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ORIGINAL ARTICLE

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Complementary antineoplastic activity of the cytosine nucleoside analogues troxacitabine (Troxatyl) and cytarabine in human leukemia cells

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Abstract *Purpose*: Troxacitabine (BCH-4556, L-(−)-OddC, Troxatyl) is a novel β-L-nucleoside analogue with potent antineoplastic activity both in vitro and in several tumor models in vivo, and is presently in phase II clinical trials. The combination of the cytosine analogues troxacitabine and araC (1-β-D-arabinofuranosylcytosine, cytarabine) has shown promising activity in patients with acute myelogenous leukemia. To further examine the interactions between these two analogues, we investigated the in vitro and in vivo effects of their combination against a human leukemia cell line, CCRF-CEM. *Methods*: The in vitro cytotoxic effect of the combination of troxacitabine and araC on the survival of CCRF-CEM cells was measured using a standard MTT assay and combination indices were generated with the CalcuSyn software. For in vivo studies, we evaluated the effect of both drugs, alone and in combination, on survival of CCRF-CEM tumor-bearing animals. Mechanistic studies addressed recovery of DNA synthesis, intracellular levels of araC metabolites, feedback inhibition by triphosphate species and pharmacokinetics of both drugs. *Results:* The combination of troxacitabine and araC in vitro was synergistic with combination indices between 0.1 and 0.7. This appeared to be related to the impact of the combination on DNA synthesis recovery, which was significantly delayed following exposure to the combination of troxacitabine and araC compared to either agent alone. Analysis of the effect of troxacitabine on the intracellular metabolites of araC revealed that troxacitabine did not inhibit araC deamination and caused a slight decrease in the overall intracellular accumulation of araCTP. The lower accumulation of araCTP could not be attributed to feedback inhibition caused by troxacitabine triphosphate on dCK. Furthermore, our in vivo experiments demonstrated that the combination of araC and troxacitabine was better at slowing down the progression of leukemia in SCID mice than either agent used alone without additive toxicities. Injections of 10 mg/kg troxacitabine i.p. daily for 5 days in combination with araC at 10 mg/kg led to an increase in median survival time of 58 days compared to 49.5 and 53.5 days for araC and troxacitabine, respectively, given as single agents. This represents an increase in life span of 17%, respectively when compared to araC alone. A pharmacokinetic study revealed that troxacitabine did not influence the disposition of araC when coadministered. *Conclusions*: Overall, our results show that the antileukemic activity of troxacitabine and araC is complementary when the two nucleoside analogues are combined in vivo. These effects appear to be related to their interaction at the level of DNA repair rather than to pharmacokinetic interactions. These results encourage the use of troxacitabine and araC in combination in patients with acute leukemia.

Keywords Troxacitabine · Troxatyl · Cytarabine · Combination · Leukemia

Abbreviations *AML* Acute myelogenous leukemia · *araC* 1-β-D-Arabinofuranosylcytosine, cytarabine · *araCMP* Cytarabine-5′-monophosphate *araCTP* Cytarabine-5′-triphosphate · *araU* 1-β-D-Arabinofuranosyluracil · *dCyd* Deoxycytidine · *CDA* Cytidine deaminase · *CI* Combination index · *dCK* Deoxycytidine kinase · *dCTP* Deoxycytidine-5′-triphosphate · *dFdC* Gemcitabine, 2′,2′-difluorodeoxycytidine · *troxacitabine TP* Troxacitabine 5′-triphosphate

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Introduction

Nucleoside analogues, such as araC (1-β-D-arabinofuranosylcytosine, cytarabine), represent a class of drug

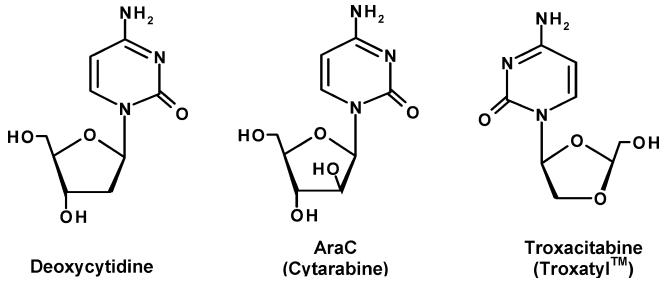

Fig. 1 Structure of deoxycytidine and its analogues, troxacitabine and araC

with an important role in the treatment of leukemia [11, 16, 26, 41]. All of these clinically approved nucleoside analogues, like all naturally occurring nucleosides, are in the β-D configuration (Fig. 1). Recently, troxacitabine (β-L-dioxolane cytidine, BCH-4556, Troxatyl), a β-L-nucleoside, was discovered and has been shown to possess potent anticancer activity both in vitro and in vivo [21, 25, 27, 31, 39, 43]. Troxacitabine is the first β-L-nucleoside to demonstrate antineoplastic activity in several tumor models and is presently being studied in phase II clinical trials [1, 8, 17, 19, 20].

Like other nucleoside analogues, troxacitabine needs to be phosphorylated intracellularly to its triphosphate form in order to be active [24, 25]. Both araC and troxacitabine are phosphorylated to their monophosphate form by deoxycytidine kinase (dCK), which demonstrates that this enzyme lacks stereo selectivity [25]. Cells deficient in dCK activity are resistant to the action of both araC and troxacitabine showing that this enzyme is required for their activity [22]. Once monophosphorylated, these antimetabolites are phosphorylated to their di- and triphosphate forms by other cellular kinases [23, 24]. The triphosphate forms of araC and troxacitabine are good substrates and inhibitors of the replicative and repair DNA polymerases in vitro, which is responsible for their antineoplastic activity [29, 32]. Troxacitabine has been shown to be a true terminator of DNA polymerization because its dioxolane sugar ring lacks the hydroxyl group necessary for chain elongation [29]. On the other hand, araC is associated with the incorporation of several natural nucleotides before causing chain termination [32]. Excision of incorporated analogues from the 3′ termini of DNA has been demonstrated in vitro and represents a way for cells to recuperate and survive the action of these nucleoside analogues [3, 33, 34]. Recently, it has been found that a human apurinic/apyrimidic DNA endonuclease (APE1) may be the major enzyme involved in the removal of troxacitabine monophosphate from DNA [3] whereas araC removal may be more dependent on a cytosolic 3′ to 5′ exonuclease [33].

Inactivation of nucleoside analogues, such as araC or gemcitabine, can occur by deamination by CDA, which is an important hurdle for their in vivo stability and clinical effectiveness [10, 12]. Resistance to araC has been demonstrated in an animal tumor model expressing high levels of CDA [21]. In contrast to dCK, CDA demonstrates stereo specificity and does not deaminate troxacitabine [25]. This explains why cells resistant to araC due to high levels of CDA are still sensitive to the antineoplastic action of troxacitabine [21]. Another characteristic that distinguishes troxacitabine from araC is the uptake of these analogues into cells. Recent results have demonstrated that the major route of cellular uptake of troxacitabine in leukemia cells is passive diffusion, which implies that deficiencies in nucleoside transport is unlikely to impair its activity [22]. On the other hand, araC uptake into leukemia cells is highly dependent on nucleoside transporters and the level of equilibration-sensitive (*es*) transporters correlates with the in vitro sensitivity of leukemia blasts from patients to araC [15].

The clinical pharmacokinetic characteristics of troxacitabine are remarkably different from other nucleoside analogues. First, disappearance of troxacitabine from plasma follows a biphasic pattern with a long terminal half-life of 40 to 80 h [1, 8, 17]. In contrast, araC has a shorter terminal half-life of 4 to 6 h in patients [2, 9, 38]. Secondly, in humans, the majority of troxacitabine (69%) is excreted in urine in the form of unchanged drug compared to 4–6% unchanged araC [9, 17]. In fact, 63–73% of araC is excreted in the urine as araU [9]. Clinical trials have established that plasma levels of troxacitabine and araC can reach levels known to cause growth inhibition in several cell lines in vitro [8, 17, 38].

Recent clinical trials have led to the observation that some cancer patients failing to respond to high-dose araC treatment are still responsive to the action of troxacitabine [17, 19]. In a phase II trial study, three complete responses and one partial remission (13%) were observed in 30 evaluable patients with AML [17]. One of five (20%) patients with refractory myelodysplastic syndromes (MDS) achieved a hematological improvement and a patient with blastic phase of chronic myeloid leukemia (CML-BP) achieved a durable and second chronic phase [17]. In a second study, of 16 assessable patients with CML-BP, six returned to a chronic phase [17, 19]. Furthermore, in 16 assessable patients with AML, two complete responses and one partial remission were observed [17, 19]. Interestingly, the combination of troxacitabine and araC in patients resulted in a complete response in patients with recurrent AML [18].

Because of this beneficial interaction between troxacitabine and araC, we set out to elucidate the mechanism of interaction between these two cytosine analogues. Since our previous preclinical studies had shown that both troxacitabine and araC have potent antitumor activity in the CCRF-CEM leukemia xenograft model [21], we chose this cell line and tumor model to determine if these nucleoside analogues would be synergistic or antagonistic when combined, and to study the mechanism by which both drugs might interact.

Materials and methods

Drugs

Troxacitabine was synthesized at Shire Biochem [31] and [^{3}H]-troxacitabine (3.9 Ci/mmol) was prepared by Moravek Biochemicals (Brea, Calif.) from material provided by Shire BioChem. AraC was purchased from Bristol-Myers Squibb (Montréal, Canada), [^{3}H]-dCyd (18.4 Ci/mmol) and [5-methyl-^{3}H]-thymidine (2 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Little Chalfont, UK) and [^{3}H]-araC (24 Ci/mmol) was purchased from Moravek Biochemicals. Tetrahydrouridine and the triphosphate nucleosides 2′-dCyd 5′-triphosphate and cytosine β-D-arabinofuranoside 5′-triphosphate were purchased from Sigma-Aldrich Canada (Oakville, Canada).

Cell culture and cytotoxicity studies

The human CCRF-CEM leukemia cell line was purchased from the American Type Culture Collection (Rockville, Md.). The cell line was cultured in RPMI 1640 containing 10% dialyzed heat-inactivated fetal bovine serum (FBS; Life Technologies, Burlington, Canada) and maintained as a suspension at 37°C in a humidified atmosphere containing 5% CO_2. Cells were subcultured twice weekly and have a doubling time of 20–24 h. Antibiotics were not used and cells were routinely checked for mycoplasma contamination by PCR analysis (mycoplasma PCR detection kit; Stratagene, La Jolla, Calif.).

The effect of the combination of troxacitabine and araC on the survival of CCRF-CEM cells was measured using a standard MTT assay. In order to determine if the combination of troxacitabine and araC was additive, antagonist, or synergistic, we used the linear curve fitting (median-effect analysis) of the CalcuSyn software (Biosoft, Ferguson, Mo.), which is based on algorithms developed by Chou and Talalay [4]. Briefly, dose-response curves were generated to determine the concentration that produced 50% cell death (IC_{50}) at 72 h continuous exposure by MTT assay. Combinations of drugs were then generated at concentrations below and above the IC_{50} values of each drug in order to determine if the combinations were additive, synergistic, or antagonistic (Fig. 2A). Combination indices (CIs) from three different experiments were generated with the CalcuSyn software for each set of combinations, in triplicate, using the results obtained from each drug alone and in combination at a constant ratio within the same experiment. A CI below 1 was defined as indicating synergy, while a CI equal to 1 was defined as indicating additivity, and CI above 1 as indicating antagonism [4].

Intracellular accumulation of araC phosphorylated metabolites

Accumulation of tritiated araC metabolites was measured by HPLC. Briefly, 5×10^6 CCRF-CEM cells were plated per flask in 5 ml RPMI containing 10% dialyzed heat-inactivated FBS and incubated at 37°C in an atmosphere containing 5% CO_2. Cells were exposed to 2 μM ^{3}H-araC and to the combination of 2 μM ^{3}H-araC and 5 μM of troxacitabine. At each time point (30, 60, and 120 min), 1 ml of medium was removed, the number of cells determined, and the sample was centrifuged, washed once in cold PBS, and transferred to an Eppendorf tube. The cell pellet was resuspended in 600 µl cold 60% methanol and stored overnight at −20°C. The tubes were then centrifuged at 13,000 *g* at 4°C for 10 min and the supernatants were transferred to clean Eppendorf tubes. Supernatants were evaporated under a gentle stream of nitrogen and stored at −20°C until HPLC analysis.

For HPLC analysis, samples were resuspended in 200 µl ddH_2O. AraC and its metabolites were separated on a C18 reversed-phase column (Zorbax XDB, YMC ODS-A, 5 µm, 120A, 250×4.6 mm; Waters Corporation, Milford, Mass.) using an Agilent 1100 HPLC system equipped with diode array UV detector and a radioactivity detector (Canberra Packard Canada, Montréal, Canada). The mobile phase was pumped at 1 ml/min and consisted of acetonitrile (A) and 10 mM phosphate buffer, pH 6.7, containing 1 mM tetrabutylammonium phosphate (B). Aqueous phase (B) was applied for 20 min, followed by a linear gradient of 0–15% A/B for 20 min. Standards (15 nmol each of araU, araC, araCMP, and araCTP) had retention times of 8.3, 13.5, 17.6, and 44.9 min, respectively. Peaks were quantified using Millennium software (Waters).

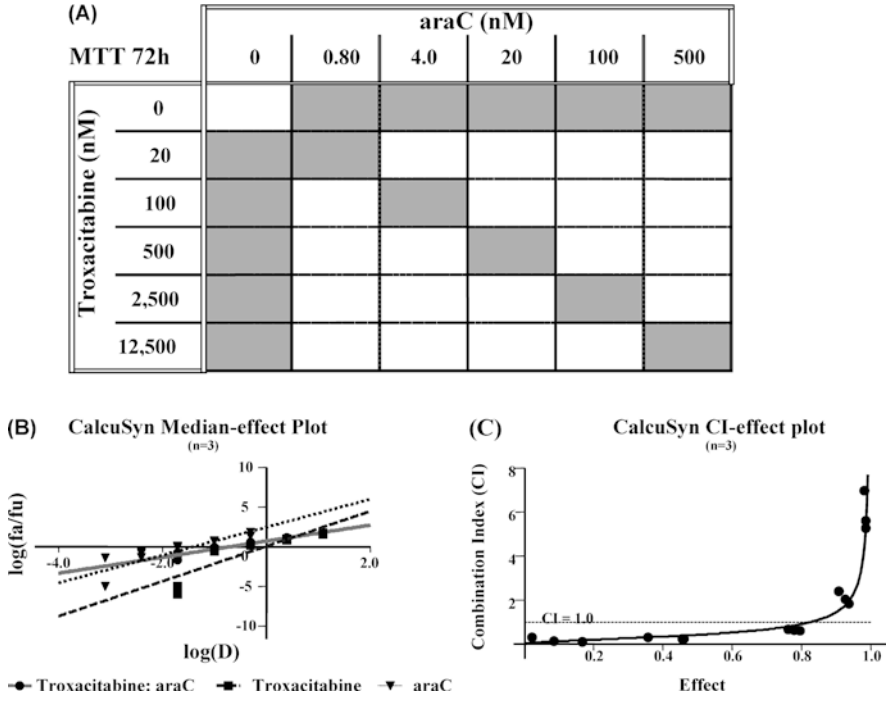

Fig. 2A–C CalcuSyn analysis of the troxacitabine/araC combination in CCRF-CEM leukemia cells. **A** Graphical representation of the cytotoxicity assay for the combination in CCRF-CEM cells. The drugs were combined at a fixed 1:25 ratio (araC to troxacitabine, respectively). **B** The CalcuSyn median-effect plot was generated from three separate experiments in triplicate with SD < 20% (*Fa* affected fraction, *Fu* unaffected fraction, *D* concentration of drug used). **C** CIs from three different experiments were generated with the CalcuSyn software for each set of combinations, in triplicate, using the results obtained from each drug alone and their effect in combination within the same experiment

Deoxycytidine kinase purification and assay

dCK was cloned using reverse transcriptase polymerase chain reaction (RT-PCR) using total RNA isolated from HT-29 human colon tumor cell line. The upper and lower primers had the following sequences: *upper* 5′-GCTCTGGGCCGCCACAAGACT-3′; *lower* 5′-GGCTGCCTGTAGTCTTCAGCA-3′. The cDNA for *dCK* was confirmed by sequencing and inserted into the pHAT10 protein expression plasmid (Clontech, Palo Alto, Calif.) using the *Sal*I and *Kpn*I sites within the multiple cloning site of the vector. The HAT-tagged dCK protein was purified according to standard procedures of the HAT Protein Expression and Purification System Protocol from Clontech and stored at −20°C in 20% glycerol and 10 m*M* dithiothreitol (DTT). The purified dCK was run on an SDS-polyacrylamide gel to verify the size and purity of the enzyme. To test the activity of the purified dCK, a radioactive biochemical assay was used. The incubation mixture contained 50 m*M* Tris (pH 7.6), 2 m*M* ATP, 5 m*M* $MgCl_2$, 100 m*M* KCl, 2 m*M* DTT, 0.5 mg/ml bovine serum albumin, and a known concentration of ^{3}H-dCyd, ^{3}H-araC, or ^{3}H-troxacitabine. The reaction was started with the addition of 2 U enzyme (1 U being defined as the quantity of protein phosphorylating 1 pmol dCyd per microgram protein per minute). After 30 min of incubation at 37°C, the reaction was stopped by the addition of 3 ml cold water and spotted on DE-81 filter discs (Whatman, VWR, Montréal, Canada). The discs were then washed with water and ethanol, dried, and counted for radioactivity in a Wallac beta counter (Turku, Finland). The assay was performed in duplicate with a standard error below 20%. The same assay was used for the feedback inhibition assay in which dCTP, araCTP, or troxacitabineTP (troxacitabine-5′-triphosphate) were used to determine the concentration that inhibited phosphorylation of substrate by 50% (IC_{50}). IC_{50} values were calculated using GraphPad Prism version 3.02 for Windows software (GraphPad Software, San Diego, Calif.). In that assay, the substrates were used at concentrations approaching their respective Km (Km for dCyd, 1.5 μ*M*; for araC, 7 μ*M*; and for troxacitabine, 28 μ*M*).

DNA synthesis recovery studies

Exponentially growing cells (2×10^5) were resuspended in RPMI plus 10% dialyzed heat-inactivated FBS and exposed for 2 h to 0.1 μ*M* araC, 1 μ*M* troxacitabine, and to the combination of araC/troxacitabine at the same concentrations. After the 2-h incubation period with araC and troxacitabine, cells were spun and resuspended in fresh medium. At several time points after drug removal (0, 2, 4, 8, and 16 h), 0.5 μCi [5-methyl-^{3}H]-TdR was added per well to measure the rate of DNA synthesis. DNA from the cells was harvested 1 h later on a glass fiber filter (Wallac, Turku, Finland) using a Tomtec cell harvester (Orange, Ct.). Filters were dried and counted in ScintiSafe Econo F scintillation liquid (Fisher Scientific, Montréal, Canada) in a Wallac beta counter (Turku).

In vivo studies

Female SCID mice at 5–6 weeks of age (Charles River, St-Constant, Canada) were injected i.p. with 3×10^7 CCRF-CEM cells. Treatment with troxacitabine and/or araC was started 20 days after CCRF-CEM tumor cell injection, once the mice had developed visible tumors at the site of inoculation. Tumor-bearing animals were randomized (ten per group) and treated by one of the following schemes: (a) control (saline), (b) araC 10 mg/kg; (c) araC 20 mg/kg; (d) troxacitabine 5 mg/kg; (e) troxacitabine 10 mg/kg; (f) araC 10 mg/kg plus troxacitabine 5 mg/kg; (g) araC 10 mg/kg plus troxacitabine 10 mg/kg; (h) araC 20 mg/kg plus troxacitabine 5 mg/kg; (i) araC 20 mg/kg plus troxacitabine 10 mg/kg. Treatments were given i.p. once a day for five consecutive days (days 20–24). In the combination studies, drugs were injected sequentially: troxacitabine first, followed by araC 4 h later.

The results are expressed as percent increase in life span (median survival time of treated animals minus that of control animals divided by the median survival time of the control group). By NCI criteria, increases in life span exceeding 25% indicate that the drug has significant antitumor activity [36]. Median survival time and *P* values were calculated using GraphPad Prism software (San Diego, Calif.) with comparison of each curve done by the log-rank test.

All animals received humane care in compliance with the Canadian Council Guidelines of the Care and Use of Experimental Animals. The Animal Care Committee of Shire BioChem approved these in vivo experimental protocols; the Canadian Council for animal care accredits our animal facility.

Pharmacokinetic analysis of troxacitabine and/or araC

The pharmacokinetic analysis of troxacitabine, araC, and combinations of the two were evaluated in female SCID mice ($n=3$ per time point). The following protocols were investigated: (1) araC 20 mg/kg i.v.; (2) troxacitabine 20 mg/kg i.v.; (3) troxacitabine 20 mg/kg i.v. followed 20 min later by araC 20 mg/kg i.v.; and (4) troxacitabine 20 mg/kg i.v. followed 3 h later by araC 20 mg/kg i.v. Plasma was collected by cardiac puncture at 2, 5, 15, 30, 60, 90, 120, 180, and 240 min following troxacitabine (protocol 2) or araC (protocols 1, 3, and 4) into heparinized tubes containing 1 m*M* final concentration of tetrahydrouridine to inhibit the deamination of araC. Mouse plasma (300 μl) was mixed with 20 μl of an internal standard and 100 μl $ZnSO_4$ (15%) and briefly vortexed. Then 900 μl acetonitrile was added and samples were vortexed for 1 min. After centrifugation at 3000 *g* for 5 min, 1.1 ml supernatant was collected and dried under a gentle stream of nitrogen. The residue was reconstituted with 80 μl ddH_2O, filtered, and 50 μl injected. A standard curve of araC was linear between 0.1 and 20 μg/ml and that of araU and troxacitabine between 0.1 and 60 μg/ml. The coefficients of variation (CV%) of the low, medium, and high controls were less than 10%. AraC, araU, and troxacitabine concentrations were determined by HPLC (Agilent 1100) and separated on a Synergi Hydro RP, 5 μm, 2.1×155 mm column. The mobile phase consisted of acetonitrile and 10 m*M* ammonium acetate buffer pH 6.7. The aqueous phase was applied for 15 min. followed by a gradient from 0 to 50% acetonitrile over 5 min. The flow rate was 0.3 ml/min. Peaks were evaluated at 276 nm. Under these conditions troxacitabine, araC, and araU eluted at 13.1, 7.3, and 11.9 min, respectively. Their limits of detection were 0.3, 0.2 and 0.2 μg/ml, respectively.

Data were analyzed by a noncompartment model and araC was analyzed by a two-compartment model using Kinetica 2000 (Innaphase Corporation, Philadelphia, Pa.). A weighting factor of $1/y^2$ was applied. Maximal plasma concentrations (Cmax) of the analogues were read directly from the data, and the area under the curve (AUC) was calculated by the log linear trapezoidal rule. The AUC was extrapolated to infinity by dividing the last measured concentration (Cn) by λz, where λz represents the terminal slope. Systemic clearance was calculated by dividing the dose by $AUC_{0\rightarrow\infty}$.

Results

In vitro combination studies

Figure 2A shows the matrix generated to study the interaction of troxacitabine and araC in vitro. The curves of the toxicity for araC and troxacitabine (Fig. 2B) were almost parallel while the curve representing their combination intersected, showing that the effect of their combination was more cytotoxic than when the agents were used alone. The IC_{50} for

troxacitabine on CCRF-CEM cells for a 72-h exposure was evaluated as 903 n*M* and that for araC as 37 n*M*. When the two drugs were used in combination, their respective IC_{50} values were lower (183 n*M* for troxacitabine and 7 n*M* for araC), demonstrating that the cytotoxicity of troxacitabine and araC was increased when they were used in combination. Also, the combination of troxacitabine and araC was synergistic at low concentrations with CIs between 0.1 and 0.7 while at higher concentrations antagonism was observed (Fig. 2C).

Analysis of araC's phosphorylated metabolites

In order to determine if troxacitabine modulates araC's intracellular metabolism, we performed HPLC analysis of CCRF-CEM cells following exposure to ^{3}H-araC alone or in combination with troxacitabine (Table 1). Exposure of CCRF-CEM cells to 2 μ*M* of ^{3}H-araC resulted in rapid intracellular accumulation of araC and its phosphorylated metabolites. Accumulation of araC increased over the 2-h period as well as the phosphorylated forms. However, rapid saturation in the level of araCTP formed inside the cells occurred (2.4 pmol/10^6 cells at 30 min). The total percentage of araU in the cells did not vary extensively during the 2-h period and remained close to 10%. In the presence of troxacitabine, a two- to threefold lower accumulation of araC was observed and hence the absolute quantity of araC metabolites was decreased. The araU ratio was unaffected by troxacitabine. Even though troxacitabine decreased the total amount of intracellular phosphorylated araC metabolites, this was not sufficient to block their cytotoxic activity since the combination of the two agents was more cytotoxic than either agent alone.

Effect of troxacitabineTP and araCTP on dCK activity

In order to determine if the lower accumulation of araCMP in the cells was due to the feedback inhibition caused by troxacitabineTP, we studied these effects using purified dCK (Table 2). The enzyme was cloned and purified using a bacterial expression system and was shown to have all the characteristics previously reported for this enzyme, assuring the quality of the enzyme [7]. dCTP was best at inhibiting its own phosphorylation (IC_{50} 12 μ*M* compared to >200 μ*M* for the analogues) and even better at inhibiting the phosphorylation of araC and troxacitabine (IC_{50} values of 1.2 and 1.7 μ*M*, respectively). On the other hand, araCTP was a poor feedback inhibitor of all nucleosides tested (IC_{50} values around or above 200 μ*M*) showing that its triphosphated form was not limiting its own accumulation in cells. TroxacitabineTP, like araCTP, was a weak inhibitor of the phosphorylation of dCyd by dCK (IC_{50} values >200 μ*M*) but was a better inhibitor of the phosphorylation of araC (25 μ*M*) and troxacitabine (43 μ*M*). This effect by troxacitabineTP could explain in part the lower accumulation of araCTP (two- to threefold) in CCRF-CEM cells when troxacitabine and araC were coincubated (Table 1).

Effect of troxacitabine and araC on DNA synthesis recovery

AraC was a better inhibitor of DNA synthesis than troxacitabine with almost 100% inhibition at 0.1 μ*M* following a 2-h exposure of the cells to the drug (Fig. 3). In comparison, troxacitabine was able to inhibit DNA synthesis by 75% at 1 μ*M*. The combination of the drugs did not achieve a higher level of inhibition since araC by itself was able to abrogate DNA synthesis completely. The main effect was observed over time. At 16 h, after removal of the drugs from the tissue culture medium, we observed that the recovery of DNA synthesis in cells treated with the combination was slower than the recovery following treatment with either agent used alone as measured by a 1-h pulse-exposure of the cells to tritiated thymidine. This demonstrates that the combination of troxacitabine and araC was better at inhibiting DNA synthesis recovery than either agent alone, which might account for the synergistic cytotoxic activity observed.

In vivo efficacy of the troxacitabine and araC combination

We studied the effect of the combination of troxacitabine and araC in an in vivo CCRF-CEM leukemia model in order to determine if the synergy observed in vitro could translate to an in vivo setting. In this model, CCRF-CEM leukemia cells were injected i.p. and treatment was started 20 days later to allow for disease

Table 1 Effect of troxacitabine on araC's phosphorylated metabolites

Drug	Time point (min)	Picomoles per 10^6 cells			
		araC	araCMP	araCDP	araCTP
^{3}H-araC (2 μ*M*)	30	0.10	0.57	0.40	2.4
	60	0.16	0.61	0.51	2.1
	120	0.38	0.80	0.86	2.4
Troxacitabine (5 μ*M*) + ^{3}H-araC (2 μ*M*)	30	0.035	0.20	0.11	0.47
	60	0.058	0.21	0.16	0.77
	120	0.15	0.37	0.24	0.86

Table 2 Feedback inhibition of dCTP, araCTP and troxacitabineTP on dCK substrates. Each value is the average of three different experiments done in duplicate

Substrate	IC_{50} (μM)		
	dCTP	araCTP	TroxacitabineTP
dCyd (2 μM)	12 ± 2^{a}	> 200	> 200
AraC (10 μM)	1.2 ± 0.3	184 ± 27	25 ± 3
Troxacitabine (20 μM)	1.7 ± 0.3	172 ± 48	43 ± 16

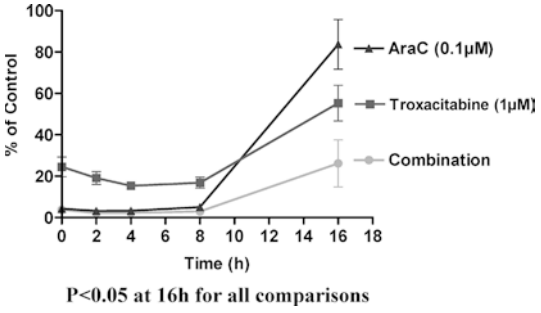

Fig. 3 Effect of araC and troxacitabine combination on recovery of DNA synthesis of CCRF-CEM. Cells were exposed for 2 h to 0.1 μM araC, 1 μM troxacitabine, and to their combination, and then resuspended in fresh medium. At 0, 2, 4, 8, and 16 h after the end of the 2-h exposure, cells were incubated with 0.5 μCi 5-^{3}H-methyl-TdR for 1 h. DNA from cells was isolated on glass fiber filters and counted in a beta counter. The value at each time point is the mean of three different experiments with SD < 20%

progression. This model was fairly aggressive, with mice dying at a median of 37 days without any treatment (Table 3). At doses of 10 and 20 mg/kg, araC was able to increase the survival time of mice by 34% and 38%, respectively ($P < 0.01$ compared to saline for both doses). Troxacitabine at doses of 5 and 10 mg/kg increased the survival time of mice by 41% and 44%, respectively ($P < 0.01$ compared to saline for both doses). These results demonstrate that both agents were efficacious in this tumor model, confirming the results of our previous studies [21].

Furthermore, our in vivo experiments demonstrate that the combination of araC and troxacitabine is better at slowing down the progression of leukemia in mice than either agent used alone. As shown in Table 3, the combination of 10 mg/kg araC and 5 or 10 mg/kg troxacitabine increased the life span of mice by 43% and 57% (compared to 34% for araC alone and 41% and 44% for troxacitabine alone; $P < 0.01$ compared to araC 10 mg/kg alone). This represents a net improvement of 7% and 17% in the overall survival of mice. The combination of 20 mg/kg araC and 5 or 10 mg/kg troxacitabine increased the life span of mice by 3% and 12%, respectively, compared with araC alone. Furthermore, the combination of troxacitabine and araC, at all the doses tested did not result in additive toxicities to the mice as measured by body weight loss. These results demonstrate that at these concentrations, sequential injections of araC and troxacitabine are better at slowing down the progression of leukemia in mice than when the two agents are used alone.

Pharmacokinetic analysis of troxacitabine and araC, alone and in combination

The plasma concentration of troxacitabine and araC after a single i.v. administration at a dose of 20 mg/kg is shown in Fig. 4A. The Tmax values of the two drugs were both 2 min and the Cmax values 32.4 mg/l and 21.6 mg/l for araC and troxacitabine, respectively. In the case of araC, a rapid and high level of accumulation of the deaminated product, araU, was observed (Cmax 11.1 mg/l at 30 min).

Interestingly, both araC and troxacitabine had similar AUC_{total} (6.6 and 7.8 mg·h/l, respectively) and half-lives (22.5 and 16.2 min). However, araC had a larger volume of distribution at 1.2 l/kg compared to 0.72 l/kg for troxacitabine, but they had a comparable clearance (60 vs 51 ml/h for araC and troxacitabine, respectively). Overall these results demonstrate that araC and troxacitabine have similar pharmacokinetic characteristics and both are cleared rapidly but by different means, araC being deaminated while troxacitabine is cleared unchanged in the urine.

The effects of troxacitabine on the pharmacokinetics of araC were evaluated using two different protocols: troxacitabine was administered 20 min (Fig. 4B) or 3 h

Table 3 Troxacitabine and araC combination studies in the human T-lymphoblastic (CCRF-CEM) xenograft tumor model. Female SCID mice (5–6 weeks old) were injected i.p. with 3×10^{7} CCRF-CEM tumor cells. Treatment with troxacitabine and/or araC was started 20 days after tumor cell injection, once the mice had developed visible tumors at the site of inoculation (*ILS* increase in life span)

Group	Dose (mg/kg) (i.p., daily for 5 days)	Median survival (days)	ILS (%)	
			Compared with saline	Compared with araC
Control (saline)	–	37.0	–	–
AraC	10	49.5	34	–
	20	51.0	38	–
Troxacitabine	5	52.0	41	–
	10	53.5	44	–
AraC + troxacitabine	10 and 5	53.0	43	7
	10 and 10	58.0	57	17
	20 and 5	52.5	42	3
	20 and 10	57.0	54	12

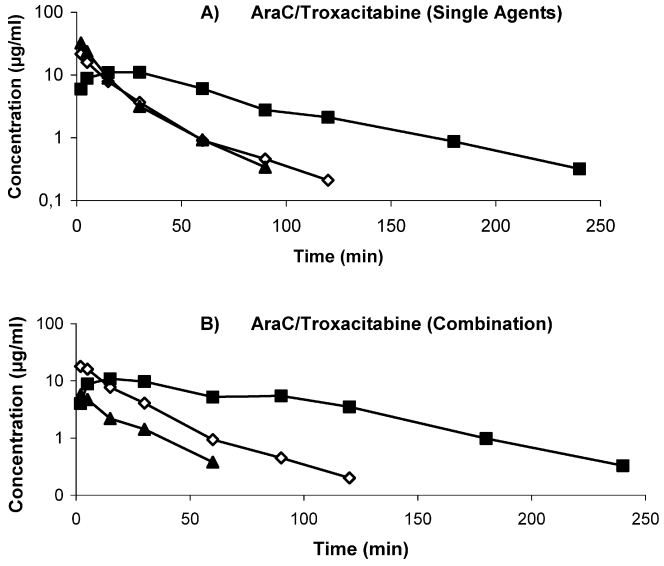

Fig. 4A, B Plasma concentration time curves of troxacitabine and araC, alone or in combination. The pharmacokinetics of troxacitabine, araC, and the combination of the two drugs were evaluated in female SCID mice ($n = 3$ per time point). **A** Treatment with a single dose of araC 20 mg/kg i.v. (protocol 1), or troxacitabine 20 mg/kg i.v. (protocol 2). **B** Treatment with troxacitabine 20 mg/kg i.v. followed, 20 min later, by araC 20 mg/kg i.v. (protocol 3). Plasma was collected by cardiac puncture at 2, 5, 15, 30, 60, 90, 120, 180, and 240 min following troxacitabine (protocol 2) or araC (protocols 1 and 3) (▲ troxacitabine, ◇ AraC, ■ AraU)

prior to araC. The deamination of araC was not influenced by administration of troxacitabine: the same amount of araU was present in both protocols (AUC_{total} 14.8 mg·h/l for araC alone compared to 16.4 and 17.3 mg·h/l when troxacitabine was injected first at 20 min and 3 h prior to araC, respectively). Also, we observed that troxacitabine did not influence the clearance, volume of distribution, or the half-life of araC ($t_{1/2}$ 19.9 and 22.2 min, when troxacitabine was administered 20 min and 3 h prior araC, respectively, compared to 22.5 min when araC was administered alone). Overall our results show that preadministration of troxacitabine did not influence the overall pharmacokinetic behavior of araC in SCID mice. Thus, these results demonstrate that the increased antitumor activity observed when araC and troxacitabine are combined is related to their respective cytotoxic effect and not due to changes in their pharmacokinetic profiles.

Discussion

Combination therapy, using chemotherapeutic agents with different modes of action, has been used extensively to improve response rates of cancer patients. Interestingly, new combination therapies are usually designed empirically based on the efficacy of single agents and with their lack of overlapping toxicities. Recently, Giles et al. have demonstrated that AML patients with refractory disease are responsive to troxacitabine even though they have been pretreated with araC [17, 19]. Also, in a phase I/II study it has been shown that combinations of troxacitabine with araC, idarubicin, or topotecan are active in patients with refractory leukemia: of 11 patients with CML-BP, 2 obtained a clinical complete response, one of which was from the araC/troxacitabine combination [20]. Also, in this study, of the 66 patients with relapsed AML treated with the combination of troxacitabine and araC, 4 were able to achieve a complete response with an additional 4 patients achieving hematological improvement [18]. We wanted to study the interaction between troxacitabine and araC in a leukemia model responsive to both agents in order to improve our understanding of why the combination of two cytosine analogues could be beneficial. For this reason, we chose the CCRF-CEM leukemia model since we had demonstrated previously that both agents are effective in this xenograft leukemia model [21].

In this study, we showed that combining araC and troxacitabine simultaneously in vitro resulted in synergy. We did observe some antagonistic effect (CIs > 1) with both drugs, but only at concentrations which by themselves caused $> 90\%$ cell kill. This could be explained by the limitation of the median-drug effect analysis method that we used, since this model does not allow evaluation of combinations that lead to complete growth inhibition or cell kill [35].

Since dCK is an important enzyme in the metabolism of pyrimidine analogues, we investigated its involvement in the metabolism of both troxacitabine and araC. dCK had a better affinity for araC than for troxacitabine (Km 7 μM and 28 μM, respectively; data not shown). Also, we observed that troxacitabine was not a substrate for thymidine kinase 1 and 2 (data not shown). The effect of the troxacitabineTP on the feedback inhibition of dCK was characterized. Feedback inhibition of dCK by dCTP has long been recognized as a mechanism to maintain an intracellular steady-state pool of dCTP [6, 28]. Our results show that phosphorylation of troxacitabine and araC by dCK was very sensitive to the feedback inhibition produced by dCTP. In fact, our experiments confirmed the observation that dCTP is the major player for the maintenance of the intracellular pool of nucleoside triphosphate and of the triphosphated analogues, araCTP and troxacitabineTP. Interestingly, our data showed that troxacitabineTP was a better feedback inhibitor than araCTP for the phosphorylation of araC and troxacitabine. However, troxacitabineTP remained a poor feedback inhibitor of dCK and would not likely limit the accumulation of araCTP or troxacitabineTP inside the cells.

The positive interactions between purine and pyrimidine nucleoside analogues have also been seen in several other studies [5, 11, 42]. Furthermore, the interaction between two pyrimidine-derived nucleoside analogues, gemcitabine and araC, has been studied both in vitro and in vivo [30, 40]. These studies demonstrated that the combination of gemcitabine and araC is synergistic

in vitro and also showed that the combination of these drugs is able to increase the life span of mice-bearing L1210 and P388 leukemia cells. Furthermore, in leukemia models their treatments gave the best results when gemcitabine was injected before araC, showing that gemcitabine is able to modulate the cytotoxic effect of araC. Indeed, the diphosphate form of gemcitabine, dFdC 5′-diphosphate, has shown the ability to inhibit ribonucleotide reductase [37]. Since dFdC 5′-diphosphate is able to decrease the pool of dCTP, and thus produce activation of dCK, it would be able to increase the phosphorylation of araC when used in combination. In fact, modulation of araC metabolism has been demonstrated with other inhibitors of ribonucleotide reductase in leukemia patients [13, 14]. On the other hand, since troxacitabine is unable to modulate the intracellular pools of dCTP or other nucleotides, we might not expect troxacitabine to increase the phosphorylation of araC inside the cells. We did indeed observe that there was a decrease in the overall accumulation of araC's metabolites in troxacitabine-treated cells. Thus, we cannot explain the additive effect of troxacitabine and araC in combination in CCRF-CEM cells by a higher accumulation of phosphorylated araC. The reduction in araC nucleotides in the presence of troxacitabine could be due to a decrease in araC uptake and/or competition for dCK. The main factor that might be contributing to the potentiation of the effects of these drugs would be their respective effect on DNA synthesis and repair.

Although both troxacitabine and araC inhibit DNA synthesis, their are also differences in the way they each can accomplish this action. Troxacitabine is a true chain terminator as opposed to araC, which can allow the incorporation of several nucleosides following its site of incorporation [23, 29, 32]. Also, there are differences that exist between the removal of incorporated troxacitabine and araC from the 3′ termini of DNA. A recent study has shown that a purified human apurinic/apyrimidic endonuclease is able to excise troxacitabine from DNA more efficiently than araC [3]. Furthermore, another study has demonstrated that a highly purified human cytosolic $3' \rightarrow 5'$ exonuclease is better at removing β-D-nucleoside from the 3′-end of DNA than β-L-nucleoside [33]. Thus, according to this latter study, araC would be a better substrate for this exonuclease than troxacitabine. Therefore, differences exist between araC and troxacitabine at the level of DNA synthesis and repair that could explain why their cytotoxic action could be complementary even though they share the same biochemical pathways. Interestingly, we observed that recovery of DNA synthesis was slower when both agents were used in combination in CCRF-CEM leukemia cells, and this could be a factor contributing to the synergy observed between these two agents in vitro.

In this study we demonstrated that the combination of troxacitabine and araC resulted in an increase in survival of mice-bearing CCRF-CEM leukemia cells. AraC at 10 mg/kg combined with troxacitabine at 10 mg/kg daily for 5 days gave an improvement of 17%, compared to treatment with araC alone, in the overall survival of mice. Also, araC at 20 mg/kg combined with troxacitabine at 10 mg/kg resulted in a 12% increase in the survival of mice compared to treatment with araC alone. Thus, the in vitro observation of a positive interaction between troxacitabine and araC was confirmed in vivo. Moreover, the combination of these two agents did not result in an increase in toxicity to the animals, which is an important consideration in the choice of any drug combination. Furthermore, the combination was beneficial in a fairly advanced disease model since treatment was started 20 days after intraperitoneal injection of the cells. Interestingly, this type of benefit is not related only to troxacitabine since it has been also observed with the combination of dFdC and araC in mouse leukemia models, suggesting that certain cytosine analogues could possess complementary mechanisms of action [30].

In conclusion, our CCRF-CEM leukemia model demonstrated a positive interaction between troxacitabine and araC in vitro and in vivo. Also, our data showed that the combination resulted in a slower recovery of cells from the DNA synthesis block created by the drugs when compared to each drug individually, and this might account for the better activity observed when the two agents are used in combination.

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