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ORIGINAL ARTICLE

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Comparative study of a novel nucleoside analogue (Troxatyl, troxacitabine, BCH-4556) and AraC against leukemic human tumor xenografts expressing high or low cytidine deaminase activity

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Abstract *Purpose*: Troxacitabine (β-L-dioxolane cytidine, BCH-4556; Troxatyl, BioChem Pharma Inc.) is a novel nucleoside analogue, which in experiments demonstrated potent antitumor activity against both leukemias and solid tumors. Since troxacitabine is a cytidine nucleoside analogue like AraC (1-β-D-arabinofuranosylcytosine), which is currently used in the treatment of acute myelogenous leukemia, we compared the in vivo antileukemic activity of troxacitabine with that of AraC in human leukemia xenograft models. *Methods*: The antiproliferative activity of troxacitabine and AraC was analyzed on hemapoietic cell lines by use of a thymidine incorporation assay. For in vivo studies, we compared troxacitabine with AraC by using equitotoxic schedules of the two nucleosides optimized for therapeutic activity. The antileukemic activity of both drugs was evaluated by measurement of their effect on the percent increased lifespan. *Results*: AraC had good in vitro antiproliferative activity ($IC_{50} = 14$ nM) but was ineffective in vivo against the HL60 promyelocyte leukemia cell line (treated vs control, T/C = 105%). Troxacitabine, which in contrast to AraC is not a substrate for cytidine deaminase, showed potent in vitro and in vivo activity in the same model ($IC_{50} = 53$ nM and T/C = 272% to 422%). The poor in vivo activity of AraC against HL60 leukemia cells could be due to the high cytidine deaminase (CDA; EC 3.5.4.5) activity in this cell line. This hypothesis was tested with CCRF-CEM T-lymphoblastoid leukemia cells which have undetectable levels of CDA activity. Short-term exposure of these leukemia cell lines to both drugs indicated that AraC was indeed significantly more effective in the CCRF-CEM cell line than in HL60. In contrast, the antiproliferative activity of troxacitabine was similar for both cell lines. These observations were extended to in vivo studies. Mice bearing CCRF-CEM tumor xenografts were treated with AraC and troxacitabine. In this model, T/C values were comparable for both drugs and ranged from 138% to 157%. *Conclusions*: Our findings indicate that troxacitabine is likely to be effective not only against solid tumors with high CDA activity but also in leukemias which have developed resistance to AraC due to increased CDA levels; this suggests that troxacitabine is a promising agent for the treatment of cancer. Indeed, significant antileukemic activity has been observed with troxacitabine in a phase I clinical trial in patients with primary refractory or relapsed acute myeloid leukemias (AML).

Key words Cytidine deaminase · Leukemia · Nucleosides · Troxacitabine · Xenografts

Abbreviations *AraC* 1-β-D-arabinofuranosylcytosine · *CDA* cytidine deaminase (EC 3.5.4.5) · *dCK* deoxycytidine kinase (EC 2.7.1.74) · *ILS* increased life span · *T/C* treated vs control

Introduction

AraC (1-β-D-arabinofuranosylcytosine, cytarabine) is part of standard therapy for acute myelogenous leukemia [1]. The cytotoxic action of AraC requires intracellular phosphorylation to form the active triphosphate nucleotide through a first rate-limiting step catalyzed by deoxycytidine kinase (dCK; EC 2.7.1.74) [10]. AraC has a relatively short half-life because of rapid deamination by cytidine deaminase (CDA; EC 3.5.4.5) leading to its uracil derivative which lacks antineoplastic activity [14]. Most leukemic patients eventually become refractory to AraC, and this leads to a poor prognosis. Resistance to AraC is likely to be multifactorial. In vitro, one mechanism of resistance to AraC seems to be a lack of dCK activity [3]. However, increased deamination, decreased

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influx or increased efflux, increased dCTP pools (by feedback inhibition of dCK), or decreased half-life of AraCTP have all been implicated [2].

With the discovery of β-L-1,3 oxathiolane cytosine (3TC, Glaxo Wellcome PLC) as a potent inhibitor of HIV-1 reverse transcriptase activity, it became evident that nucleoside analogues in the unnatural configuration were recognized and activated by cellular metabolic enzymes. This led us to evaluate a series of compounds in the L configuration and to observe that β-L-dioxolane cytidine (BCH-4556; troxacitabine; Troxatyl, BioChem Pharma) not only had antiviral, but also significant cytotoxic activity [12]. Recently, we and others have shown that troxacitabine has significant antitumor activity against experimental solid tumor xenograft models [9, 11, 16]. Grove and Cheng [8] have shown that troxacitabine is resistant to deamination. Therefore, we have compared the antileukemic activity of troxacitabine with that of AraC in cells having high or low CDA activity. Troxacitabine was as effective as AraC in human leukemia xenografts with low levels of CDA, but was significantly more effective than AraC in human leukemia xenografts having high levels of CDA. Our results indicate that troxacitabine may have clinical potential against solid tumors and leukemias that are AraC refractory due to high CDA activity.

Material and methods

Drugs

Troxacitabine was synthesized by BioChem Pharma [12] and AraC was obtained from Bristol-Myers Squibb (Montréal, Québec, Canada).

Cell culture and cytotoxicity studies

The human hematopoietic cell lines HL60 and CCRF-CEM were obtained from the American Type Culture Collection (ATCC; Rockville, Md.). These cell lines were routinely maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and incubated under humidified air with 5% CO_2 at 37 °C. Antibiotics were not used and cells were routinely checked for mycoplasma contamination by PCR analysis (Mycoplasma PCR detection kit, Stratagene; La Jolla, Calif.). For cytotoxicity studies, cells in logarithmic growth were plated in 96 well plates at a density of 10^4 cells per well. Cells were treated for either 1 h or with a continuous exposure to troxacitabine or AraC at different concentrations (final drug concentrations in media were in 10-fold increments ranging from 10^{-14} to 10^{-6} M). As a measure of proliferation, [*methyl*-^{3}H]thymidine (18.5 kBq per well, specific activity 7.4×10^{10} Bq/mmol; Amersham) was added to the cultures during the final 18 h of a total incubation period of 3 days. At the end of the incubation period, cells were aspirated directly onto glass-fiber filters. Membranes were dried and placed in plastic sample bags containing 6 ml of scintillation cocktail. The incorporation of [^{3}H]thymidine was measured with a β-scintillation counter (1450 Microbeta, Wallac, Finland). Results were expressed in percent relative to untreated cells. Each point represents a mean value of six measurements. The IC_{50} was estimated from individual inhibition curves.

In vivo studies

Female CB17/IcrIco-SCID mice (3–5 weeks old) were injected i.p. with 1.5×10^7 HL60 or 3.0×10^7 CCRF-CEM tumor cells. Treatment with troxacitabine or AraC was started 10 days after HL60 tumor cell injections or 20 days after CCRF-CEM tumor cell injections, once the mice had developed visible tumors at the site of inoculation. Tumor-bearing animals were randomized (12 per group) and treated by one of the following schemes: (a) control (saline i.p.); (b) troxacitabine (10 mg/kg per day i.p); (c) troxacitabine (25 mg/kg per day i.p.); (d) AraC (25 mg/kg per day i.p); and (e) AraC (100 mg/kg per day i.p). For both in vivo studies, treatment was given once a day for 5 consecutive days (days 10–14 for the HL60 study and days 20–24 for the CCRF-CEM study).

Results are expressed as percent of mean survival time of treated animals over mean survival time of the control group (treated vs control, T/C, %) and increased lifespan (mean survival time of treated animals minus that of control animals over the mean survival time of the control group; increased life span, ILS, %). By NCI criteria, T/C exceeding 125% and ILS exceeding 25% indicate that the drug has significant antitumor activity [15]. Animals in complete remission, free of detectable tumor, were killed at day 98 and were considered cured. Statistical analysis of the efficiency of the treatment was performed with the unpaired Student's *t* test (at $P < 0.05$ to $P < 0.001$).

All animals received humane care in compliance with the Canadian Council Guidelines on the care and use of experimental animals. These in vivo experimental protocols were approved by the animal care committee of BioChem Pharma; our animal facility is accredited by the Canadian Council for animal care.

Deoxycytidine kinase (dCK) and cytidine deaminase (CDA) assays

Enzyme activities were determined essentially as described by Ruiz van Haperen et al. [17, 18]. Cells were harvested, centrifuged, and cell pellets were stored at −70 °C until use (for up to 2 months). The cell pellets were thawed on ice and suspended in 0.3 M Tris-HCl, pH 8.0, containing 50 μM β-mercaptoethanol at a concentration of 25×10^6 cells per ml and centrifuged at 10,000 *g*. The protein content of the cellular extracts was quantified with the standard Bio-Rad Bradford protein assay. For dCK activity, 25 μl of enzyme cellular extract was incubated at 37 °C for up to 90 min with an equal volume of substrate mixture composed of two volumes of 50 mM ATP in 25 mM $MgCl_2$, pH 7.4; two volumes of [5-^{3}H]deoxycytidine (50 μM deoxycytidine containing 226.5 kBq of [5-^{3}H]deoxycytidine); and one volume of Tris-HCl buffer. The substrate (deoxycytidine) was separated from the product (dCMP) by thin layer chromatography and was quantified as described previously [17]. Experiments were performed with and without 1 mM thymidine to assess for mitochondrial TK2 contribution in dCK activity. For CDA activity, 100 μl of the cellular extract was incubated at 37 °C for up to 60 min in the presence of 20 μl of 5 mM deoxycytidine and 80 μl of CDA buffer (0.1 M Tris-HCl, pH 8.0 containing 50 μM β-mercaptoethanol). The substrate (deoxycytidine) was separated from the product (uridine) by HPLC as previously described [17].

Results and discussion

The in vitro antiproliferative activity of troxacitabine was compared to that of AraC (Table 1). AraC was slightly more potent than troxacitabine in inhibiting the proliferation of the two human leukemic cell lines tested after 72 h continuous exposure. The IC_{50} for AraC in the HL60 cell line was 14.6 nM, compared to 53 nM for troxacitabine. In the CCRF-CEM cell line, these values

Table 1 Comparative in vitro antiproliferative activity of troxacitabine and AraC in HL60 promyelocyte leukemia and CCRF-CEM T-lymphoblastoid cell lines

Cell line	Troxacitabine (IC_{50}, nM)[a]	AraC(IC_{50}, nM)
HL60, 1 h	362 ± 74	581 ± 223
HL60, 72 h	53.2 ± 10.9	14.6 ± 3.4
CEM, 1 h	510 ± 162	33 ± 0.24
CEM, 72 h	13.8 ± 5.9	4.6 ± 0.9

[a] IC_{50} was measured after 1 h or 72 h exposure to drugs. Antiproliferative activity of the drugs was determined by adding [^{3}H]thymidine during the final 18 h of a total incubation period of 3 days. The values represent the means ± SD of two experiments where each data point is an average of six measurements

were 4.6 nM and 13.8 nM, respectively. The antitumor activity of the two deoxycytidine nucleoside analogues was compared in vivo by use of equitoxic treatment regimens.

In the first study, HL60 cells were inoculated into SCID mice and treatment with AraC (25 or 100 mg/kg/day × 5, i.p.) or troxacitabine (10 and 25 mg/kg/day × 5, i.p.) was started 10 days after tumor inoculation, once animals had developed disseminated tumors (survival curves are depicted in Fig. 1, top panel, and the results are summarized in Table 2). All of the saline control group mice died between days 17 and 18 after tumor inoculation. At the time of death, SCID mice with leukemia were noted to have massive hepatosplenomegaly, enlarged kidneys, and large masses in the pelvic fat. There was generalized enlargement of peritoneal and mediastinal lymph nodes. The mice developed large amounts of viscous and bloody ascites.

AraC treatment at 25 mg/kg (once a day for 5 days) had no effect whereas the 100 mg/kg dose resulted in a slight delay in mortality (average survival time of 23 days compared to 18 days for the control group). Out of 12 animals, two survived for longer periods (41 and 51 days), but at the time of death had ascites and small tumors located in the intestine. However, T/C and ILS values for this group were not increased to statistically significant levels at 105% and 5.6%, respectively. Troxacitabine was very effective at the two doses tested. Average survivals of 50 and 79 days were obtained at 10 and 25 mg/kg dose regimens, respectively. A T/C of 272% and ILS of 172% were achieved in the 10 mg/kg troxacitabine treated group. These values were 422% and 322%, respectively, for the 25 mg/kg treated group. In the 25 mg/kg dose regimen, five animals out of 12 had complete remissions (no detectable tumor) when killed.

In the second study, CCRF-CEM cells were inoculated into SCID mice and treatment with AraC (at 25 or 100 mg/kg/day × 5, i.p.) or troxacitabine (at 10 and 25 mg/kg/day × 5, i.p.) started after 20 days, once animals had developed disseminated tumors (survival curves are depicted in Fig. 1, bottom panel, and the results are summarized in Table 2). All of the saline control group mice died between days 34 and 54 (average survival time of 40 days) after tumor inoculation.

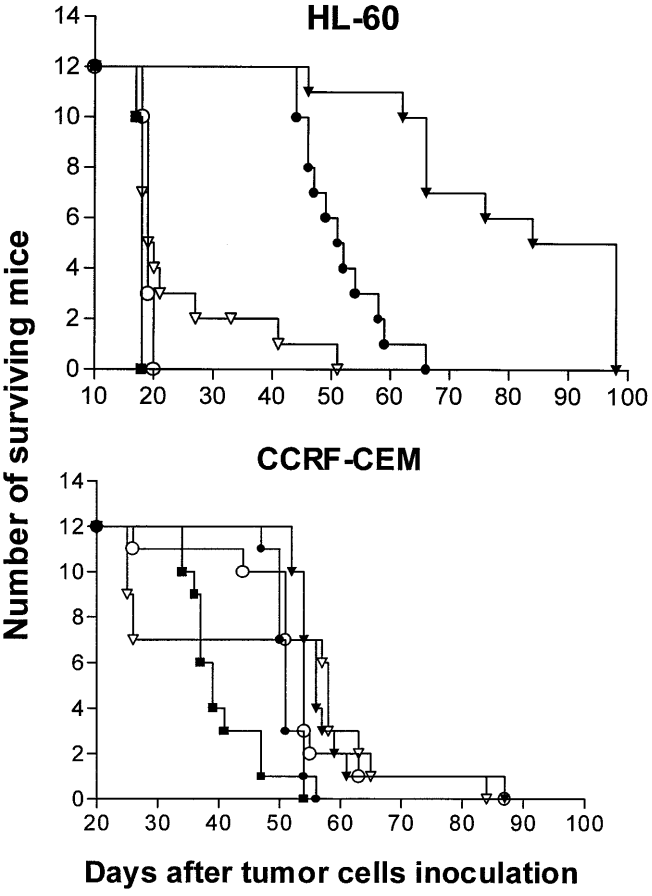

Fig. 1 Comparative effects of troxacitabine and AraC on life span of mice bearing leukemias. *Top panel:* Female CB17/IcrIco-SCID were injected i.p. with 1.5×10^7 HL60 tumor cells (day 0). Treatment with troxacitabine or AraC was started 10 days after tumor cell inoculation, for 5 consecutive days: ■ saline (i.p.); ○ AraC (25 mg/kg/day, i.p.); ▽ AraC (100 mg/kg/day, i.p.); ● troxacitabine (10 mg/kg/day, i.p.); and ▼ troxacitabine (25 mg/kg/day, i.p.). *Bottom panel:* Female CB17/IcrIco-SCID were inoculated i.p. with 3.0×10^7 CCRF-CEM tumor cells (day 0). Treatment with troxacitabine or AraC was started 20 days after tumor cell inoculation, for 5 consecutive days: ■ saline (i.p.); ○ AraC (25 mg/kg/day, i.p.); ▽ AraC (100 mg/kg/day, i.p.); ● troxacitabine (10 mg/kg/day, i.p.); and ▼ troxacitabine (25 mg/kg/day, i.p.)

AraC was effective in this model. T/C values of 146% and 157% and ILS values of 46% and 57% were obtained in the groups treated with 25 mg/kg and 100 mg/kg, respectively. Troxacitabine was equally active. At the low dose of 10 mg/kg per day, a T/C of 138% with an ILS of 38% was obtained. A dose of 25 mg/kg per day of either AraC or troxacitabine gave very similar T/C and ILS values (approximately 150% and 50%, respectively; Table 2). At day 87, the experiment was terminated and one animal in each of the higher-dose treatments of troxacitabine and AraC had major remissions (these mice had very little ascites and no other sign of tumor mass in their kidneys, intestines, or liver).

To compare the toxicity profiles of AraC and troxacitabine, we administered 25, 50, 100, and 200 mg/kg per day for 5 days to non-tumor-bearing SCID mice. None of the animals in the AraC-treated groups lost weight,

Table 2 Comparative in vivo antitumor activity of troxacitabine and AraC in HL60 promyelocyte and CCRF-CEM T-lymphoblastoid human leukemia xenografts

Groups	Cell lines innoculated	Mice per group	Dose and route	Schedule	Range survival time (days)	Median survival time (days)	ILS (%)	T/C (%)
Control	HL60	12	Saline/i.p.	qd × 5	17–19	18		
Troxacitabine	HL60	12	10 mg/kg/i.p.	qd × 5	44–66	49	172	272
Troxacitabine	HL60	12	25 m/kg/i.p.	qd × 5	44–98	76	322	422
AraC	HL60	12	25 mg/kg/i.p.	qd × 5	17–20	19	5.6	106
AraC	HL60	12	100 mg/kg/i.p.	qd × 5	17–51	19	5.6	106
Control	CCRF-CEM	12	Saline/i.p.	qd × 5	34–54	37		
Troxacitabine	CCRF-CEM	12	10 mg/kg/i.p.	qd × 5	47–56	51	37.8	138
Troxacitabine	CCRF-CEM	12	25 mg/kg/i.p.	qd × 5	52–87	56	51.4	152
AraC	CCRF-CEM	12	25 mg/kg/i.p.	qd × 5	44–87	54	45.9	146
AraC	CCRF-CEM	12	100 mg/kg/i.p.	qd × 5	25–82	58	56.8	157

Female CB17/IcrIco-SCID mice (3–5 weeks old) were injected i.p. with 1.5×10^7 HL60 or 3.0×10^7 CCRF-CEM tumor cells. Treatment with troxacitabine or AraC was started 10 days after HL60 tumor cell injections or 20 days after CCRF-CEM tumor cell injections, once mice had developed visible tumors at the site of inoculation
T/C, treated vs control; ILS, increased life span

but at 200 mg/kg per day the animals did not gain weight during the treatment period and had gained between 1 to 2 g after 11 days. Troxacitabine did not cause weight loss at 25 and 50 mg/kg per day, but some weight loss was observed at 100 mg/kg per day and daily treatment with 200 mg/kg was lethal after 8 days. These results suggest that the two compounds have comparable toxicities.

AraC and troxacitabine must be phosphorylated to the triphosphate for incorporation into the DNA chain and to inhibit DNA synthesis [7]. The rate-limiting step in the phosphorylation cascade is the conversion to the monophosphate by dCK [10]. On the other hand, CDA catalyzes the inactivation of deoxycytidine and analogues to their deaminated products. Deamination of AraC leads to AraU (1-D-arabinofuranosyluridine) which lacks antitumor activity [14]. Troxacitabine, on the other hand, is resistant to deamination [8]. The levels of activity of these two enzymes may therefore be important in determining the amount of active phosphorylated metabolites formed. Deoxycytidine kinase and cytidine deaminase activities were measured in the two cell lines (values are reported in Table 3). As expected for leukemic cell lines, CCRF-CEM cells had high levels of dCK activity and undetectable, by HPLC, levels of CDA activity. Interestingly, HL60 had a profile of activity reminiscent of solid tumors, which, in general, have high levels of CDA activity [17]. This could explain the lack of antitumor activity of AraC in the HL60 tumor model compared to its significant activity in the CCRF-CEM tumor model (this report) as well as in L1210 and P388 leukemic models [20].

Table 3 Deoxycytidine kinase (dCK) and cytidine deaminase (CDA) activities[a]

Cell line	Activity[a]		
	dCK + TK2	dCK	CDA
HL60	2.88 ± 0.4	0.74 ± 0.3	304 ± 14
CCRF-CEM	7.48 ± 3.22	5.85 ± 2.9	ND[b]

[a] Activities are expressed as nmole product formed/h/mg protein and are the means of three experiments ± SD
[b] ND, not detected by HPLC

To evaluate if high CDA activity interferes with AraC efficacy, we compared the in vitro antiproliferative activity of both drugs after a short exposure (1 h, Table 1). A short exposure is likely to reflect the in vivo situation where compounds are metabolized and excreted. In the HL60 cell line, troxacitabine was slightly more potent, having an IC_{50} value of 362 nM while that of AraC was 581 nM. In contrast, in the CCRF-CEM cell line, AraC was clearly more effective (IC_{50} values of 33 nM compared to 510 nM for troxacitabine). This indicates that in the presence of undetectable or low CDA activity AraC is quite effective and this may explain the low activity of AraC in solid tumors, which in general have high CDA levels [13, 17]. This difference was not observed with a continuous exposure of AraC, due to the continuous supply of unmetabolized compound. Since troxacitabine is resistant to deamination, it remains potent in tumor lines with high CDA activity. Interestingly, the antiproliferative activity of 2′-deoxy-2′-methylidenecytidine (DMDC), a new antitumor nucleoside resistant to deamination, is also modulated by cytidine deaminase activity [4, 13].

Troxacitabine is a potent antitumor compound. Not only did we observe significant antileukemic activity as well as complete cures in the HL60 xenograft model, but previous studies demonstrated potent antitumor activity in several solid tumor xenograft models [9, 11, 16]. Furthermore, when compared to gemcitabine, troxacitabine was significantly more effective in the Panc-01 xenograft model [19]. Gemcitabine, which is also a substrate of CDA, although with less affinity than AraC [10], was shown to be less effective in several tumors with high CDA levels [13]. These observations, combined with our results, suggest a therapeutic advantage of troxacitabine over current clinical nucleoside analogues.

Troxacitabine has been studied in a phase I clinical trial in patients with acute myelogenous leukemia; it was

given as an intravenous infusion over 30 min daily for 5 days every 3–4 weeks [5]. Results suggest that troxacitabine had significant antileukemic activity in AraC-refractory patients and is now being evaluated in phase II. Six pilot phase II trials are also being performed in different solid tumors.

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