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ORIGINAL ARTICLE

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All-*trans*-retinoic acid increases cytotoxicity of 1-β-D-arabinofuranosylcytosine in NB4 cells

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Abstract *Purpose*: Clinically, the benefits of combining all-*trans*-retinoic acid (ATRA) with chemotherapy have been well documented in the treatment of acute promyelocytic leukemia (APL). Changes in nucleoside transporter expression and activity have been shown to occur in NB4 cells in vitro following treatment with ATRA. In this study we investigated whether ATRA treatment increases sensitivity to ara-C in NB4 cells. Specifically, we examined the role of ATRA-associated changes in nucleoside transporter expression and activity in eliciting ara-C cytotoxicity. *Methods*: Cellular uptake of [^{3}H]-ara-C and nucleoside transporter abundance were determined in untreated cells and cells treated with 1 μ*M* ATRA for 12–72 h using an inhibitor and oil stop procedure, and an equilibrium [^{3}H]-NBMPR binding assay, respectively. Cytotoxicity of ara-C and the apoptotic response prior to and following ATRA treatment were determined using the MTT viability assay and the TUNEL assay, respectively. *Results*: ATRA treatment increased ara-C cytotoxicity and potency, ara-C transport, and augmented ara-C-induced apoptosis. The combination effect was supraadditive under some conditions and sequence-dependent whereby the maximum effect was seen when the addition of ATRA preceded the addition of ara-C, and when ara-C administration closely followed ATRA administration. *Conclusions*: The ATRA-induced increase in cytotoxicity of ara-C was, in part, the result of an increase in the functional expression of nucleoside transporters, and a role for bcl-2 was also indicated. Our results would suggest that timing of ara-C therapy should be tied to maximal *es* transporter expression, which is likely to be 24 h after ATRA treatment begins. It remains to be seen whether the response in the clinic can be further enhanced in APL by taking advantage of ara-C transporter regulation by ATRA.

Keywords Nucleoside transport · ATRA · Ara-C · Acute promyelocytic leukemia · bcl-2

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Introduction

The majority of nucleoside analogs used in antineoplastic and antiviral therapies execute their biological activity intracellularly and must be metabolically activated making their transportability across plasma membranes critical to their pharmacological action [13, 60]. However, due to their hydrophilic nature, they do not readily permeate the lipid bilayer and require mediated transport. Multiple distinct transport proteins in mammalian cells mediate the uptake and release of nucleosides and nucleoside analogs [13, 16, 38, 60]. Nucleoside transport (NT) systems have been demonstrated to influence the accumulation and retention of several cytotoxic nucleosides in leukemia cells [1, 13, 41, 60, 77, 83], two characteristics of drug metabolism that are critical to the overall cytotoxic efficiency of an analog.

1-β-D-Arabinofuranosylcytosine (ara-C) is a prototypical nucleoside analog (for a recent review see reference 36) and one of the most effective nucleoside analogs used in the treatment of acute myelogenous leukemia (AML) [30]. Ara-C is a prodrug that must be converted to its lethal derivative ara-CTP to exert its toxic effects [31, 35]. Conversion to ara-CTP is the result of sequential phosphorylation steps that are catalyzed by deoxycytidine kinase and pyrimidine nucleotide kinases [69]. The pharmacological action of ara-CTP in part involves its incorporation into nascent DNA strands, thereby slowing and eventually terminating strand elongation [55]. Ultimately, ara-C and other cytotoxic agents kill neoplastic cells by triggering apoptosis [39, 50, 51].

Classification of NT processes is based on functional and pharmacological characteristics. (For detailed descriptions of the various NT processes, see the following reviews: references 4, 13, 38, 64, 68.) Briefly, two equilibrative, bidirectional facilitated diffusion processes, *es* and *ei*, are distinguished by their sensitivity to nitrobenzylthioinosine (NBMPR), a transport inhibitor, and exhibit broad permeant selectivity. The concentrative Na^+-dependent processes driven by the sodium electrochemical gradient (*cif*, *cit*, *cib*, *cs*, *csg*) are distinguishable on the basis of substrate specificity and inhibitor sensitivity. The characteristics of this complex family of membrane proteins are presently being delineated through isolation and functional expression of cDNAs encoding the various nucleoside transporter proteins (reviewed in references 15 and 17).

Nucleoside transporters can be pharmacologically manipulated to increase drug uptake, inhibit uptake of naturally occurring nucleosides, and to inhibit drug efflux. Administration of the NT blocker dipyridamole following ara-C leads to synergistic antileukemic effects in HL-60 cells and L5178Y cells, presumably by preventing the efflux of drug [19, 90]. The adenosine analogs, fludarabine (fluroarabinosyladenosine) and cladribine (2-chlorodeoxyadenosine) (FI and 2-CdA) are used successfully to treat chronic lymphocytic leukemia (CLL), and their mechanism of action in vitro includes increasing the level of expression of nucleoside transporters of CLL cells [67], presumably elevating drug entry [32]. Ara-C and FI are taken into leukemia cells by *es* transporters and the in vitro sensitivity of cells to these drugs has been correlated with the cell surface abundance of these transporters [60]. Tamoxifen inhibits NT via *es* routes and inhibits the binding of NBMPR to human MCF breast cancer cells [12, 34] blocking the salvage pathway so important for nucleoside metabolism and cell replication.

Treatment success with ara-C is confounded by development of drug resistance [23, 24, 36] involving any of the many metabolic steps intrinsic to the pathway of drug activation [91] including transport [36]. In the past, efforts to increase the therapeutic efficiency of ara-C have included strategies to increase the cellular accumulation and retention of ara-CTP, since a close correlation has been found between this and ara-C cytotoxicity [19, 56, 74, 76] and clinical response [7, 24, 44, 49, 69, 74, 75]. More recently sensitivity to ara-C has been shown to be increased by the use of anti-signaling drugs, and growth regulators, such as hematopoietic growth factors and retinoic acids [29, 59, 89].

In vitro all-*trans*-retinoic acid (ATRA) has been shown to potentiate the cytotoxicity of ara-C in HL-60 cells and AML blasts [2, 30, 42]. Clinically, the benefits of combining ATRA with chemotherapy (CT) have been well documented in the treatment of acute promyelocytic leukemia (APL). APL is a specific type of AML characterized by a t(15:17) translocation [22]. NB4 cells provide a valuable tool for the in vitro study of APL disease and contain the characteristic 15;17 translocation [58]. ATRA can differentiate abnormal promyelocytes into mature granulocytes in vitro (in primary cultures and in established cell lines, including NB4 cells [58]) and in vivo can induce complete remission (CR) in 80–90% of patients [9, 10, 18, 20, 43]. However, resistance to this therapy develops rapidly [27].

NB4 cells express multiple NT processes, simultaneously (namely, *es*, *ei*, *cif*, and *csg*) [28, 73] and transporter abundance and activity are regulated during ATRA-induced differentiation of these cells (Flanagan and Meckling, submitted for publication). Specifically, increases in total transport rates of guanosine, and uridine initially following ATRA treatment, were attributed to an increase in both *es* and *csg* activity, and occurred concomitantly with an increase in the number of [^{3}H]-NMBPR binding sites present in plasma membranes.

In this study we investigated the effect of ATRA on the cytotoxicity of ara-C in NB4 cells. We hypothesized that treatment with ATRA would induce a subsequent increase in ara-C transport and intracellular accumulation as a result of the ATRA-induced increase in [^{3}H]-NBMPR binding sites, thereby potentiating the cytotoxic effects of ara-C. Direct examination of the transport of ara-C in both untreated and ATRA-treated cells was performed and the degree of correlation between transporter numbers and ara-C cytotoxicity was assessed.

Materials and methods

Cell culture

NB4 cells were maintained in Iscove's modified Dulbecco's medium (IMDM) (Gibco/BRL, Burlington, Ontario, Canada) with 10% fetal bovine serum (ICN Biochemicals, Mississauga, Ontario, Canada) supplemented with penicillin and streptomycin (50 U/ml) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. An initial mycoplasma infection with *M. arginini* was treated with BM cyclin (Boehringer Mannheim) and cells were subsequently screened for mycoplasma on a routine basis. The cells were routinely passaged in tissue culture flasks (Fisher Scientific, Whitby, Ontario, Canada) from passage 5 to 40. Cell counts and cell volumes were determined using a Beckman Coulter Z2 particle count and size analyzer. Cell viability was determined by a trypan blue dye exclusion assay and was >85% in all experiments.

ATRA treatment

Cells were seeded at 2.0×10^5 cells/ml and treated with 1 μ*M* ATRA, solubilized in ethanol at 0.1% v/v, for 0 (control), 12, 24, 48 and 72 h. At the appropriate time point, a nitroblue tetrazolium salt reduction (NBT) assay was performed. A 500-μl aliquot of NBT solution (Sigma Diagnostics) containing 300 n*M* 12-tetradecanoylphorbol-13-acetate (TPA) was incubated with 5.0×10^5 cells for 30 min. After 30 min the number of NBT-positive cells (cells reducing nitroblue tetrazolium salt, stained dark blue) were counted and expressed as a percentage of total cells. ATRA-treated cells were harvested at the appropriate time points for use in transport studies and viability assays.

Transport studies

Control cells and ATRA-treated cells were harvested at various times (12, 24, 48 and 72 h) following treatment by centrifuging at 1000 rpm for 8 min in a Hermle Z 383 K centrifuge (Mandel

Scientific, Guelph, Ontario, Canada). The resulting pellets were washed twice in 25 ml Na^+ buffer (3 m*M* K_2HPO_4, 1.8 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 144 m*M* NaCl, 20 m*M* Tris; pH 7.4, osmolarity 300 ± 10 osm) or Na^+ replacement choline buffer (3 m*M* K_2HPO_4, 1.8 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 140 m*M* choline Cl, 20 m*M* Tris; pH 7.4, osmolarity 300 ± 10 osm) and centrifuged at 1000 rpm for 8 min. The final pellets were resuspended in the appropriate buffer to a final density of $7.0–8.0 \times 10^6$ cells/ml. The cells were used immediately in the transport assay or after a 15–20 min incubation with 1 μ*M* NBMPR, a NT inhibitor. Employing rapid assay technology, the uptake of [^{3}H]-ara-C was determined using an inhibitor and an oil stop procedure at 22°C [66], as previously explained in detail [28]. Uptake curves were generated over a 10-min time course (0, 5, 10, 15, 30, 60,120, 300, 600 s). Each time-point was performed in triplicate. The intracellular volume of NB4 cells was determined from the cell volume distributions recorded by a Beckman Coulter Z2 particle counter and size analyzer [11] and was consistently 1.2–1.4 μl for control cells and 0.9–1.2 μl for ATRA-treated cells. Initial rates were estimated from computer-generated (TableCurve, Jandel Scientific, Corte Madera, Calif.) best-fit equations applied to the Na^+, Na^+ NBMPR, choline, and choline NBMPR uptake curves followed by determination of transport processes, *ci*, *cs* ,*es* and *ei*, by subtraction of the curve-defined initial rates, as previously described in detail [28].

Quantification of cell viability by the MTT assay

To measure the effect of ara-C on cell survival by the MTT assay, cells from each treatment group (untreated/control, and 12-, 24-, 48- and 72-h ATRA-pretreated) were transferred into each well of a 96-well plate following the serial dilution of ara-C (dissolved in the IMDM medium with 10% bovine serum and 5% penicillin and streptomycin, 50 U/ml) to give a final volume of 200 μl/well, 10,000 cells/well, and 20 μ*M* as the highest obtainable concentration of ara-C . Simultaneously treated cells (ATRA and ara-C) were treated with 1 μ*M* ATRA at the time of plating. Plates were incubated for 24 h at 37°C, then centrifuged for 8 min at 1200 rpm at room temperature followed by aspiration of medium from each well. A 50-μl sample of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in PBS) in IMDM at 1:9 with 1% fetal bovine serum was added to each well and the plates were incubated for an additional 4 h at 37°C. To each well was added 100 μl 2-propanol, with added HCl at 0.03% v/v, to dissolve the blue insoluble MTT formazan produced by mitochondrial succinate dehydrogenase. Absorbance was measured at 570 nm in a microplate spectrophotometer and the percentage of viable cells was calculated as the ratio of the absorbance measured in control cells (those not treated with drug) to that measured in treated cells.

Immunoblot analysis of NB4 cells

Proteins from whole cell lysates were separated by SDS-PAGE on 10% acrylamide gels and transferred to a nitrocellulose membrane (Amersham) on a semidry transfer apparatus for 2 h at 200 mA. Whole cells were suspended in Laemmli buffer, sonicated for 45 s and boiled for 5 min before use [57]. For determination of bcl-2 protein expression, gels were loaded on the basis of cell number. In all cases the efficiency of protein transfer and equality of loading between samples was estimated by staining the membranes with Fast Green (0.1% Fast Green, 20% methanol, 5% acetic acid). Membranes were blocked with 5% milk powder in Tris-buffered saline (TBS) overnight at 4°C or for 1 h at room temperature, washed with TBS with 0.05% Tween-20 (TBS-T), and incubated with primary antibody for 1 h at room temperature. The bcl-2 primary antibody was a mouse monoclonal that recognizes the 26 kDa species and was used at a 1:500 dilution (BD Biosciences, Mississauga, Ontario). Membranes were then washed with TBS-T for 30 min. followed by incubation with secondary antibody conjugated to horseradish peroxidase (anti-mouse IgG at a dilution of 1:10,000; Bio-rad Laboratories, Mississauga, Canada) for 1 h at room temperature. Polypeptides were detected using an ECL kit (Amersham) and quantified by autoradiography and densitometry.

Cell cycle distribution and apoptosis analysis by flow cytometry

Flow cytometric discrimination of apoptotic from healthy cells and cell cycle analysis of the ATRA-treated and ara-C-treated cell cultures were carried out by total DNA staining with propidium iodide. A total of 5 ml cell suspension was harvested from each flask and centrifuged at 1000 rpm at 4°C for 8 min. Cell pellets were washed in cold PBS centrifuged at 1000 rpm for 8 min and fixed in 80% ethanol for 24–48 h at 20°C. On the day of analysis, 1.5×10^6 of the fixed cells from each treatment group (control, treated for 24 h with 1 μ*M* ara-C, treated for 12, 24, 48 or 72 h with ATRA) were centrifuged and washed in 1 ml PBS plus 0.1% Triton-X-100 plus 0.1% bovine serum albumin. Following centrifugation at 1000 rpm for 8 min at 4°C, pellets were resuspended in 100 μl PBS and 400 μl PBS containing 12 μg/ml propidium iodide and 240 μg/ml RNAse giving a final concentration of 10 μg/ml propidium iodide and 200 μg/ml RNAse. Cells were left to incubate for 30 min at room temperature then transferred to 12×75 mm Falcon tubes for determination of total DNA by red fluorescence and flow cytometry. Evidence of apoptosis and the percentage of cells in each phase of the cell cycle were determined using the Windows MultiCycle software (Phoenix Flow Systems, San Diego, Calif.).

DNA fragmentation detection and quantification

A terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was used to detect and quantify DNA fragmentation (Roche Molecular Biochemicals). The assay kit identifies the DNA strand breaks by labeling the free 3′-OH termini with fluorescein-labeled nucleotides. Analyses were performed on harvested cells following the treatments described in Fig. 1. Cells were prepared according to the kit instructions. A negative control was also prepared during each assay to correct for background fluorescence. Cells were then analyzed by flow cytometry.

NBMPR binding assay

NBMPR binds tightly to the *es* nucleoside transporter [14] and has been used in its radioactive form as a probe for enumeration of the *es* transporter in many cell types [40, 46, 47, 65, 79]. For both control and ATRA-treated cell experiments, cells were treated and harvested as described for determination of transport rates. Plasma membrane fractions were obtained from harvested NB4 cells by sucrose gradient centrifugation [3, 48] as described in detail by us (Flanagan and Meckling, submitted for publication) and others [61]. Plasma membrane preparations (about 10 μg protein/ml final concentration) were subjected to [^{3}H]-NBMPR equilibrium binding analysis to quantify the number of binding sites and the affinity of these sites for NBMPR as previously described in detail by us (Flanagan and Meckling, submitted for publication) and others [61]. Specific binding was defined as the difference between the binding of [^{3}H]-NBMPR to cells in the presence and absence of excess non-isotopic NBMPR. Binding characteristics, K_d and B_{max}, were estimated by application of a two-site binding model equation (GraphPad Prism) to the bound-versus-free curve.

Determination of intracellular [^{3}H]-ara-C incorporation

Cells were incubated for 24 h in the presence of 1 μ*M* [^{3}H]-ara-C in the absence of ATRA (ara-C alone or 0 h ATRA), following 12, 24, 48 or 72 h pretreatment with 1 μ*M* ATRA, or treated

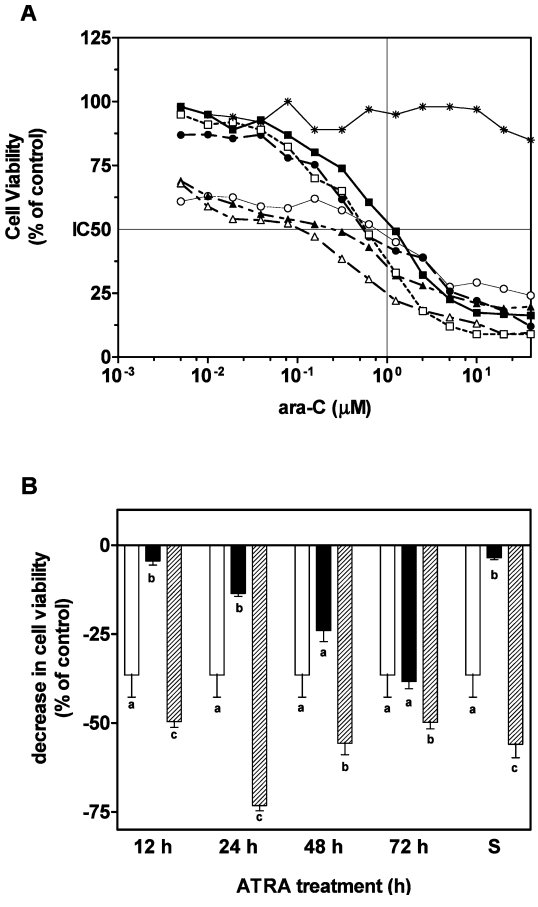

Fig. 1A, B ATRA increases ara-C cytotoxicity. **A** The effect of ara-C on NB4 cell survival rate was measured using the MTT viability assay to assess mitochondrial succinate dehydrogenase activity. Cells were incubated for 24 h in the presence of various concentrations of ara-C in the absence of ATRA (■ ara-C alone, * ara-C in the presence of 1 μ*M* NBMPR), or following a 12-h (●), 24-h (△), 48-h (▲) or 72-h (○) pretreatment with 1 μ*M* ATRA or treated simultaneously with various ara-C concentrations and 1 μ*M* ATRA (□). The percentages of viable cells were calculated from the ratios of the absorbance measured in control cells (those not treated with ATRA or ara-C) to that measured in treated cells. **B** The relative contribution of ATRA and ara-C to the decrease in cell viability. Cells were preincubated with 1 μ*M* ATRA for 12, 24, 48 or 72 h and then treated with ara-C for 24 h or were coincubated for 24 h with both agents (*S*). Cell viabilities in the presence of ara-C (*clear bars*), ATRA (*black bars*), and both ara-C and ATRA (*hatched bars*) are expressed as the percentage decrease compared to the viability of control cells. Statistical significance was determined using the Student-Newman-Keuls Multiple Comparisons test ($P < 0.5$) for each discrete data set (12, 24, 48 and 72 h, and *S*). Bars not sharing a letter are significantly different

simultaneously with 1 μ*M* [^{3}H]-ara-C and 1 μ*M* ATRA, or left untreated (control cells). Following the various treatments, cells were harvested by centrifugation at 1000 rpm for 8 min and washed twice in cold PBS. Each treatment was performed in triplicate and pellets were pooled for determination of intracellular incorporation of transported [^{3}H]-ara-C. Combined pellets were extracted with equal volumes of H_20 and 1 *M* perchloracetic acid as previously described by us (Flanagan and Meckling, submitted for publication). Nucleoside metabolites were detected by high-pressure liquid chromatography (HPLC), using a C18 reverse-phase column and two mobile phases with a flow rate of 0.6 ml/min at room temperature. Mobile phase I comprised 60 m*M* KH_2PO_4 and 5 m*M* tetrabutylammonium phosphate (PIC-A reagent, Waters), pH 6.0, and mobile phase II comprised 30% methanol plus 70% mobile phase I. The gradient eluent was as follows: 45-min linear gradient from 100% mobile phase I to 100% mobile phase II, 1 min mobile phase II, a 3-min linear gradient to return to 100% mobile phase I, and 2 min mobile phase I. Eluents were collected every 20 s during the sample run from 25 to 35 min (ara-CTP came off the column at approximately 30 min). Each collection was added to a new scintillation vial to which scintillation cocktail (ScintiVerse, Fisher Scientific, Whitby, Ontario, Canada) was added. The radioactivity of the samples was determined using a Beckman (Fullerton, Calif.) liquid scintillation spectrophotometer. Peaks were identified by comparison of retention times to those of standards.

Results

Treatment of NB4 cells with 1 μ*M* ATRA increased ara-C cytotoxicity

ATRA potentiated the decrease in cell viability following treatment with ara-C

Tabulation of cell viability following the various treatment regimens suggested that ara-C concentration, ATRA exposure time, and dosing schedule (simultaneous vs consecutive treatment) each influenced cell viability when the agents were used in combination (Table 1). At 0.1 μ*M* and 1 μ*M* ara-C, the addition of ATRA increased ara-C cytotoxicity when added consecutively or simultaneously at all exposure times compared to ara-C alone. Cultures pretreated with ATRA showed a progressive decrease in cell viability as exposure time increased from 0 h to 24 h with the maximal decrease observed in cultures pretreated for 24 h. Cell viability began to recover in cultures pretreated for

Table 1 Effect of ATRA on ara-C-induced cytotoxicity. Cytotoxicity was measured using the MTT viability assay to assess mitochondrial succinate dehydrogenase activity and is expressed as a percentage of control (drug and ATRA-free) cells. IC_{50} values were estimated from computer generated (TableCurve, Jandel Scientific, Corte Madera, Calif.) best-fit equations for the percentage viability (as a percentage of control) vs concentration of ara-C (see Fig. 1A). Values are the means ± SEM of at least three separate experiments performed in quadruplicate. Statistical significance was determined using the Student Neuman Keul's Multiple Comparisons test ($P < 0.5$) for the cytotoxicity values and IC_{50} values

Treatment	0.1 μ*M* ara-C	1.0 μ*M* ara-C	IC_{50} for ara-C
Ara-C	89.6 ± 2.0^a	64.0 ± 3.0^a	1.21 ± 0.15^a
12 h 1 μ*M* ATRA then ara-C	76.5 ± 1.5^b	51.0 ± 1.5^b	0.71 ± 0.08^b
24 h 1 μ*M* ATRA then ara-C	53.3 ± 0.5^d	26.0 ± 1.5^d	0.12 ± 0.01^d
48 h 1 μ*M* ATRA then ara-C	61.9 ± 1.8^c	42.0 ± 0.4^c	0.41 ± 0.10^c
72 h 1 μ*M* ATRA then ara-C	59.7 ± 1.4^c	47.5 ± 1.2^b	0.75 ± 0.20^b
1 μ*M* ATRA plus ara-C simultaneously	77.5 ± 1.5^b	41.6 ± 1.6^c	0.65 ± 0.04^b

Values in the same column not sharing a letter are significantly different

48 and 72 h compared to those pretreated for 24 h. Exposure to 1 μ*M* ATRA for 24 h followed by ara-C resulted in much higher drug toxicity than exposure to ara-C alone or ATRA pretreatment for shorter or longer periods. At 0.1 μ*M* ara-C, simultaneous treatment with both agents was modestly advantageous over ara-C alone but was significantly less toxic than sequential ATRA/ara-C treatment. At 1.0 μ*M* ara-C, simultaneous treatment was superior to ara-C alone and to lengthy ATRA pretreatments, but was significantly less toxic than observed in 24-h pretreated cultures.

ATRA decreased the IC_{50} for ara-C-induced death of NB4 cells

Decreases in IC_{50} values for ara-C following the addition of ATRA were influenced by the time length of exposure to ATRA and the dose schedule of the two modalities. IC_{50} values were decreased by 41%, 90%, 65%, 38%, and 46% with 12, 24, 48 and 72 h pretreatment and simultaneous treatment, respectively, in comparison to the values in ATRA-free cultures (Fig. 1A, Table 1). The largest difference observed com pared to ara-C alone occurred in cultures pretreated with ATRA for 24 h in which almost a tenfold decrease in the IC_{50} value for ara-C toxicity was demonstrated.

Combination therapy with ATRA and ara-C has an additive or synergistic effect on cell viability

Ara-C alone at 1 μ*M* produced a decrease in cell viability of approximately 35% while the ATRA-induced decrease in cell viability was approximately 5%, 12%, 22%, 35% and 5% in cultures that were pretreated for 12, 24, 48 or 72 h with 1 μ*M* ATRA, or treated simultaneously with both agents, respectively (Fig. 1B). The effect of the combination was additive at both 12 h and 48 h and synergistic at 24 h and with simultaneous treatment. Cultures treated with ATRA alone for more than 48 h showed decreased cell viability to the same extent as those treated with ara-C alone.

ATRA induces G_1 arrest and a small apoptotic cell population in NB4 cells

The cell cycle distribution of NB4 cells following exposure to ATRA showed no obvious changes early in the treatment time course. Following a 48-h exposure the number of cells arrested in the G_1 phase increased and the percentage of cells in S or G_2/M decreased (Table 2). This pattern was most distinct by 72 h of treatment. A qualitative representation of the cell cycle distribution is presented in Fig. 2A. This G_1 arrest reflected the decrease in cell growth that was observed in cultures following ATRA treatment (Fig. 2B). However, quantitative analysis of 10,000 cells in each treatment group showed that less than 3% of cells were undergoing apoptosis (Table 2) following up to 3 days of ATRA treatment, as indicated by the presence of a minor sub-G_1 peak (Fig. 2A).

Table 2 Distribution of NB4 cell cycle phases after treatment with ATRA or ara-C. The DNA content of cells following the indicated treatments was analyzed by flow cytometry after total DNA staining with propidium iodide. Evidence of apoptosis and the percentage of cells in each phase of the cell cycle were determined using Windows MultiCycle software (Phoenix Flow Systems, San Diego, Calif.)

Treatment	% G_1	% G_2/M	% S	% apoptosis
Control/untreated (0 h)	42	34	25	0.0
Preincubation with 1 μ*M* ATRA				
12 h	45	29	26	0.0
24 h	36	38	25	0.5
48 h	51	26	21	1.2
72 h	62	22	16	2.6
1 μ*M* Ara-C, 24 h	63	7.1	30	31.5

ATRA increases the apoptotic cell population following exposure to ara-C

Cultures treated with 1 μ*M* ara-C alone contained approximately 45% TUNEL-positive cells. An increase in positive cells was observed in cultures consecutively or simultaneously treated with both ATRA and ara-C. The time of exposure to ATRA influenced the number of positive cells present in the cultures with a maximum number reached at 24 h. In the presence of NBMPR, the percentage of positive cells was the same as that in control cultures (Fig. 3A). Figure 3B provides a qualitative picture of DNA fragmentation. Individual dot plots represent fluorescing cells detected within the treatment groups indicated. Ara-C-treated cells displayed a pattern characteristic of apoptosis, that is the appearance and accumulation of a population of smaller cells with increased DNA fragmentation or apoptotic cells as represented by the decreased forward scatter (FS), suggestive of cell shrinkage, and increased side scatter (SSC), suggestive of increased fluorescence. In cultures pretreated with ATRA followed by ara-C the number of cells making up this second population of cells increased progressively to 24 h. Beyond 24 h the second population was less distinct. In cultures treated with both ara-C and NBMPR, no substantive apoptotic population was apparent.

ATRA increases the number of plasma-associated [^{3}H]-NBMPR binding sites expressed in NB4 cells

NB4 cells exhibit two NBMPR binding sites, distinguished by their binding affinities, within all membrane fractions studied including plasma membranes (Flanagan and Meckling, submitted for publication) [73]. Since [^{3}H]-NBMPR binding is not *es*-specific but presumably measures all sensitive transporter-specific sites, we are unable

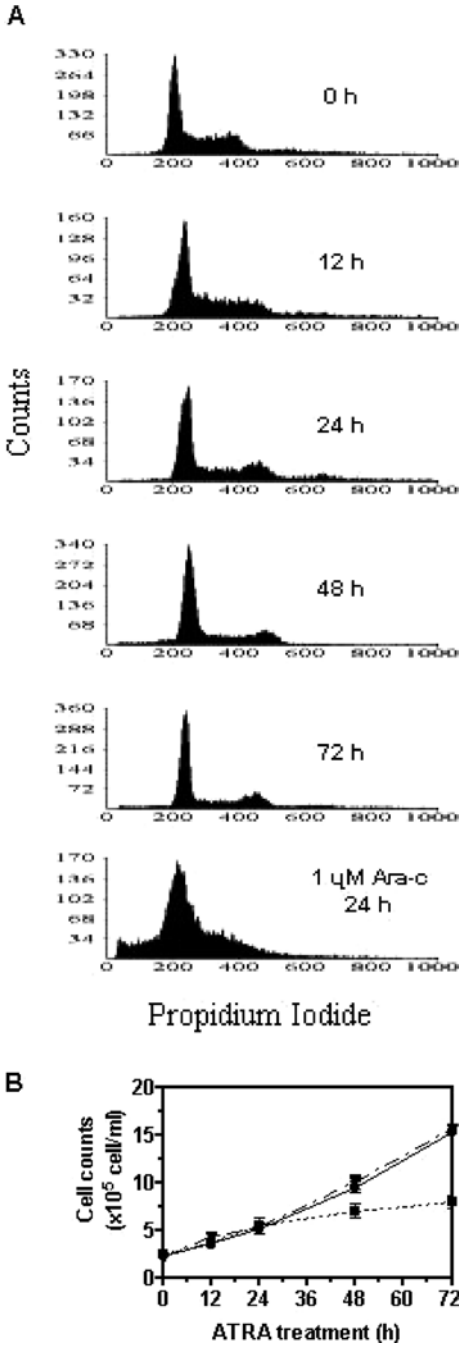

Fig. 2A, B ATRA induces a G_1 arrest in absence of apoptosis. **A** Aliquots of NB4 cells were collected at various times following ATRA treatment (control/0 h, 12, 24, 48 and 72 h, and following 24 h ara-C treatment). Apoptosis and cell cycle distribution were analyzed by flow cytometry after total DNA staining with pro pidium iodide. The histograms presented are representative of at least three separate experiments. **B** Cell numbers were determined in the presence (■) or absence (●) of 1 μ*M* ATRA and in ethanol (▲). Aliquots of cells were harvested at the indicated times and cell numbers were determined using a Coulter counter. The data points represent the averages of total cell numbers ± SEM of at least three separate experiments

to distinguish which NBMPR binding sites represented *es* transporters and which represented the *csg* transporters. Transport of ara-C in NB4 cells occurred via the *es* system only (Fig. 4) and in this study the combined total number of binding sites from each of the binding site populations was used to represent the total number of plasma-associated [^{3}H]-NBMPR binding sites available per cell. The number of [^{3}H]-NBMPR binding sites within both populations increases following treatment with ATRA (Flanagan and Meckling, submitted for publication). In the present study, the [^{3}H]-NBMPR binding characteristics of isolated plasma membranes from NB4 cells were again determined to confirm the previous results (data not shown). Figure 5A displays the increase in total number of [^{3}H]-NBMPR binding sites present in a cell as a function of ATRA exposure.

Correlation between ara-C sensitivity and [^{3}H]-NBMPR binding site content in NB4 cells

In Fig. 5A the number of [^{3}H]-NBMPR binding sites present in a cell is plotted as a function of ATRA or NBMPR exposure and is presented together with plots of coordinate changes in the viability of parallel cell populations that were exposed to 1 μ*M* ara-C under identical cell culture conditions with or without ATRA or NBMPR exposure. It is presumed that at 1 μ*M* NBMPR, virtually all sensitive transporter sites are functionally blocked. Cell viability decreased with an increase in cellular [^{3}H]-NBMPR binding sites. However, the coordinate relationship was lost at 48 h, at which time binding site numbers continued to increase while the curve describing cell viability began to move in the same direction as change in transporter number. Chemosensitivity experiments showed that 1 μ*M* NBMPR protected NB4 cells against ara-C cytotoxicity: the viability of cells exposed to both NBMPR and ara-C was 92% of the viability of drug-free controls (see IC_{50} curve of Fig. 1A and Table 1). When the data presented in Fig. 5A were plotted as the differential cell kill by ara-C (difference between cell viability in cultures receiving the various treatments and cell viability in NBMPR/ara-C-free cultures) vs the number of transporters (influenced by the presence of NBMPR and exposure to ATRA; Fig. 5B) a close correlation was evident (correlation coefficient 0.90).

Figure 5C shows a plot of IC_{50} values for ara-C cytotoxicity (Table 1) vs the B_{max} for [^{3}H]-NBMPR binding and reveals a positive correlation between sensitivity to ara-C and the expression of transporter sites ($r^2 = 0.75$).

Uptake of ara-C in NB4 cells occurs via equilibrative routes

Ara-C uptake in untreated NB4 cells occurred almost exclusively via *es* transporters (Fig. 4A i and ii), resembling the uptake of the related metabolite, cytidine [28]. The elimination of the Na^+ gradient by replacement with choline had little effect on ara-C uptake (Fig. 4A). At 5 μ*M*, ara-C uptake in control cells consistently

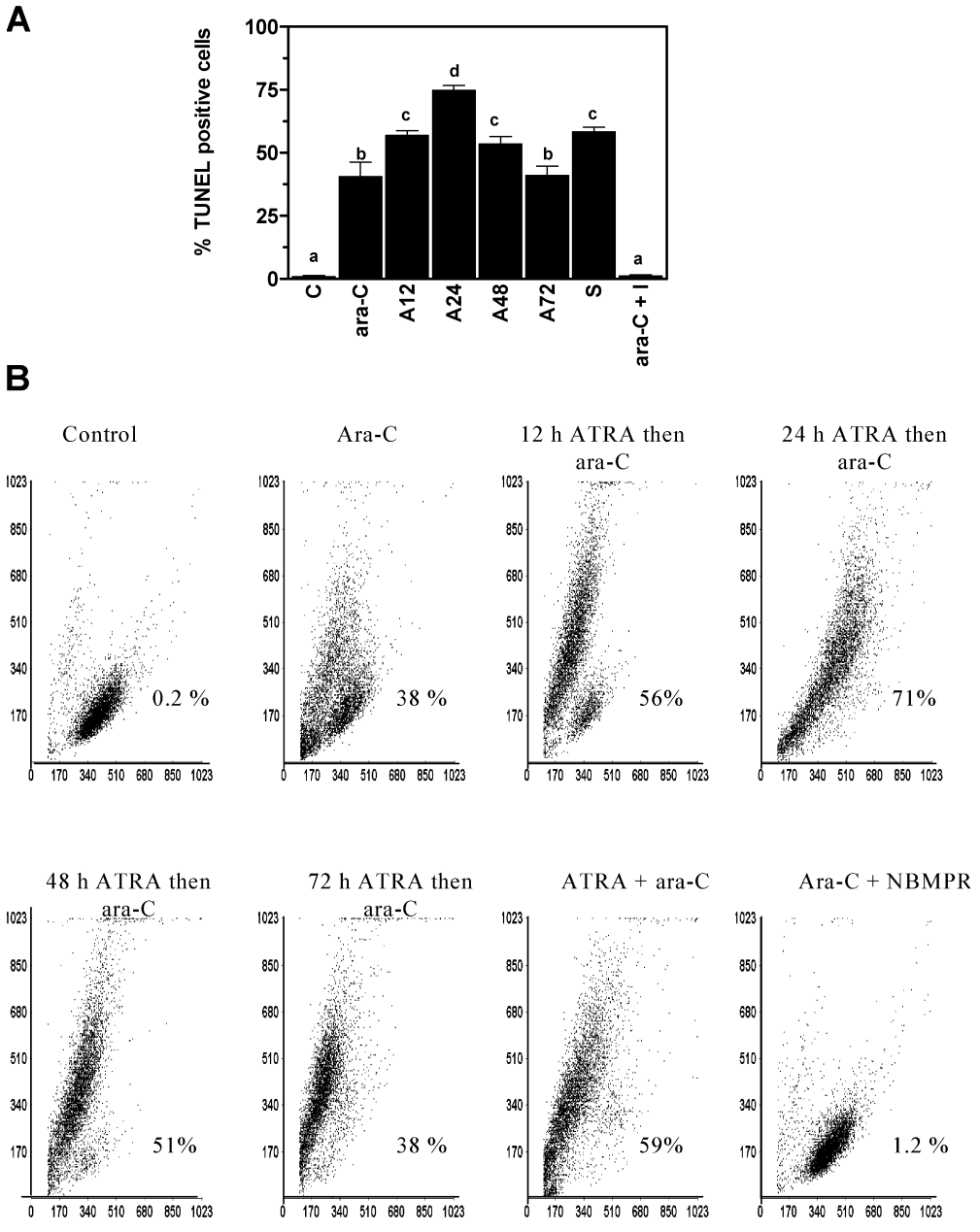

Fig. 3A, B Combining ATRA with ara-C increased apoptosis. **A** A graphical representation of the quantification of DNA fragmentation in cultures of (in sequence from left to right) untreated control cells (*C*), cells treated with 1 μ*M* ara-C (*ara-C*), cultures pretreated with 1 μ*M* ATRA for 12, 24, 48, or 72 h, followed by 1 μ*M* ara-C (*A12*, *A24*, *A48*, and *A72*), cultures coincubated with 1 μ*M* ara-C plus 1 μ*M* ATRA simultaneously (*S*), and cultures incubated with 1 μ*M* ara-C plus 1 μ*M* NBMPR (*ara-C + I*). All treatment cultures were incubated for 24 h following the addition of 1 μ*M* ara-C. Quantification of DNA fragmentation was performed using a TUNEL assay. Statistical significance was determined using the Student-Newman-Keuls Multiple Comparisons test ($P < 0.5$). Bars not sharing a letter are significantly different. The data shown are the means ± SEM of at least three separate experiments. **B** Qualitative picture of DNA fragmentation. The *x*-axis represents FS (forward scatter) and the *y*-axis SSC (side scatter) of fluorescing cells. The percentage of TUNEL-positive cells in each treated culture is shown at the lower right within each separate dot plot

reached equilibrium in all experiments within approximately 10 s (Fig. 4A ii) and was saturable by 10 min (Fig. 4A i). A small amount of ara-C uptake, approximately 5% of the total, occurred with choline plus NBMPR treatment. However, this process was unsaturable and likely represented diffusion.

Total initial transport rate of ara-C increases in NB4 cells following treatment with ATRA

The total initial transport rate of ara-C as measured in Na^+ buffer increased over the ATRA treatment time course (Fig. 4B). The increase in initial transport rate was observed as early as 12 h, had increased approximately threefold by 24 h, and continued to increase reaching a maximum of fourfold by 48 h of treatment. Resolution of the individual transport processes revealed that an increase in *es* activity was responsible for the increase in total ara-C transport (Fig. 4C).

Intracellular incorporation of [^{3}H]-ara-C increased following ATRA treatment

In the presence of ATRA, total intracellular incorporation of [^{3}H]-ara-C substantially increased as early as 12 h, and had reached a maximum concentration by 24 h (Fig. 6A). This pattern of increase was reflected by an increase in the intracellular level of the active metabolite [^{3}H]-ara-CTP (Fig. 6B). ATRA treatment beyond 24 h resulted in a decline from peak intracellular

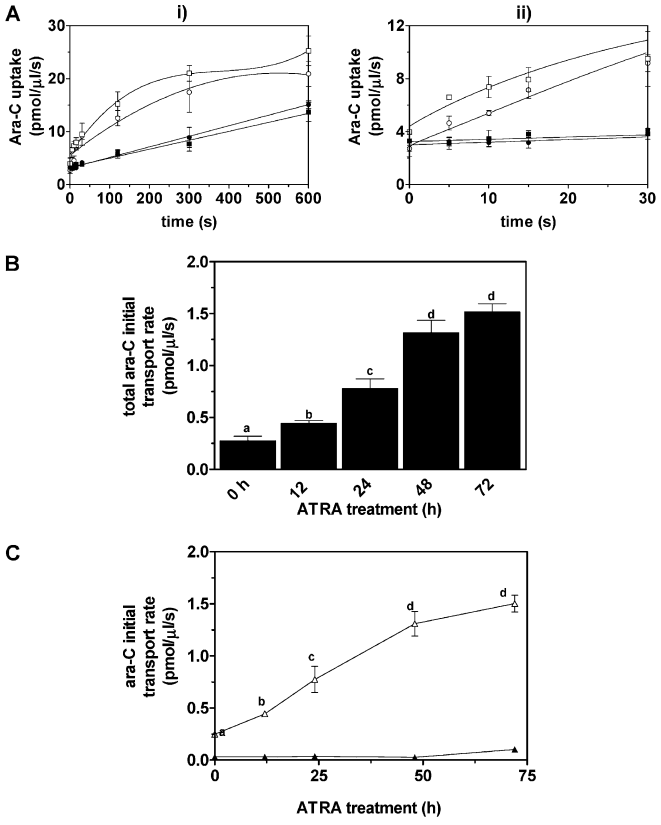

Fig. 4A–C Uptake of ara-C occurs via equilibrative processes in NB4 cells. Time course of (**A**) Ara-C uptake in NB4 cells for the periods 0–600 s (*i*) and 0–30 s (*ii*). Transport assays were carried out as described in detail previously [28]. Uptake was measured in the absence (● ○) or presence (■ □) of Na^+ with (● ■) or without (○□) 1 µ*M* NBMPR. The data shown are the means ± SEM of triplicate assays and are representative of at least three separate experiments. **B**, **C** Transport of ara-C following treatment with 1 µ*M* ATRA for various times (0, 12, 24, 48 and 72 h). Initial transport rates were estimated from computer-generated best-fit equations over the linear portion of the time-course curve in the presence of 5 µ*M* ara-C throughout the ATRA time course. **B** Changes in transport activity are presented as changes in total initial transport rates vs duration of ATRA treatment and as changes in initial transport rates of individual transporter processes (**C**). At each time point in the ATRA treatment (0, 12, 24, 48 and 72 h), the individual transport processes were resolved as described previously [28]. Each data point in **C** and each bar in **B** represents the average ± SEM of at least three separate experiments. The average initial transport rates of the individual transport processes at each time point in the ATRA treatment time course together comprise a discrete data set. **B**, **C** Statistical significance was determined using the Student-Newman-Keuls Multiple Comparisons test ($P < 0.5$) for each discrete data set. Those bars/data points not sharing a letter are significantly different

accumulation to levels similar to those measured in cultures treated with ara-C only (0-h cultures).

Expression of the oncogene bcl-2 is down-regulated in NB4 cells following treatment with ATRA

To determine the level of bcl-2 protein expressed in NB4 cells, whole cell lysates were prepared as described in Materials and methods, and Western blot analysis was performed. The level of bcl-2 protein expressed in NB4 cells following treatment with ATRA was determined (Fig. 7). Cells were treated with 1 µ*M* ATRA and harvested at 0 h (lane 1), 12 h (lane 2), 24 h (lane 3), 48 h (lane 4), and 72 h (lane 5). Immunodetectable bcl-2 protein levels decreased as the duration of ATRA treatment increased. The level of bcl-2 protein decreased within 24 h of ATRA treatment and continued to decrease for the remainder of the 72-h time course.

Discussion

In this study ara-C cytotoxicity to NB4 cells was augmented by the addition of ATRA. Specifically, the treatment combination resulted in a greater decrease in cell viability, increased drug potency, and augmentation of ara-C-induced apoptosis than when either modality was used singly, a phenomenon also seen in HL-60 cells [30]. The overall effect of the combination on cell viability was at least additive, with some treatments synergistic, depending on the nature of ATRA administration. Pretreatment with ATRA for 24 h followed by ara-C gave the best cell kill. The increase in ara-C toxicity observed in the presence of ATRA was not entirely explained by either a change in drug transport or intracellular incorporation of [^{3}H]-ara-CTP since transport continued to increase following 48 and 72 h ATRA treatment, but toxicity of ara-C began to decrease to levels below those observed with 24 h ATRA treatment. Additionally, while toxicity remained elevated with 48 and 72 h treatment compared to that following treatment with ara-C only, the intracellular accumulation of ara-CTP in these cultures was not different from that in control cultures.

Clinical results of the combination therapy of ATRA and CT clearly demonstrate superiority over CT alone in terms of relapse and survival in newly diagnosed APL (for a recent review see reference 27). Results have suggested that the combination not only increases CR rates but also has an additive or synergistic effect in reducing the incidence of relapse in APL [26]. Currently, the dose of CT administered with ATRA in clinical studies is being reconsidered [78], specifically as to whether it could be reduced during consolidation therapy while maintaining the high therapeutic index observed at standard doses [25]. Our study demonstrated in vitro that the degree of cell death reached at a low dose of ara-C (0.1 µ*M*) when used in the presence of ATRA is similar to that observed at a standard dose (1.0 µ*M*) of ara-C used alone. The greatest effect on cell viability however, was observed with the combination ara-C at 1.0 µ*M* and ATRA, suggesting that a standard dose approach is most efficacious in combined therapy regimens; ara-C concentrations found in the plasma with low to standard-dose treatment regimens are ≤ 1 µ*M* [60]. Additionally, as the concentration of ara-C increased, the decrease in cell viability reached a

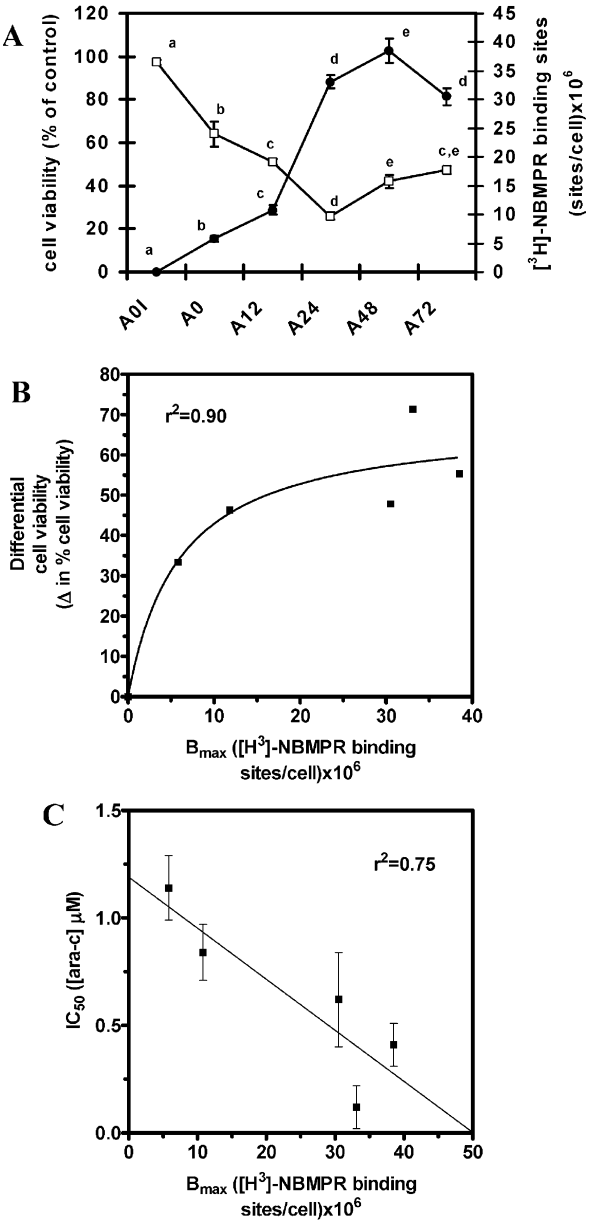

Fig. 5A–C Relationship between [^{3}H]-NBMPR binding sites and cell viability of ara-C-treated cells. **A** Cell viability and the number of plasma membrane-associated [^{3}H]-NBMPR binding sites were determined following various treatments: untreated/control in the absence of ATRA (*A0*), 1 μM NBMPR in the absence of ATRA (*A0I*), or 1 μ*M* ATRA for 12, 24, 48 and 72 h (*A12*, *A24*, *A48*, *A72*). Plasma membrane-associated [^{3}H]-NBMPR binding sites were enumerated by using an equilibrium [^{3}H]-NBMPR binding assay (●). Cell viability following incubation with 1 μ*M* ara-C, after each indicated treatment, is expressed as a percentage of control (NBMPR-, ara-C-, and ATRA-free cultures; □). Cell viability was determined using the MTT viability assay. Cell viability and [^{3}H]-NBMPR binding site values are means ± SEM of quadruplicate determinations of at least three separate experiments. Statistical significance was determined using the Student-Newman-Keuls Multiple Comparisons test ($P < 0.5$) for viability values and binding site values, each of which comprises a discrete data set. Values not sharing a letter within the same data set are significantly different. **B** Data shown in **A** was plotted as the differential cell kill by ara-C [the difference between cell survival in the presence of 1 μ*M* NBMPR plus 1 μ*M* ara-C (*A0I + 1* μM ara-C) and in the presence of 1 μ*M* ATRA (*A0*, *A12*, *A24*, *A48*, *A72*) plus 1 μ*M* ara-C] vs the number of plasma-associated [^{3}H]-NBMPR binding sites present following the indicated treatments and prior to treatment with ara-C. **C** Correlation between maximal [^{3}H]-NBMPR binding sites and sensitivity of cells to ara-C. The *x*-axis represents the number of plasma-associated [^{3}H]-NBMPR binding sites per cell enumerated following the indicated treatments. Chemosensitivity is shown as the IC_{50} for ara-C in a 24-h MTT assay (Table 1)

maximum of approximately 90% at about 10 μ*M* ara-C and an increase in the concentration of ara-C beyond this point had little effect whether administered singly or in combination with ATRA. Concentrations exceeding 10 μ*M* ara-C likely do not provide additional benefit because the phosphorylation capacity of a cell determines the net rate of ara-C uptake [83]. Intracellular activating enzymes have likely been saturated so that further increases in the ara-CTP pool are unlikely.

In addition to dosage, dose scheduling is important to the therapeutic efficiency of any one drug. In a recent clinical trial of newly diagnosed APL patients in whom ATRA was given alone until CR was achieved followed by CT (ATRA → CT) or CT was initiated just 3 days following the start of ATRA treatment (ATRA + CT), the CR rate was similar between the two groups but the relapse rate at 2 years was significantly less in the ATRA + CT group [26]. Our in vitro studies reflect the clinical experience whereby the addition of ATRA was most effective when added prior to ara-C administration and where ara-C administration closely followed (within 24 h) ATRA administration. When pretreatment with ATRA was beyond 24 h, the effect between combination and single use began to narrow. The simultaneous use of both modalities was not different from 48-h ATRA pretreatment followed by ara-C, but was less effective than the close administration of both modalities. Studies with HL-60 cells have suggested that the combination effect is greatest when ATRA follows ara-C treatment as opposed to administration of ATRA prior to ara-C or their simultaneous use [2]. While this finding may provide mechanistic clues, in the clinic ATRA is most often given immediately upon confirmation of an APL diagnosis to help alleviate accompanying coagulopathy and improve morbidity before CT begins [27]. Furthermore, the NB4 cell line was originally established from the leukemia cells of a patient with APL [58] and expresses the disease-specific t(15;17) chromosomal translocation of APL, thus providing a better model for APL than HL-60 cells which lack the characteristic chromosomal translocation [21].

The activity of ara-C depends on conversion to its active triphosphate form, ara-CTP, and transportability across the plasma membrane is critical to its pharmacological action. NBMPR is a tightly bound, inhibitory ligand of the *es* nucleoside transporter [14] and has been employed in the past as a probe for enumeration of the *es* transporter in many cell types [41, 47, 48, 65, 79]. Studies of the NBMPR-sensitive nucleoside transporter

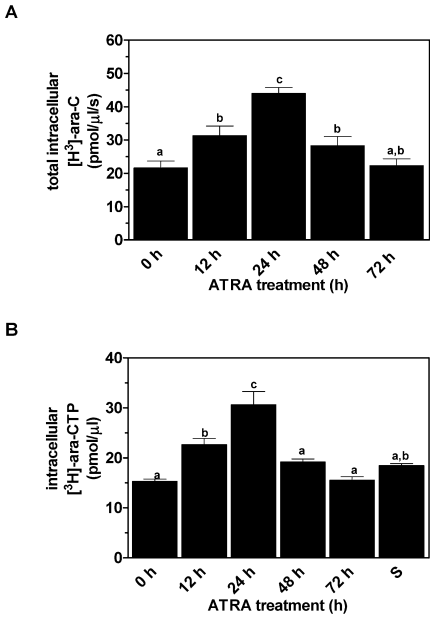

Fig. 6A, B Intracellular incorporation of [^{3}H]-ara-C increases following ATRA treatment. **A** Uptake of [^{3}H]-ara-C was determined for control cells (0 h ATRA treatment) and for cells treated with 1 μ*M* ATRA for the indicated times. Total intracellular [^{3}H]-ara-C incorporation was measured in the presence of Na^+ after 10 min transport incubation with 5 μ*M* [^{3}H]-ara-C. **B** Intracellular [^{3}H]-ara-CTP levels were determined by HPLC where the peaks were identified by comparison of retention times with those of standards. Cells were incubated for 24 h with 1 μ*M* [^{3}H]-ara-C in the absence of ATRA (control), following pretreatment with ATRA for various times (12, 24, 48 and 72 h), or treated simultaneously with ara-C and ATRA (*S*). Statistical significance was determined using the Student-Newman Keuls Multiple Comparisons test ($P < 0.5$). Bars not sharing a letter are significantly different

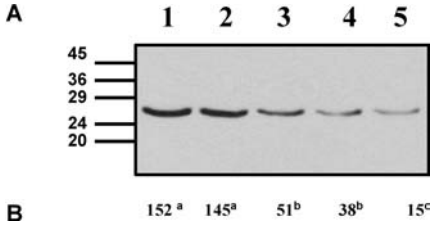

Fig. 7 Bcl-2 expression in NB4 cell lysates decreased following ATRA treatment. The expression of bcl-2 protein was determined after cells were treated for 0, 12, 24, 48, and 72 h with 1 μ*M* ATRA (*lanes 1–5*). Total cellular proteins of whole cell lysates from approximately 500,000 NB4 cells were obtained from each treatment group, lysed and analyzed by SDS-PAGE and Western blotting. Molecular weight markers (kDa) are shown on the left. Autoradiograms were developed after 5 min of exposure, using ECL detection. The blot shown represents a compilation of at least three separate experiments. The line labeled *B* indicates the mean densities of the bands in arbitrary units. Statistical significance was determined using the Student-Newman-Keuls Multiple Comparisons test ($P < 0.5$, $n = 3$). Values not sharing a letter are significantly different

[83, 84, 85] have suggested that nucleoside transporter expression is necessary for the effective treatment of AML and ALL with ara-C. Correlations between the cellular content of transporters and ara-C influx [84], ara-CTP formation [86], and ara-C cytotoxicity [33] have been demonstrated, and the efficiency of ara-C uptake by leukemic blast cells is related to clinical outcome in AML patients receiving standard-dose ara-C [84]. The level of nucleoside transporter expression differs among hematopoietic malignancies and within the same malignancy [45, 71, 84, 86, 87, 88]. In general AML responds better to ara-C treatment than ALL and it has been suggested that this differential response is in part due to the much lower expression of nucleoside transporter sites in blasts from ALL than in those from AML. Furthermore, interpatient variations in the number of NT expressed may account for differences in response to treatment within the same disease.

In the present study we demonstrated that with the increase in number of [^{3}H]-NBMPR sites following increased exposure time to ATRA, cell viability concomitantly decreased (Fig. 5A) and that there are strong correlations between cell viability and expression of [^{3}H]-NBMPR binding sites and between ara-C potency and [^{3}H]-NBMPR binding sites ($r^2 = 0.90$ and $r^2 = 0.75$, respectively; Fig. 5B, C). The increase in [^{3}H]-NBMPR binding sites was functionally reflected by increased transport rates of ara-C (Fig. 4B, C). However, the loss of the relationship between transporter abundance and activity and ara-C cytotoxicity with ≥48 h ATRA treatment suggested that changes in transporter number and activity are not solely responsible for the increased cytotoxicity to ara-C. Sensitivity of another cytosine analog, gemcitabine, in combination with a thymidylate synthase (TS) inhibitor is also correlated with *es* NT numbers [72] where inhibition of TS leads to an increased number of *es* transporters at the cell surface [13]. This combination effect was, like ATRA and ara-C, sequence-dependent, whereby the maximum effect was seen when addition of the TS inhibitors preceded addition of gemcitabine.

The enhancement of both cellular accumulation and retention have been previously found to have a close correlation with ara-C cytotoxicity [56, 74, 76] and clinical response [7, 44, 49, 69, 75]. Total intracellular incorporation of [^{3}H]-ara-C initially increased following ATRA treatment, an increase that was revealed within the active metabolite pool, [^{3}H]-ara-CTP (Fig. 6A, B). Beyond 24 h, although ara-C transport rates remained elevated above control levels, total intracellular [^{3}H]-ara-C incorporation began to decline and the increase in ara-C cytotoxicity in the presence of ATRA was no longer correlated with ara-CTP concentration. The decline in incorporation was likely due to the bidirectional nature of the *es* transporter system, permitting the efflux of drug. Similarly, the ATRA-induced increase in cytotoxicity to ara-C demonstrated in Hl-60 cells is not a result of an increase in CTP levels [30]. Upon differentiation to neutrophils, HL-60 cells exhibit increased activity of the ara-CTP catabolic enzyme, cytidine deaminase, thereby prohibiting an increase in ara-CTP accumulation [81]. It is possible that in NB4 cells an

increase in the activity of this catabolic enzyme occurs with an increase in duration of ATRA exposure, thereby decreasing attainable levels of active ara-CTP and the augmentation effect seen at earlier ATRA treatment time-points regardless of the continued increase in transport rate. The sustained increase in transport at the later time-points may be the result of the maintenance of the ara-C gradient across the membrane established by the increased rate of catabolism of the parent form, ara-C. Furthermore, deoxycytidine kinase activity in many cell lines is increased in proliferating cells [80], but whether this enzyme decreases with changes in differentiation state has not been demonstrated but would affect achievable levels of ara-CTP.

The cytotoxic effect of ara-C is specific for cells in S phase [36, 55]; it is not toxic to nonproliferating cells. In this study, the observed decrease in percentage of cells in S phase and the concomitant increased percentage of cells in G_1 as the ATRA exposure time increased (Table 2) suggests that changes in cell cycle distribution may contribute to the decrease in cytotoxicity to ara-C of NB4 cells treated with ATRA for prolonged times compared to briefly treated cells.

It is widely recognized that ara-C and other cytotoxic agents kill neoplastic cells by triggering apoptosis [39, 50, 51] and the susceptibility of cells to apoptosis depends on the status of genes involved in the regulation of various components of the cell death machinery [37]. The decision of a cell to die upon exposure to ara-C is ultimately determined by whether a threshold level of DNA damage is exceeded. Components of various signal transduction pathways and expression levels of oncogenes influence the threshold [36]. The proto-oncogene bcl-2 is a suppressor of cell death and human leukemic cells genetically modified to overexpress bcl-2 display resistance to ara-C-induced apoptosis [63]. ATRA has been shown to reduce the expression of bcl-2 and to increase ara-C cytotoxicity in AML cells lines [8, 42], and in normal and AML progenitors isolated from bone marrow or peripheral blood [2, 62]. Bcl-2 levels have been correlated to clinical response in terms of survival and remission duration, especially in those patients within the cytogenetic subgroup t(15:17) of APL [8, 54, 70].

In our study, ATRA-induced differentiation of NB4 cells was concomitant with downregulation of bcl-2 protein as has been previously demonstrated [6]. The decrease was notable by 24 h, a time coincident with the maximal decrease in cell viability observed in cultures treated with ara-C and ATRA combined. However, an appreciable level of apoptosis in cultures treated solely with ATRA took much longer (Table 2, Fig. 2A). A decrease in bcl-2 protein does not accompany ara-C-induced apoptosis of NB4 cells [5]. Presumably, by decreasing the expression of bcl-2 with the use of ATRA, we can decrease the threshold at which ara-C-damaged DNA leads to apoptosis. This may help to explain why with 48 h and 72 h of ATRA treatment, ara-C cytotoxicity was elevated above that in cultures treated with ara-C alone despite the absence of a difference in ara-CTP levels. Evidence in this experiment is indirect, but direct evidence for a role of bcl-2 in the increased cytotoxicity of ara-C in the presence of ATRA has been observed in other models using bcl-2 antisense oligonucleotide assays [42, 52, 53]. Furthermore, bcl-2 antisense therapy has been clinically applied with some success [82].

The ATRA-induced increase in cytotoxicity of ara-C in NB4 cells is, in part, the result of an increase in the functional expression of nucleoside transporters and a role for bcl-2 is also presented. Our results would suggest that timing of ara-C therapy should be tied to maximal *es* transporter expression, which is likely to be 24 h after ATRA treatment begins. It remains to be seen whether the response in the clinic can be further enhanced for APL taking advantage of ara-C transporter regulation by ATRA.

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