 1
Tyrphostin + Ara-C low conc.	18 ± 8*	11 ± 3	7 ± 9
Tyrphostin + Ara-C high conc.	64 ± 2	31 ± 8*	71 ± 2
CGP 52411	—	13 ± 3	—
Ara-C low conc.	14 ± 4	13 ± 2	21 ± 2
Ara-C high conc.	62 ± 3	25 ± 3	69 ± 1
CGP + Ara-C low conc.	31 ± 5**	35 ± 14	15 ± 5
CGP + Ara-C high conc.	70 ± 4	46 ± 3*	66 ± 2
NDGA	—	29 ± 6	—
Ara-C low conc.	15 ± 4	13 ± 3	14 ± 8
Ara-C high conc.	53 ± 17	24 ± 4	68 ± 8
NDGA + Ara-C low conc.	28 ± 5*	47 ± 6	26 ± 5*
NDGA + Ara-C high conc.	60 ± 12	49 ± 10	69 ± 4

Cytotoxicity was measured using the 3-(4,5 dimethylthiazol-2-yl-)5 diphenyltetrazolium bromide (MTT) assay and is expressed as percentage reduction in cell number compared with untreated controls. Details are described under Materials and Methods. Values are the mean ± standard deviation of three to five experiments, each conducted in octuplets. Drugs were used in HL-60, HL-60/ara-C and U937 cells, respectively, at the following concentrations: HU 100 μmol/l in all three lines; F-Ara-A 1, 100 and 1 μmol/l; 2-CdA 0.05, 100 and 0.05 μmol/l; ATRA 1 μmol/l; staurosporine 0.01 μmol/l; tyrphostin A48 50 μmol/l; CGP 52411 10 μmol/l; NDGA 10 μmol/l in all three lines. HU, hydroxyurea; F-ara-A, arabinosyl-2-fluoroadenine; 2-CdA, 2-chlorodeoxyadenosine; ATRA, all-*trans* retinoic acid; NDGA, nordihydroguaiaretic acid.

†No reduction in cell number. ‡Ara-C 'low conc.' was 0.1 μmol/l in HL-60, 100 μmol/l in HL-60/ara-C and 0.5 μmol/l in U937 cells. §Ara-C 'high conc.' was 10 μmol/l in HL-60, 2500 μmol/l in HL-60/ara-C and 5 μmol/l in U937 cells.

Asterisks indicate that values are significantly different from the sum of the cytotoxicities produced by the two drugs alone, e.g. in HL-60 cells HU + Ara-C low conc. value (39 ± 13) was compared with HU alone (14 ± 7) + Ara-C low conc. (7 ± 10). *$P<0.05$, **$P<0.01$.

cytidine kinase activity which was observed in these cells. Enzyme activity was 2.4% of that seen in wild-type HL-60 cells when measured at low, and 31% when determined at high deoxycytidine concentrations. This discrepancy suggests that, in terms of kinetic properties, the deoxycytidine kinase in HL-60/ara-C differs from that in wild-type cells.

In the combination treatments, ara-C was used at two concentrations, which, when employed alone, caused 0–27% and 18–73% cytotoxicity, respectively. Modulators were employed at concentrations which on their own displayed little or no toxicity. The most toxic modulator used was NDGA at 10 μmol/l, which killed 29% of HL-60/ara-C cells under the conditions of the assay. The results are shown in Table 1. HU was the most potent modulator of the ribonucleotide reductase inhibitors, sensitising all three cell lines against ara-C. 2-CdA and F-Ara-A were efficacious in U937 cells, the former also at low ara-C concentrations in HL-60, and the latter also at high ara-C concentrations in HL-60/ara-C cells. Of the antisignalling drugs, ATRA, staurosporine, CGP 52411, tyrphostin A48 and NDGA augmented ara-C toxicity (Table 1), but quercetin and genistein did not (results not shown). ATRA was exquisitely active in all three cell lines. Ara-C-sensitising potencies of the kinase inhibitors differed between cell types. CGP 52411, tyrphostin A48, staurosporine and NDGA were effective in HL-60 cells. CGP 52411 and tyrphostin A48 were also effective in HL-60/ara-C cells, whilst staurosporine and NDGA were also effective in U937 cells. In contrast, CGP 52411 and tyrphostin A48 failed to increase ara-C cytotoxicity in U937 cells.

Effects on ara-C-induced apoptosis

Incubation of HL-60 cells with ara-C induced apoptosis as assessed by the occurrence of a sub-G1 peak in the flow cytogram of the cells, DNA fragmentation and cell shrinkage. We wanted to test the hypothesis that the sensitisation of HL-60 cells to ara-C described above for HU, ATRA and certain kinase inhibitors was the consequence of an increase in cellular susceptibility towards ara-C-induced apoptosis. Figure 1 shows the effect of the modulators on the abundance of ara-C-induced hypodiploid cells (sub-G1 peak); Table 2

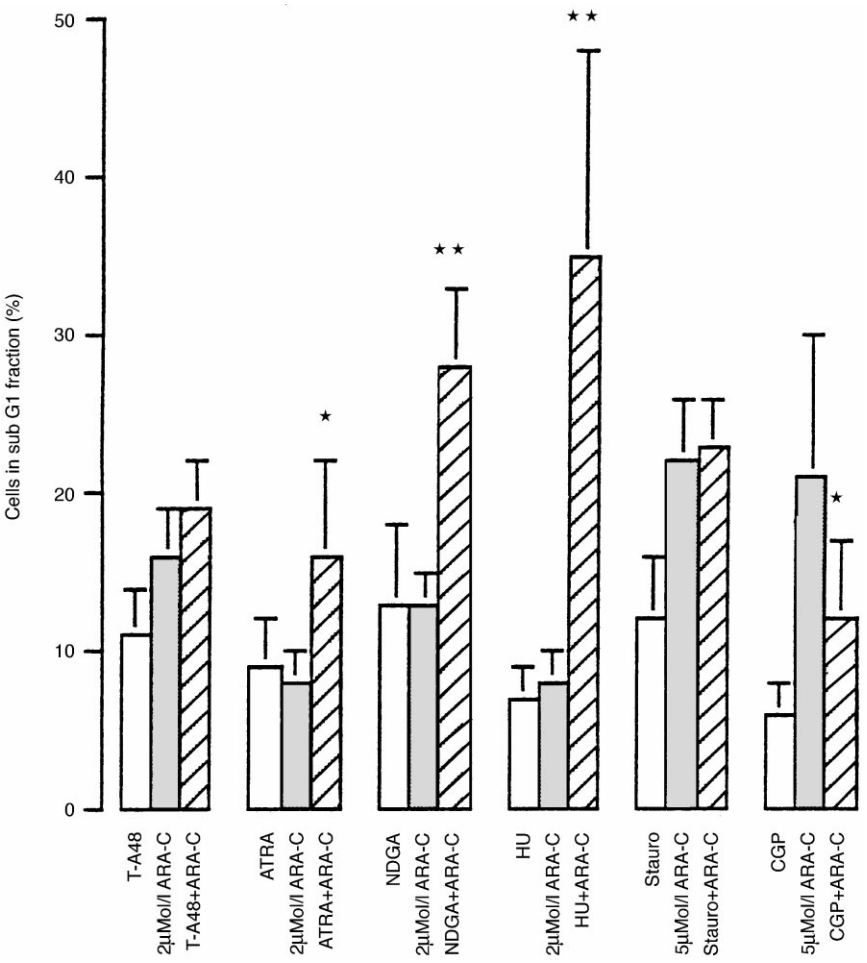

Figure 1. Effect of 1-β-D-arabinofuranosylcytosine (ara-C) alone, tyrphostin A48 (T-A48, 50 μmol/l), all-*trans* retinoic acid (ATRA, 50 μmol/l), nordihydroguaiaretic acid (NDGA, 10 μmol/l), hydroxyurea (HU, 100 μmol/l), staurosporine (Stauro, 0.05 μmol/l) and CGP 52411 (10 μmol/l) alone or in combination with ara-C on percentage of cells in sub-G1 (hypodiploid cells) as detected by flow cytometry. Cell cycle distribution in untreated controls was as follows: sub-G1 8 ± 3%, G1 49 ± 7%, S 22 ± 3%, G2 21 ± 6%. The increase in sub-G1 peak observed with combinations of ara-C with ATRA, NDGA or HU were compensated for by corresponding reductions in cell abundance in S and G2 (ATRA: S 17 ± 3%, G2 14 ± 5%, NDGA: S 12 ± 4%, G2 11 ± 4%; HU: S 6 ± 2%, G2 7 ± 3%). For details of the flow cytometric method see Materials and Methods. Values are the mean ± three separate observations, asterisks indicate that the value is significantly different from that for cells exposed to ara-C alone (*$P < 0.05$, **$P < 0.01$).

highlights alterations by the modulators of ara-C-induced decrease in cell volume, and Figure 2 demonstrates their influence on ara-C-induced DNA laddering. ATRA, NDGA and HU increased all three indicators of ara-C-induced apoptosis, and tyrphostin A48 exacerbated ara-C-elicited cell shrinkage (Table 2) and DNA fragmentation (Figure 2a). In contrast, CGP 52411 and staurosporine did not increase the effect of ara-C on cell cycle distribution, cell volume or DNA fragmentation. Thus, the results presented in Figures 1 and 2 and Table 2 suggest that ATRA, NDGA and HU augmented ara-C-induced apoptosis as assessed by three criteria and tyrphostin A48 by two, whilst staurosporine and CGP 52411 failed to enhance ara-C-induced apoptosis. CGP 52411 actually decreased ara-C-induced hypodiploid cells (Figure 1) and DNA laddering at the 4 h time point (Figure 2b); after a longer incubation time (12 h) CGP 52411 did not modulate ara-C-mediated DNA fragmentation (Figure 2c). Furthermore, the effect of CGP 52411 on ara-C-induced events was specific, as it did not alter apoptosis elicited by etoposide (result not shown).

DISCUSSION

The major objective of this study was to compare stringently a series of known and novel agents for their potency to sensitise leukaemia cells to ara-C cytotoxicity. Our results allow the following conclusions to be made as to the sensitivity of the chosen leukaemia cell fine panel towards the investigated drug combinations. Of the ribonucleotide reductase inhibitors, HU was a more efficacious modifier of ara-C cytotoxicity than 2-CdA or F-Ara-A. It sensitised not only U937 and HL-60 cells to ara-C, but also the ara-C-resistant HL-60 counterpart, which is consistent with a previous observation in ara-C-resistant HL-60 cells [38]. ATRA and the kinase-inhibitory antisignalling drugs CGP 52411, tyrphostin A48, NDGA and staurosporine were effective ara-C sensitisers exhibiting potencies comparable with those of the ribonucleotide reductase inhibitors.

The results described above for staurosporine and ATRA are in accordance with previous reports [9, 11, 12], but the lack of ara-C sensitisation by quercetin is inconsistent with results obtained by Teofili and colleagues [7]. Whilst these

*Table 2. Effect of hydroxyurea (HU), all-*trans *retinoic acid (ATRA), staurosporine, tyrphostin A48, CGP 52411 and nordihydroguaiaretic acid (NDGA) alone and in combination with 1-β-D-arabinofuranosylcytosine (ara-C) on HL-60 cell volume (percentage decrease compared with untreated cells)*

HU	8 ± 2
Ara-C (2 μM)	—†
HU + Ara-C	30 ± 7**
ATRA	9 ± 2
Ara-C (2 μmol/l)	—
ATRA + Ara-C	12 ± 2*
Staurosporine	—
Ara-C (5 μmol/l)	12 ± 3
Staurosporine + Ara-C	7 ± 8
Tyrphostin A48	9 ± 3
Ara-C (2 μmol/l)	5 ± 3
Tyrphostin + Ara-C	12 ± 6*
CGP 52411	6 ± 6
Ara-C (5 μmol/l)	14 ± 8
CGP + Ara-C	13 ± 9
NDGA	15 ± 3
Ara-C (2 μmol/l)	—
NDGA + Ara-C	24 ± 5**

Decrease in cell volume was measured as described under Materials and Methods. Values are the mean ± standard deviations of three to four experiments, the volume of untreated cells was 1068 ± 94 fl. Drug concentrations used were: HU 100 μmol/l; ATRA 50 μmol/l; staurosporine 0.05 μmol/l; tyrphostin A48 50 μmol/l; CGP 52411 10 μmol/l; NDGA 10 μmol/l.

†No change in cell volume.

Asterisks indicate that values are significantly different from the volume of cells exposed to ara-C alone, $*P < 0.05$, $**P < 0.01$.

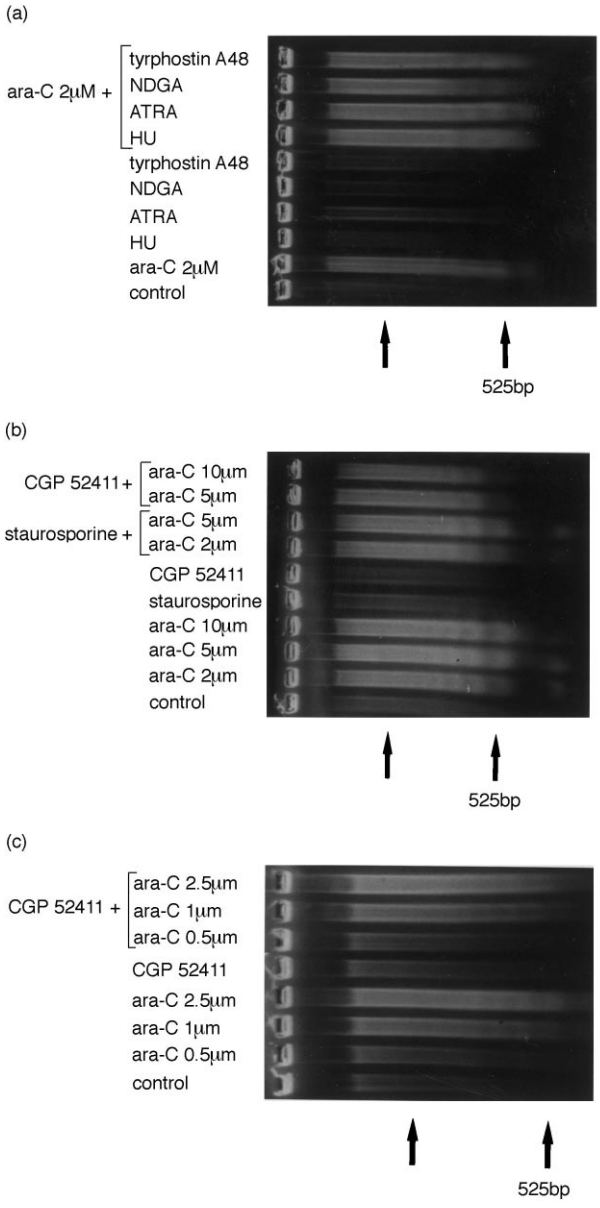

Figure 2. Effect of 1-β-D-arabinofuranosylcytosine (ara-C) alone and in combination with tyrphostin A48 (50 μmol/l), nordihydroguaiaretic acid (NDGA, 10 μmol/l), all-*trans* retinoic acid (ATRA, 50 μmol/l), hydroxyurea (HU, 100 μmol/l) (a), or with staurosporine (0.05 μmol/l) (b) and CGP 52411 (10 μmol/l) (b, c) on ara-C-induced DNA fragmentation in HL-60 cells. Incubation time was 4 h (a, b) or 12 h (c). DNA fragmentation was assessed as described under Materials and Methods, the gels shown are representative of two separate experiments.

authors found potentiation of ara-C cytotoxicity by quercetin, we did not detect any evidence of sensitisation. This discrepancy is difficult to rationalise, as the same cell type was used (HL-60) in both studies. It is conceivable that the effect of quercetin is so marginal that it is only detectable when the interaction between the drugs is evaluated by the isobole method [39], as carried out by Teofili and colleagues [7]. Whatever the reason for the disparity, in the experiments described above, CGP 52411, tyrphostin A48, NDGA and staurosporine were undoubtedly far superior to quercetin in this leukaemia cell panel. Inhibition of EGF receptor PTK by CGP 52411 and tyrphostin A48 [24, 25] could be considered as a potential mechanism of ara-C sensitisation. However, this notion is untenable because HL-60 cells lack EGF receptors [40]. The nature of the protein kinase(s), the inhibition of which renders cells more vulnerable to the cytotoxic attack of ara-C, and how such inhibition might impact on mechanisms of ara-C action, remain to be elucidated.

The results outlined above allow the tentative conclusion that sensitisation of HL-60 cells towards ara-C by tyrphostin A48, ATRA, NDGA and HU may be explained, at least in part, by the enhancement of ara-C-induced apoptosis. Intriguingly, CGP 52411 exerted a paradoxical effect on ara-C-induced apoptosis with inhibition after incubation for 4 h, although not after 12 h. Staurosporine did not enhance ara-C-induced apoptosis, which is surprising in view of two previous reports, according to which it augmented apoptosis in HL-60 cells [8, 41]. As the conditions described by these authors were somewhat dissimilar in terms of incubation time and drug concentration to those used in the experiments described here, it appears that induction of apoptosis by staurosporine is exceedingly sensitive to even subtle experimental differences. The mechanism which underlies the heightened proclivity of cells to succumb to ara-C-mediated cytotoxicity and apoptosis does not appear to be inevitably linked to augmentation of cellular ara-CTP levels as shown for staurosporine [8]. Ultimately it will be desirable to find out whether or not antisignalling agents other than staurosporine affect ara-C metabolism.

From the clinical viewpoint, the results presented above suggest that HU and ATRA might be considered for co-administration with ara-C in the treatment of acute promyelocytic leukaemia as well as perhaps other AML subtypes. For HU, this proposition is propitious in the light of its relatively low rate of toxic side-effects *vis-a-vis* those of 2-CdA and F-Ara-A [42]. In addition, the kinase inhibitors CGP 52411, tyrphostin A48, NDGA and staurosporine may possess clinical potential as sensitisers of ara-C cytotoxicity. As toxicity has been described for the latter two compounds [43, 44], CGP 52411 and tyrphostin A48 may be superior in terms of clinical applicability. Consistent with the results described above, staurosporine, and its less toxic *N*-benzoyl cogener CGP 41251, were recently found to enhance ara-C-induced apoptosis in a multidrug-resistant HL-60 subline [45].

Novel strategies to overcome resistance to ara-C are the subject of considerable research efforts, and recent proposals proffer the use of an anti-bcl-2 antisense oligonucleotide [46] and gene therapy with a human deoxycytidine kinase gene [47]. The results presented here suggest that new antisignalling drugs, which are small molecules, remain a fertile hunting ground in the search for novel ara-C combination partners, eventually leading to the enlargement of the therapeutic armamentarium to combat AML.

1. Kufe D, Spriggs D, Egan EM, Munroe D. Relationship among ara-CTP pools, formation of (ara-C) DNA, and cytotoxicity of human leukemic cells. *Blood* 1984, **64**, 54–58.
2. Plunkett W, Iacoboni S, Keating MJ. Cellular pharmacology and optimal therapeutic concentrations of 1-β-D-arabinofuranosylcytosine 5′-triphosphate in leukemic blasts during treatment of refractory leukemia with high-dose 1-β-D-arabinofuranosylcytosine. *Scand J Haematol* 1986, **34**(Suppl. 44), 51–59.
3. Boos J, Hohenlöchter B, Schulze-Westhoff P, *et al.* Intracellular retention of cytosine arabinoside triphosphate in blast cells from children with acute myelogenous and lymphoblastic leukemia. *Med Pediatr Oncol* 1996, **26**, 397–404.
4. Zühlsdorf M, Vormoor J, Boos J. Cytosine arabinoside resistance in childhood. *Leukemia* (in press).
5. Gandhi V, Estey E, Keating MJ, Plunkett W. Fludarabine potentiates metabolism of cytarabine in patients with acute myelogenous leukemia. *J Clin Oncol* 1993, **11**, 116–124.
6. Gandhi V, Estey E, Keating MJ, Chucrallah A, Plunkett W. Chlorodeoxyadenosine and arabinosylcytosine in patients with acute myelogenous leukemia: pharmacokinetic, pharmacodynamic, and molecular interactions. *Blood* 1996, **87**, 256–264.
7. Teofili L, Pierelli L, Iovino MS, *et al.* The combination of quercetin and cytosine arabinoside synergistically inhibits leukemic cell growth. *Leukemia Res* 1992, **16**, 497–503.
8. Grant S, Turner AJ, Bartimole TM, Nelms PA, Joe VC, Jarvis WD. Modulation of 1-[β-D-arabinofuranosyl] cytosine-induced apoptosis in human myeloid leukemia cells by staurosporine and other pharmacological inhibitors of protein kinase C. *Oncol Res* 1994, **6**, 87–99.
9. Grant S, Jarvis WD, Swerdlow PS, *et al.* Potentiation of the activity of 1-β-D-arabinofuranosylcytosine by the protein kinase C activator bryostatin 1 in HL-60 cells: association with enhanced fragmentation of mature DNA. *Cancer Res* 1992, **52**, 6270–6278.
10. Jarvis WD, Povirk LF, Turner AJ, *et al.* Effects of bryostatin 1 and other pharmacological activators of protein kinase C on 1-[β-D-arabinofuranosyl] cytosine-induced apoptosis in HL-60 human promyelocytic leukemia cells. *Biochem Pharmacol* 1994, **47**, 839–852.
11. Lishner M, Curtis JE, Minkin S, McCulloch EA. Interaction between retinoic acid and cytosine arabinoside affecting the blast cells of acute myeloblastic leukemia. *Leukemia* 1989, **3**, 784–788.
12. Yang GS, Minden MD, McCulloch EA. Influence of schedule on regulated sensitivity of AML blasts to cytosine arabinoside. *Leukemia* 1993, **7**, 1012–1019.
13. Kharbanda S, Emoto Y, Kisaki H, Saleem A, Kufe D. 1-β-D-arabinofuranosylcytosine activates serine/threonine protein kinases and c-*jun* gene expression in phorbol ester-resistant myeloid leukemia cells. *Mol Pharmacol* 1994, **46**, 67–72.
14. Emoto Y, Kisaki H, Manome Y, Kharbanda S, Kufe D. Activation of protein kinase C-δ in human myeloid leukemia cells treated with 1-β-D-arabinofuranosylcytosine. *Blood* 1996, **87**, 1990–1996.
15. Yuan ZM, Kharbanda S, Kufe D. 1-β-D-arabinofuranosylcytosine activates tyrosine phosphorylation of $p34^{cdc2}$ and its association with the Src-like $p56/p53^{lyn}$ kinase in human myeloid leukemia cells. *Biochemistry* 1995, **34**, 1058–1063.
16. Dou QP, An B, Yu C. Failure to dephosphorylate retinoblastoma protein in drug-resistant cells. *Biochem Biophys Res Commun* 1995, **214**, 771–780.
17. Dou QP, Lui WYL. Activation of cyclin E-dependent kinase by DNA damage signals during apoptosis. *Cancer Res* 1995, **55**, 5222–5225.
18. Brach MA, Kharbanda SM, Herrmann F, Kufe DW. Activation of the transcription factor NFκB in human KG-1 myeloid leukemia cells treated with 1-β-D-arabinofuranosylcytosine. *Mol Pharmacol* 1992, **41**, 60–63.
19. Brach MA, Herrmann F, Kufe DW. Activation of the AP-1 transcription factor by arabinofuranosylcytosine in myeloid leukemia cells. *Blood* 1992, **79**, 728–734.
20. Henschler R, Brennscheidt U, Mertelsmann R, Herrmann F. Induction of c-*jun* expression in the myeloid leukemia cell line KG-1 by 1-β-D-arabinofuranosylcytosine. *Mol Pharmacol* 1991, **39**, 171–176.

21. Gunji H, Kharbanda S, Kufe D. Induction of internucleosomal DNA fragmentation in human myeloid leukemia cells by 1-β-D-arabinofuranosylcytosine. *Cancer Res* 1991, **51**, 741–743.
22. Akiyama T, Ishida J, Nakagawa S, *et al.* Genistein, a specific inhibitor of tyrosine-specific protein kinase. *J Biol Chem* 1987, **262**, 5592–5595.
23. Meggio F, Donella Deana A, Ruzzene M, *et al.* Different susceptibility of protein kinases to staurosporine inhibition. Kinetic studies and molecular bases for the resistance of protein kinase CK2. *Eur J Biochem* 1995, **234**, 317–322.
24. Traxler P, Lydon N. Recent advances in protein tyrosine kinase inhibitors. *Drugs of the Future* 1995, **20**, 1261–1274.
25. Gazit A, Yaish P, Gilon C, Levitzki A. Tyrphostins: synthesis and biological activity of protein tyrosine kinase inhibitors. *J Med Chem* 1989, **32**, 2344–2352.
26. Domin J, Higgins T, Rozengurt E. Preferential inhibition of platelet-derived growth-factor (PDGF)-stimulated DNA synthesis and protein tyrosine phosphorylation by nordihydroguaiaretic acid. *J Biol Chem* 1994, **269**, 8260–8267.
27. Bhalla K, Nayak R, Grant S. Isolation and characterization of a deoxycytidinekinase-deficient human promyelocytic leukemic cell line highly resistant to 1-β-D-arabinofuranosylcytosine. *Cancer Res* 1984, **44**, 5029–5037.
28. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Meth* 1983, **65**, 55–63.
29. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987, **47**, 936–942.
30. Habtemariam S. Catechols and quercetin reduce MTT through iron ions: a possible artefact in cell viability assays. *Phytother Res* 1995, **9**, 603–605.
31. Pagliacci MC, Spinozzi F, Migliorati G, *et al.* Genistein inhibits tumour cell growth *in vitro* but enhances mitochondrial reduction of tetrazolium salts: a further pitfall in the use of the MTT assay for evaluating cell growth and survival. *Eur J Cancer* 1993, **29A**, 1573–1577.
32. Gorczyka W, Gong J, Ardelt B, Traganos F, Darzynkievicz Z. The cell cycle related changes in susceptibility of HL60 cells to apoptosis induced by antitumour agents. *Cancer Res* 1992, **52**, 6270–6278.
33. Gandhi V, Plunkett W. Modulation of arabinosylnucleoside metabolism by arabinosylnucleotides in human leukemia cells. *Cancer Res* 1988, **48**, 329–334.
34. Ruiz van Haperen VWT, Veeman G, Vermorken JB, Pinedo HM, Peters GJ. Regulation of phosphorylation of deoxycytidine and 2′,2′-difluorodeoxycytidine (gemcitabine); effects of cytidine 5′-triphosphate and uridine 5′-triphosphate in relation to chemosensitivity for 2′,2′-difluorodeoxycytidine. *Biochem Pharmacol* 1996, **15**, 911–918.
35. Arner ESJ, Spasukoukotskaja T, Eriksson S. Selective assays for thymidine kinase 1 and 2 and deoxycytidine kinase and their activities in extracts from human cells and tissues. *Biochem Biophys Res Commun* 1992, **188**, 712–718.
36. Sorenson CM, Bertrand R, Pommier Y. Apoptosis of events associated with cell cycle arrest at G2 phase and cell death induced by cisplatin. *J Natl Cancer Inst* 1990, **82**, 749–755.
37. Ormerod MG. *Flow Cytometry—A Practical Approach*. Oxford, Oxford University Press, 1990, 69–87.
38. Bhalla K, Swerdlow P, Grant S. Effects of thymidine and hydroxyurea on the metabolism and cytotoxicity of 1-β-D-arabinofuranosylcytosine in highly resistant human leukemia cells. *Blood* 1991, **78**, 2937–2044.
39. Beerenbaum MC. What is synergy? *Pharmacol Rev* 1989, **41**, 93–141.
40. Chen LL, Gansbacher B, Gilboa E, *et al.* Retroviral gene transfer of epidermal growth factor receptor into HL60 cells results in a partial block of retinoic acid-induced granulocytic differentiation. *Cell Growth Diff* 1993, **4**, 769–776.
41. Bouffard DY, Momparler RL. Comparison of the induction of apoptosis in human leukemic cell lines by 2′,2′-difluorodeoxycytidine (gemcitabine) and cytosine arabinoside. *Leukemia Res* 1995, **19**, 849–856.
42. Donehower RC. An overview of the clinical experience with hydroxyurea. *Semin Oncol* 1992, **19**, 11–19.
43. Katz M, Saibil F. Herbal hepatitis: Subacute hepatic necrosis secondary to chaparral leaf. *J Clin Gastroenterol* 1990, **12**, 203–206.
44. Meyer T, Regenass U, Fabbro D, *et al.* A derivative of staurosporine (CGP 41251) shows selectivity for protein kinase C inhibition and *in vitro* anti-proliferative as well as *in vivo* anti-tumour activity. *Int J Cancer* 1989, **43**, 851–856.
45. Hunakova L, Sulikova M, Duraj J, Sedlak J, Chorvath J. Stimulation of 1-(beta-D-arabinofuranosyl)cytosine (ara-C)-induced apoptosis in multidrug resistant human promyelocytic leukemia cell lines with protein kinase inhibitors. *Neoplasma* 1996, **5**, 291–295.
46. Keith FJ, Bradbury DA, Zhu YM, Russell NH. Inhibition of bcl-2 with antisense oligonucleotides induces apoptosis and increases the sensitivity of AML blasts to ara-C. *Leukemia* 1995, **9**, 131–138.
47. Manome Y, Wen PY, Dong Y, *et al.* Viral vector transduction of the human deoxycytidine kinase cDNA sensitizes glioma cells to the cytotoxic effects of cytosine arabinoside *in vitro* and *in vivo*. *Nature Med* 1996, **2**, 567–573.

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