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Synergistic anti-tumor activity of a novel immunomodulator, BCH-1393, in combination with cyclophosphamide

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Abstract

N,*N*-dimethylaminopurine pentoxycarbonyl D-arginine (BCH-1393) is a novel low molecular weight synthetic immunomodulator that has been shown to significantly stimulate cytotoxic T-lymphocyte responses both in vitro and in vivo (Zacharie B, Gagnon L, Attardo G, Connolly TP, St-Denis Y, Penney CL. Synthesis and activity of 6-substituted purine linker amine immunostimulants. J. Med. Chem. 1997;40:2883–94). Prompted by this evidence, we extended evaluation of BCH-1393 for anticancer activity in syngeneic mouse experimental tumor models. Consistent with previous findings, in vitro assessment of BCH-1393 activity demonstrated a significant increase in the CTL responses in the range of 10^{-9}–10^{-5} M. Treatment of mice with four consecutive daily intraperitoneal injections at 25 and 50 mg/kg resulted in a significant increase of the relative percentage of blood CD4+, CD8+, NK and monocyte subsets without any evidence of toxicity. In vivo anti-tumor activity of BCH-1393 was evaluated, either alone or in combination with subtherapeutic doses of cyclophosphamide (Cy), against weakly immunogenic mouse breast carcinoma DA-3 and strongly immunogenic colon adenocarcinoma MC38. Daily intraperitoneal injection of BCH-1393 at 50 mg/kg alone was well tolerated but produced a relatively weak anti-tumor effect in both tumor models. However, a significant inhibition of tumor outgrowth and suppression of established tumor growth was observed when BCH-1393 was administered in combination with subtherapeutic doses of Cy. Combination treatment of 50 mg/kg BCH-1393 with 100 mg/kg Cy (given as single intravenous bolus injection) starting 2 days prior to DA-3 tumor cell inoculation prevented tumor outgrowth in 70–80% of treated mice. In the remaining 20–30% of mice that had developed tumors, a nearly complete (90%) tumor growth inhibition was observed at days 22–24 post tumor implant. In the MC38 tumor model, combination treatment of established tumors with BCH-1393 and Cy (CTX) at 50 mg/kg resulted in a significant delay in tumor growth compared to CTX treatment alone. The

Abbreviations: CTL, cytotoxic T-lymphocytes; MLR, mixed lymphocyte reaction; LAK, lymphokine activated killer cells; IL-2, interleukin 2; MIMP, methyl inosine monophosphate; TP5, thymopentin; Cy, cyclophosphamide; CTX, cytoxan; FITC, fluorescein; PE, phycoerythrin; s.c., subcutaneous; i.p., intraperitoneal; I.V., intravenous; T/C, treatment over control.

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observed concomitant anti-tumor activity of BCH-1393 with cyclophosphamide warrants further investigation of this immunomodulator as an adjunctive treatment of cancer.

Keywords: Immunomodulator; Adjunctive cancer treatment; Breast; Colon carcinoma; Murine tumor models

1. Introduction

The current direction of cancer immunotherapy research is to identify more effective and less toxic treatment modalities. The main efforts have centered around the use of a combination of biological response modifiers and cytotoxic agents [1,2], adoptive transfer of cytotoxic lymphocytes [3,4] and more recently gene therapy [5,6] and dendritic cell based immunotherapy [7,8]. However, these modalities are expensive, difficult to administer and remain mostly unproven. Another approach has been to combine immunotherapy with existing standard treatment modalities such as whole-body irradiation or systemic chemotherapy [9,10]. Overall, these studies indicate a statistically significant benefit of the combined chemo-immunotherapy regimen compared to either therapy alone [11].

The use of low dose Cy has gained therapeutic acceptance as a means to down-regulate suppressor cells and thereby enhance the activity of a CTL stimulant [12,13]. Clinical trials utilizing low-dose Cy with various cytokines such as rIL-2 or IFN-γ are currently well accepted and have shown efficacy in the treatment of advanced melanoma and renal cell carcinoma [12,13]. Such therapy can lead to increased LAK and T-cell activation in responding renal cell carcinoma patients [14] and induce a durable complete response in metastatic melanoma patients [15].

A large number of immunomodulators have been used in experimental models and human trials as agents that activate non-specific immune mechanisms. These include bacterial preparations such as BCG, *C. parvum* or streptococcal extracts [16–18], viral products [19,20] and interferon inducers such as bropiramine [21,22] or nucleosides such as 7-thia-8-oxoguanosine [23] and 7-allyl-8-oxoguanosine (loxoribine) [24]. A number of immunomodulators can also stimulate a specific immune response acting primarily on the T-cell lineage in a manner similar to thymic hormones and are so classified as 'thymomimetics'. Among this class of molecules, levamisole, methyl inosine monophosphate (MIMP), thymopentin (TP5) and tucaresol have demonstrated significant anti-tumor activity and are now in clinical trials [25–27].

In an effort to further enhance the immunostimulatory activity, a synthetic purine derivative BCH-1393 (*N*,*N*-dimethylaminopurine pentoxycarbonyl D-arginine) was developed and tested for T-cell immune stimulation [28]. Herein, we show that BCH-1393 (Fig. 1) exhibits potent activation of murine CD8+ cytotoxic T-lymphocytes (CTL) and inhibits growth of syngeneic mouse breast and colon carcinomas when coadministered with Cy.

2. Experimental procedures

2.1. Therapeutic agents

BCH-1393 (*N*,*N*-dimethylaminopurine pentoxycarbonyl D-arginine) was synthesized as described [28] at Biochem Pharma. Cyclophosphamide (Cy) was purchased from Sigma, Canada. Cytoxan (CTX: cyclophosphamide for injection, U.S.P. with mannitol) was purchased from Bristol Laboratories of Canada. 5-Fluorouracil (5-FU) was purchased from David Bull Laboratories, Montreal, Canada.

2.2. Cytotoxic T-lymphocyte/mixed lymphocyte reaction (CTL/MLR) assay

Splenocyte cell suspensions were prepared by homogenization of spleens from 6–8-week old female C57BL/6 (H-2[b]) and DBA/2 (H-2[d]) mice in tissue culture medium (RPMI-1640) supplemented with 10% fetal calf serum and 2 mM L-glutamine. The homogenate was centrifuged on a density gradient (Lympholyte M, Cedarlane) (500 × g, 20 min), and mononuclear leukocytes were collected and washed three times in phosphate buffered saline (PBS) and resuspended in RPMI for evaluation of cell viability by trypan blue exclusion. DBA/2 spleen cells were irradiated (3000 rads) for the unidirectional MLR and for priming CTL response. The assay was performed as follows: splenocytes (10^7 cells from C57BL/6 and DBA/2 mice) were incubated in a 5% CO_2 humidified incubator at 37°C for 5 days, collected and an aliquot was pulsed with 0.1 μCi of [^{3}H] thymidine (Amersham, Oakville, Ontario). After 6 h these cells were collected by filtration, and newly synthesized DNA was measured by determination of the uptake of tritiated thymidine, measured by scintillation counting.

Cells were simultaneously assayed for cell-mediated cytolysis by incubation of 5×10^5 cells from the culture with sodium chromate labeled $Na_2{}^{51}CrO_4$; (Amersham, Oakville, Ontario) P815 mastocytoma cells (5×10^3 cells) for 4 h. After the incubation, chromium in the supernatant was quantified and the percent specific lysis calculated using the expression:

$$\%\text{Specific Lysis} = \frac{(\text{ER} - \text{SR})}{(\text{TR} - \text{SR})} \times 100\%$$

where ER = Cpm experimental release; TR = Cpm total release; SR = Cpm spontaneous release.

2.3. Immunophenotyping assay

Female, 6–8-week old, C57BL/6 mice were injected i.p. for one or four consecutive days with BCH-1393 at different concentrations. Immunophenotyping was also performed on immunosuppressed animals. Immunosuppression was achieved with 80 mg/kg of 5-fluorouracil (5-FU) or 100 mg/kg of cyclophosphamide (Cy) injected i.p. on day 0. Mice were sacrificed on day 5 by cardiac puncture. Gross pathological observations were recorded at the end of the experiment. Blood and spleen were collected and cell suspension prepared and lysed in ACK buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3) for 5 min. The cells were washed three times in phosphate buffered saline, pH 7.4 (PBS) and resuspended in tissue culture medium. The cells were then incubated for 45 min on ice with fluorescein (FITC) or phycoerythrin (PE) conjugated cell surface markers according to the manufacturers (Gibco/BRL, Cedarlane, Boehringer Mannheim) recommendation. The cells were then washed in PBS, fixed with 1% paraformaldehyde and analyzed with a Coulter XL flow cytometer. Analysis of the cell subsets was undertaken by determination of standard cell surface markers which were as follows: CD3 (T-cells), TCR (T-cell receptor), CD4 (T helper),

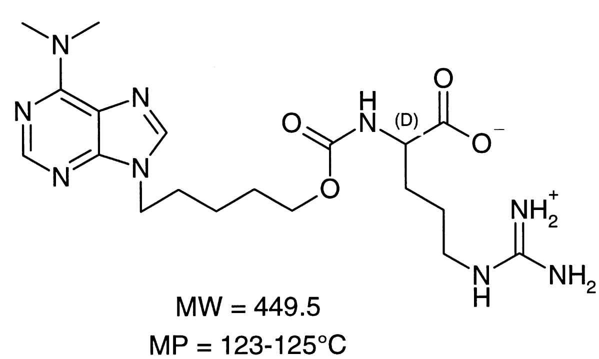

Fig. 1. Structure of the immunomodulator *N*,*N*-dimethylaminopurine pentoxycarbonyl D-arginine (BCH-1393).

CD8 (T cytotoxic/suppressor), CD45 (tyrosine phosphatase; activation marker), CD11b (macrophage), NK (NK cells) and Ly5 (B-cells).

2.4. Animals

Six to 8-week old female C57BL/6 and BALB/c mice were purchased from Charles River Canada (Montreal, Quebec, Canada). Mice were housed five per cage in a pathogen-free animal facility and fed standard mouse chow with tap water to drink, ad libitum.

2.5. Tumors and tumor cell culture

The mouse mammary adenocarcinoma (DA-3) was kindly provided by Dr Diane Lopez, Miami School of Medicine, Miami, Florida. This tumor arose from a preneoplastic lesion treated with 7,12-dimethylbenzanthracene in female BALB/c mice. MC38 colon adenocarcinoma was induced by 1,2-dimethylhydrazine in C57BL/6 mice and was kindly provided by Dr James Young (National Cancer Institute, Bethesda, MD). Both DA-3 and MC38 cell lines were maintained as monolayer cultures in plastic flasks in RPMI-1640 containing 0.1 mM nonessential amino acids, 0.1 mM sodium pyruvate, 2 mM L-glutamine, and 100 μg/ml gentamicin sulfate. This was further supplemented with 50 μM 2-mercaptoethanol and 10% fetal bovine serum. The DA-3 tumors were serially passaged in vivo by s.c. inoculation of 5×10^5 viable tumor cells to produce localized tumors in BALB/c mice. Solid MC38 tumors were produced by s.c. injection of 5×10^5 viable tumor cells in C57BL/6 mice.

2.6. Tumor implantation and treatment validation

The respective tumor cell lines were passaged in vitro and single cell suspensions were prepared from tissue culture flasks by trypsinization. Tumor cells were adjusted to the required concentration and injected s.c. in 0.1 ml volume into the shaved flank region. The animals were then serially monitored by manual palpation for the evidence of tumor nodule. Serial tumor volumes were obtained by bi-dimensional diameter measurements with calipers, using the formula $0.4\ (a \times b^2)$ where 'a' was the major tumor diameter and 'b' the minor perpendicular diameter. In general, tumors were palpable 5–7 days post-inoculation. In the animals where treatment resulted in complete tumor regression, tumors were considered below detection level and were measured as 1×1 mm for two perpendicular diameter sizes. Those values were averaged with the values for the animals that did develop tumors.

2.7. Efficacy evaluation in the DA-3 breast carcinoma model

Two treatment regimens (Programs A and B) were used for anti-tumor efficacy evaluation of BCH-1393 in combination with Cy in the DA-3 tumor model. BALB/c mice were injected s.c. with tumor cells on day 0 (D0). Treatment with BCH-1393 (25 and 50 mg/kg, i.p.) was done either alone or following pretreatment with 100 mg/kg Cy administered i.v. at D0 as single bolus injection. BCH-1393 was given on days D-2, D-1, D0, D1 and then continued either daily (D1–D21: program B) or every second day (D1–D21: program A). Mice were monitored for tumor incidence, tumor size and body weights three times per week for 3 weeks from D0 until D36.

2.8. Efficacy evaluation in the MC38 colon carcinoma model

Treatment schedule for anti-tumor efficacy evaluation of BCH-1393 and Cytoxan (CTX) in the MC38 colon carcinoma model was carried out against established solid tumors that developed 5 days following s.c. injection of C57BL/6 female mice with 5×10^5 MC38 tumor cells. BCH-1393 was given at 50 mg/kg i.p. on day 5 when palpable tumors were first evident and continued until day 28. The treatment was given daily either alone or in combination with CTX which was administered at 50 mg/kg i.v. on days 7 and 15, respectively, post tumor cell inoculation.

2.9. Statistics

The immunophenotyping data were presented as mean % cells positive ± STD. The means were compared using unpaired Student's *t*-test. The differences were considered significant at $p \leq 0.05$. Tumor volumes of control and treatment groups were analyzed using Student's *t* test at $p < 0.05$–0.001. The % T/C was calculated as the ratio of mean tumor volume at termination date in the treatment group divided by the respective volume in the control group expressed as a percentage. By NCI criteria the product is considered effective if % T/C is ≤ 40%.

3. Results

3.1. Immunological effects of BCH-1393

In vitro assessment of BCH-1393 immunostimulatory effect on murine splenocytes was undertaken using CTL/MLR, mitogenicity and NK-cell assays. In vitro stimulation of splenocytes with BCH-1393 resulted in a significant increase of CTLs in a mixed lymphocyte reaction. The dose response profile for BCH-1393 stimulation is shown in Fig. 2. Maximum augmentation of CTL effect was achieved at 10^{-7} M concentration of BCH-1393 resulting in over 80% increase in antigen-specific CTL activity against P815 target cells ($p < 0.05$ vs non-stimulated group). Treatment with BCH-1393 did not result in significant stimulation of NK and B-cells, but moderately enhanced T-cell proliferative activity when used in the presence of concanavalin A (data not shown).

In view of the significant in vitro stimulatory activity on CTLs by BCH-1393, it was of interest to determine if this activity would translate into an in vivo effect. Therefore, immunophenotyping

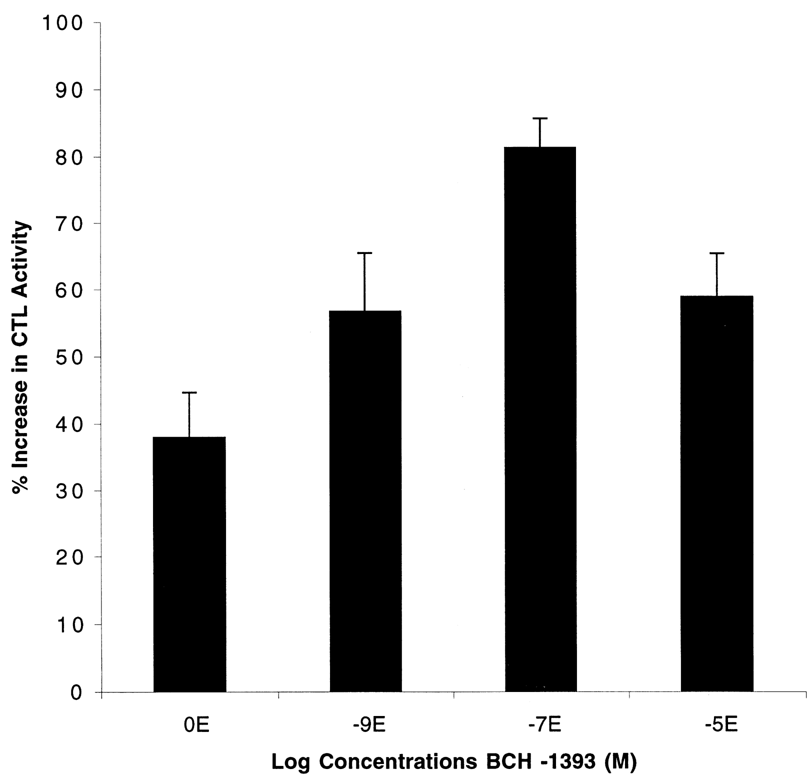

Fig. 2. Cytotoxic T-lymphocyte/mixed lymphocyte reaction (CTL/MLR) assay. Dose dependent augmentation of C57 BL/6 splenocyte CTL activity against P815 tumor cell targets by BCH-1393 (10^{-5}–10^{-9} M).

experiments were undertaken utilizing normal status or immunosuppressed mice, in the presence or absence of BCH-1393, to determine the increase or decrease of immune cell subset populations, *relative to each other*. The results of the analysis are presented in Tables 1–3. In normal non-immunosuppressed mice, BCH-1393 significantly increased the relative percentage of CTL (CD8+ CD45+, $p < 0.041$), NK (CD3+ or CD3−, $p < 0.021$) and monocyte (CD11b+, $p < 0.015$) populations of the peripheral blood (Table 1). Assessment of cell populations in the spleen showed an increase in the relative percentage of TCR (T-cells, $p < 0.035$) and a decrease of Ly5 (B-cells, $p < 0.034$) cell subsets (Table 1).

To achieve immunosuppression, a single bolus injection of high dose 5-FU at 80 mg/kg or Cy at 100 mg/kg was administered i.p. on day 0 1 h prior to BCH-1393 injection. In the 5-FU-immunosuppressed mice, BCH-1393 significantly increased the CD8+ CD45+ ($p < 0.022$) and NK+ ($p < 0.01$) cells in the peripheral blood (Table 2). Splenocyte immunophenotyping analysis showed a significant increase in CD4+ ($p < 0.04$) and a significant decrease in NK+ ($p < 0.001$) cell subtypes. Furthermore, a CD8+ CD45+ augmentation in the peripheral blood ($p < 0.05$) was also observed in the Cy-immunosuppressed mice (Table 3).

Table 1
Summary of BCH-1393 immunophenotyping data, four doses ($n = 10$)

Cell subsets		0 mg/kg	25 mg/kg	50 mg/kg
Blood immunophenotyping BCH-1393[a]				
CD8+ CD45+	Mean	8.25	11.7	10.1
	STD	1.59	5.54	2.16
	p value		0.108	0.041
NK+ CD3−	Mean	6.01	5.90	8.14
	STD	0.98	1.39	1.35
	p value		0.500	0.005
NK+ CD3+	Mean	3.43	5.84	3.25
	STD	0.76	2.08	0.57
	p value		0.021	0.289
CD4− CD11b+	Mean	9.60	13.7	9.68
	STD	2.79	2.68	3.59
	p value		0.015	0.404
Spleen immunophenotyping BCH-1393				
TCR	Mean	38.9	39.8	45.1
	STD	3.83	7.61	7.34
	p value		0.421	0.035
Ly5	Mean	55.5	54.1	50.4
	STD	3.44	7.30	6.72
	p value		0.367	0.034

[a] Groups of 10 C57BL/6 mice were injected i.p. for four consecutive days with BCH-1393.

3.2. Anti-tumor effects of BCH-1393

To confirm that BCH-1393 does not produce direct cytotoxicity against tumor cells, an MTT assay [29] was undertaken utilizing a number of mouse and human tumor cell lines (DA-3, MC38, B16-F10, KB, KBV and HT-29) in the presence of BCH-1393 at various concentrations. No cytotoxicity effect was observed at concentrations of up to 100 μM BCH-1393 (data not shown).

In vivo assessment of BCH-1393 anti-tumor effect in the DA-3 breast carcinoma model was carried out either alone or in combination with subtherapeutic dose of Cy. Our results showed that repeated treatment with 50 mg/kg i.p. in combination with Cy (single bolus i.v. injection of 100 mg/kg at D0) resulted in both significant inhibition of tumor outgrowth and subsequent retardation of established tumor growth. As shown in Table 4, a significant reduction in tumor incidence ($p < 0.005$) was observed in BCH-1393 and Cy treated mice compared to control-untreated mice (2/10 vs 10/10, respectively) using both treatment regimens (programs A and B) at day 22 post tumor cell implantation. Treatment of mice with similar doses of either BCH-1393 or Cy alone did not result in a significant reduction of tumor incidence, indicating a synergistic effect between the two agents. Furthermore, in addition to the significant delay in tumor outgrowth in those mice that had developed tumors, a rapid and complete tumor regression occurred from the time of tumor onset at days 4 and 5 till day 10 after tumor cell inoculation. Eight out of 10 (80%) and 7 out of 10 (70%) animals remained tumor free by day 24, respectively, in each of treatment regimens A and B (Figs. 3 and 4). Treatment with BCH-1393 alone at 25 or 50

Table 2
Summary of BCH-1393 immunophenotyping data, 5-fluorouracil + BCH-1393 ($n = 10$)

Cell subsets		5-FU alone (80 mg/kg)	5-FU + BCH-1393 (25 mg/kg)	5-FU + BCH-1393 (50 mg/kg)
Blood immunophenotyping BCH-1393[a]				
CD8+, CD45+	Mean	6.66	10.1	8.65
	STD	2.09	2.69	1.39
	p value		0.005	0.022
NK+	Mean	3.24	3.58	4.12
	STD	0.66	1.01	0.74
	p value		0.38	0.01
Spleen immunophenotyping BCH-1393				
CD4+, CD45−	Mean	10.0	13.2	12.1
	STD	1.98	3.19	2.27
	p value		0.015	0.04
NK+	Mean	4.22	3.32	3.17
	STD	0.50	0.45	0.36
	p value		< 0.001	< 0.001

[a] Groups of 10 immunosuppressed C57BL/6 mice (80 mg/kg 5-FU, i.p.) were injected i.p. for four consecutive days with BCH-1393.

mg/kg did not result in a significant anti-tumor effect with 10 out of 10 (100%) animals developing large tumors by day 22, respectively, for both doses. Treatment with Cy alone given at 100 mg/kg as a single bolus i.v. injection also did not result in a significant anti-tumor effect (Figs. 3 and 4). As shown in Figs. 3 and 4, DA-3 tumors in groups 7 and 8 slowly resumed growth after day 24 when treatment with BCH-1393 was discontinued, indicating a requirement for repeated treatment with BCH-1393 to sustain complete tumor growth regression. These results demonstrate a highly potent anti-tumor activity of BCH-1393 when co-administered in combination with Cy against DA-3 breast carcinoma.

Efficacy validation of BCH-1393 and CTX in the MC38 colon carcinoma model also revealed a potent anti-tumor activity. Results in Fig. 5 show that tumor-bearing mice injected daily with BCH-1393 (at 50 mg/kg on days 4–28) in combination with CTX (at 50 mg/kg given twice on days 7 and 15) showed a highly significant delay in tumor progression compared to control saline-treated mice ($p = 0.0009$; T/C = 13%). Treatment with CTX alone also resulted in tumor growth inhibition but was less effective ($p = 0.01$; T/C = 34%), whereas BCH-1393 given alone at 50 mg/kg was completely ineffective against early established MC38 tumors.

Table 3
Summary of BCH-1393 immunophenotyping data cyclophosphamide + BCH-1393 ($n = 3$)

Cell subsets		CY alone (100 mg/kg)	CY + BCH-1393 (25 mg/kg)	CY + BCH-1393 (50 mg/kg)
Blood immunophenotyping BCH-1393[a]				
CD8+ CD45+	Mean	15.1	13.3	20.8
	STD	3.89	0.07	0.85
	p value		0.33	0.05

[a] Groups of three immunosuppressed C57BL/6 mice (100 mg/kg Cy, i.p.) were injected i.p. for four consecutive days with BCH-1393.

Table 4
Effect of CY and BCH-1393 treatment on DA-3 tumor outgrowth[a]

Group/day	4	6	8	10	12	14	16	18	20	22
Gp 1: saline s.c.	10/10[b]	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
Gp 2: CY (100 mg/kg i.v.)	8/10	8/10	8/10	8/10	8/10	8/10	8/10	8/10	8/10	8/10
Gp 3: BCH-1393 (25 mg/kg i.p.) Program A	9/10	10/10	9/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
Gp 4: BCH-1393 (25 mg/kg i.p.) Program B	8/10	9/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
Gp 5: BCH-1393 (50 mg/kg i.p.) Program A	7/10	8/10	9/10	9/10	9/10	9/10	9/10	9/10	10/10	10/10
Gp 6: BCH-1393 (50 mg/kg i.p.) Program B	5/10	6/10	7/10	7/10	7/10	7/10	7/10	7/10	7/10	7/10
Gp 7: CY 100 mg/kg i.v.) + BCH-1393 (50 mg/kg i.p.) Program A	6/10	6/10	5/10	3/10**	2/10***	2/10***	2/10***	2/10***	2/10***	2/10***
Gp 8: CY 100 mg/kg i.v.) + BCH-1393 (50 mg/kg i.p.) Program B	4/10*	4/10*	4/10*	3/10**	3/10**	3/10**	3/10**	3/10**	3/10**	2/10***

[a] $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ by Chi-square analysis.
[b] Tumor incidence = No. of tumor-bearing mice / No. of total mice injected with tumor cells.

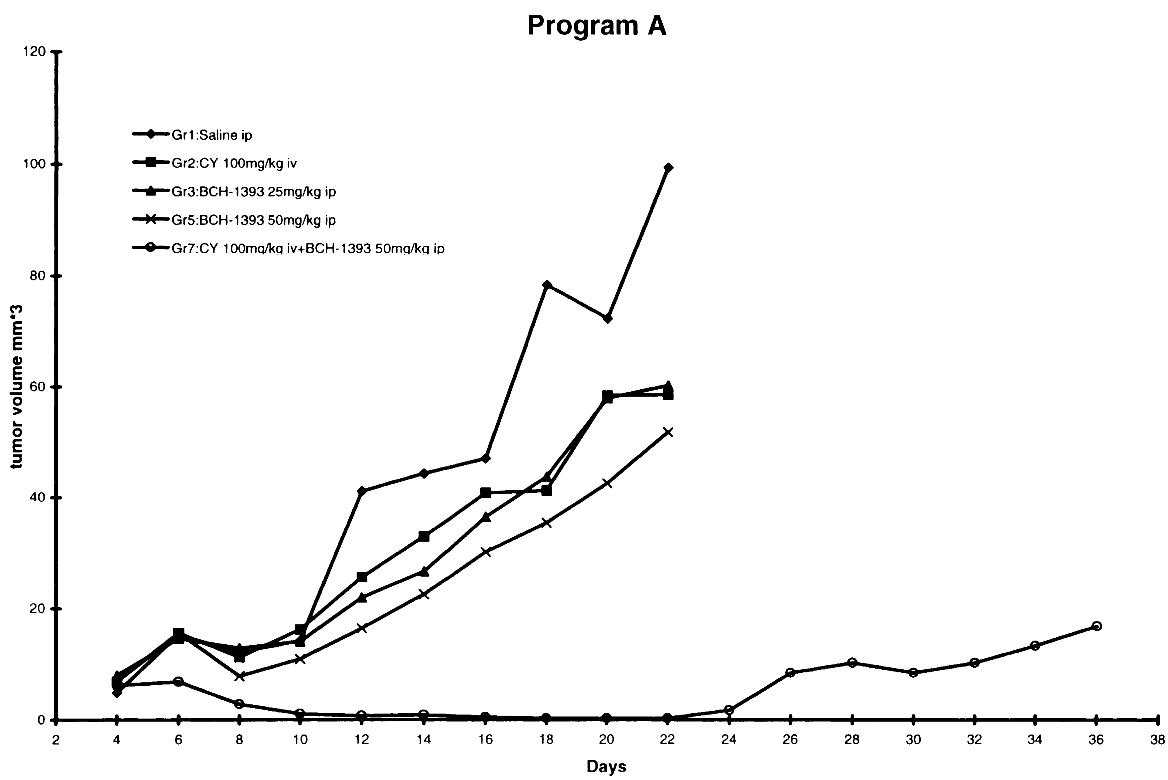

Fig. 3. Mice were injected s.c. with 5×10^5 DA-3 tumor cells on day 0 (D0). Treatment with BCH-1393 (25 and 50 mg/kg, i.p.) was done either alone or following pretreatment with 100 mg/kg Cy administered i.v. at D0 as single bolus injection. BCH-1393 was given on days D-2, D-1, D0 and then continued every second day (D1–D21).

Collectively, these results demonstrate augmentation of the anti-tumor effect of BCH-1393 against breast and colon tumors when used in combination with subtherapeutic doses of cyclophosphamide.

4. Discussion

In the present study it is demonstrated that a novel immunostimulant BCH-1393 (*N,N*-dimethylaminopurine pentoxycarbonyl D-arginine) can augment the therapeutic effect of the alkylating agent Cy in the treatment of mouse breast and colon carcinomas. This concomitant anti-tumor effect was achieved in combination with suboptimal doses of Cy. In contrast to human patients, established solid tumors of breast carcinoma and liver metastasis of colon carcinoma in mice can be cured with high doses of cytotoxic agents alone [11,30,31]. The optimal therapeutic effect of BCH-1393 and Cy on inhibiting the outgrowth of DA-3 breast carcinoma was obtained in prophylaxis regimens with multiple treatments initiated 2 days before tumor cell implantation. Obviously, the experimental approach used in the present study required the presence of progressively growing established tumors. Thus, the combination treatment with BCH-1393 and Cy was initiated post tumor cell inoculation in the MC38 colon carcinoma model when early solid tumors were present. Under these conditions, which mimic to a certain extent the clinical situation, the combination of BCH-1393 and Cy had a clear-cut synergistic therapeutic effect. It is possible that the success of this

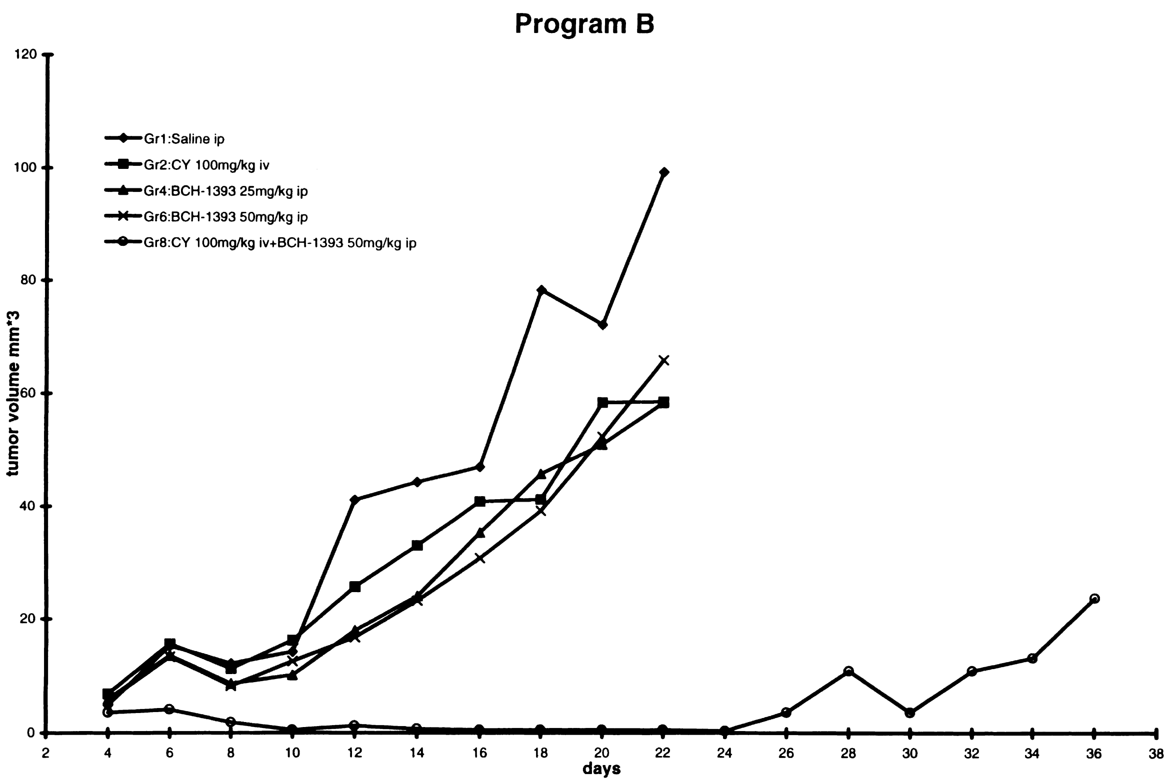

Fig. 4. Mice were injected s.c. with 5×10^5 DA-3 tumor cells on day 0 (D0). Treatment with BCH-1393 (25 and 50 mg/kg, i.p.) was done either alone or following pretreatment with 100 mg/kg Cy administered i.v. at D0 as single bolus injection. BCH-1393 was given on days D-2, D-1, D0 and then continued daily (D1–D21).

combined modality treatment is based on the ability of the two agents to abrogate tumor-mediated immunological aberrations and regulate immune response. Such aberrations occur in tumor-bearing mice with excessive antibody production and presence of antigen-antibody complexes that have been found to induce energy and immune suppression. In other instances tumor-mediated changes in lymphocyte subsets can lead to altered lymphocyte functions [32]. A number of studies provide evidence for the presence of tumor-derived suppressive factors. Both suppressor T-cells and macrophages (via prostaglandin-s)have been observed to account for such changes [33,34]. The action of low-dose cyclophosphamide appears to be mediated through a specific and selective impairment of suppressor T-cells generated in the course of tumor progression [35,36]. On the contrary, BCH-1393 was found to be a potent activator of CTL and to significantly expand other T-cell subsets, NK cells and macrophages. Increases in relative CTL numbers were observed in both normal and immunosuppressed animals. Thus, it appears that a successful therapeutic effect is achieved synergistically by Cy's specific reduction of suppressor lymphocytes and by the increase of immunopotentiating effect of BCH-1393 leading to enhanced immune parameters and reduced tumor growth. However, further evidence demonstrating increase in tumor specific splenocyte cytotoxicity against relevant tumors as well as functional immunophenotypic analysis will be needed to validate this hypothesis.

Apart from direct anti-tumor activity, some antineoplastic agents can profoundly affect the host immune competence. While most chemotherapeutic agents are immunosuppressive

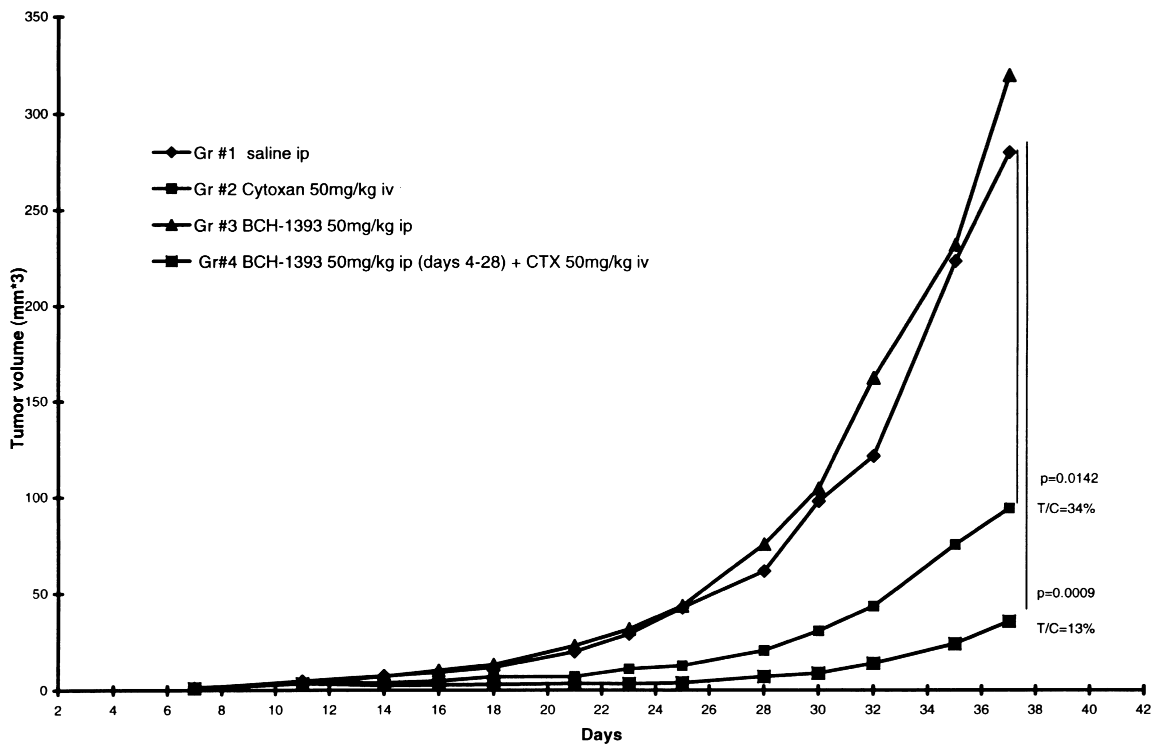

Fig. 5. Mice were injected s.c. with 5×10^5 MC38 tumor cells on day 0 (D0). Treatment with BCH-1393 (50 mg/kg i.p.) started at day 5 (D-5) when palpable tumors were evident. BCH-1393 was administered daily either alone or in combination with CTX from day D-5 till D-28. CTX (50 mg/kg) was injected i.v. twice as single bolus injections given on days D-7 and D-15, respectively.

[37,38], some anti-cancer drugs have been shown to augment several parameters of host immunity in different experimental tumor systems [37,39]. Pretreatment of mice with adriamycin was shown to enhance the production of natural killer cells, frequency of LAK cells, and tumor-specific T-cell-mediated delayed type hypersensitivity response [40]. In particular, adriamycin has been shown to increase cell mediated cytotoxicity and production of IL-2 in mice and in cancer patients [41,42]. Such an immuno-potentiating effect of chemotherapeutic agent on immune cells cannot be ruled out by administration of low subtherapeutic doses of Cy. Treatment with BCH-1393, an immunostimulant with in vitro activity similar to that of IL-2, may also directly complement the antitumor effect of Cy.

The DA-3 mammary adenocarcinoma has been previously reported to be a weakly immunogenic tumor [43]. Chemotherapy and immunotherapy generally produce a greater antitumor response against tumors that are highly immunogenic [30]. Thus, at least some of the antitumor effects of combined BCH-1393 and Cy treatment might result from the development of tumor specific immunity. The mechanisms by which Cy and BCH-1393 promote the development of an immune response in tumor-bearing mice have not been extensively investigated. One possibility is that the cells of the immune system are optimally exposed to the tumor antigens released during pretreatment of tumor by Cy, whereas BCH-1393 may cause clonal expansion of the sensitized effector CTL cells that mediate tumor eradication. This is supported by the fact that BCH-1393 increases the CTL response when used between 10^{-9} and 10^{-5} M concentrations. Further, this potent in vitro activity is reflected by a significant increase in relative percentage of CTL in vivo. As noted above, Cy may enhance immune response to tumor by eliminating the development of suppressor cell populations that downregulate a positive immune response. Watson et al. [43] have demonstrated the presence of Mac-1^+2^+I-A^+, Thy-1^-L3T4^-, Lyt-2^-Ig^- macrophage cells with suppressive functions in the DA-3 tumor-bearing mice. Furthermore, it was found that DA-3 tumor releases high amounts of GM-CSF which appears to contribute to the expansion of this cell population [44,45]. GM-CSF can also lead to increased levels of PGE_2 which in its turn suppresses immune functions.

Both BCH-1393 and Cy have been evaluated as single agents for antitumor therapy based on the hypothesis that they normalize immune competence of tumor-bearing hosts. Our data shows a superior anti-tumor effect of BCH-1393 and Cy in treatment of established MC38 colon carcinoma tumors, compared to that of Cy treatment alone. The positive interaction of BCH-1393 and Cy could be attributed to the immunorestorative effects of both agents on a depressed cell-mediated immune response associated with progression of established tumors. However, despite significant reduction of tumor load and subsequent regression as a consequence of the combined BCH-1393 and Cy therapy, tumors resume growth upon withdrawal of treatment. Similar to treatment of patients with Cy as a single treatment modality, multiple rounds of chemoimmunotherapy might be necessary to further enhance and sustain long-term therapeutic effect.

Our data indicate a potential role for BCH-1393 in the regulation of antitumor immune response. BCH-1393 may directly correct tumor-related immunological defects and restore that pathway to its functional capacity. This study suggests that concomitant treatment with this immunomodulator could be useful as an adjunct to Cy chemotherapy.

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