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Cyclocreatine in cancer chemotherapy

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Abstract Cyclocreatine, an analog of creatine, is an efficient substrate for creatine kinase, but its phosphorylated form is a poor phosphate donor in comparison with creatine phosphate. Cyclocreatine was not very cytotoxic upon 24 h of exposure of human SW2 small-cell lung cancer cells to concentrations of up to 5 m*M*. However, combinations of cyclocreatine (0.5 m*M*, 24 h) with each of four antitumor alkylating agents, *cis*-diamminedichloroplatinum(II), melphalan, 4-hydroperoxycyclophosphamide, and carmustine, resulted in additive to greater-than-additive cytotoxicity toward exponentially growing SW2 cells. The greatest levels of synergy were seen at higher concentrations of 4-hydroperoxycyclophosphamide and carmustine as determined by isobologram analysis. In vivo cyclocreatine (0.5 or 1 g/kg) was more effective if given i.v. rather than i.p. The longest tumor-growth delays, up to 10 days, were produced by extended regimens of cyclocreatine. Cyclocreatine was an effective addition to therapy with standard anticancer agents including *cis*-diamminedichloroplatinum(II), cyclophosphamide, Adriamycin, or 5-fluorouracil. No additional toxicity was observed when 10 days of cyclocreatine treatment was given with full standard-dose regimens of each drug. The resultant increases in tumor-growth delay were 1.7- to 2.4-fold as compared with those obtained for each of the drugs alone. These results indicate that cyclocreatine may be an effective single agent and an effective addition to combination chemotherapy regimens.

Key Words Cyclocreatine · Creatine kinase · Combination chemotherapy

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Introduction

It has been recognized since the elegant studies of Otto Warburg [25] that many neoplastic tissues have altered respiratory metabolism and energy production. One of the key enzymes involved in the cellular energy homeostasis is creatine kinase (EC 2.7.3.2), which catalyzes the reversible transfer of a high-energy phosphate bond from creatine phosphate to adenosine diphosphate (ADP) [24]. The creatine kinase system is involved in energy buffering as well as energy transport. The creatine phosphate molecule seems to serve as an energy carrier connecting sites of energy production with sites of energy utilization via the subcellularly compartmentalized creatine kinase isoenzymes. The creatine kinase isoenzymes are subject to complex regulation. The brain isoform creatine kinase B has been shown to be induced by the transforming domains of the adenovirus oncogenic product Ela, suggesting a role for this enzyme in the metabolic events that take place during transformation [10]. Creatine kinase seems to be connected to intracellular signal transduction via protein kinase C-dependent pathways, suggesting a potential physiological role in the maintenance of adenosine triphosphate (ATP) in the stimulation pathways [5, 12, 15, 24]. The muscle isoform (creatine kinase M) is regulated by p53 and is induced during muscle differentiation [26, 27].

The level of creatine kinase activity has been measured in 23 human tumor cell lines and found to be markedly increased in 10 and moderately increased in 5 of the lines [11]. Three of three small-cell lung-carcinoma cell lines had markedly increased creatine kinase activity levels and three of three colon adenocarcinoma lines had marked to moderate increases in creatine kinase activity as compared with "normal" cell lines [11]. The brain isoform (creatine kinase B) has been identified as the most predominant form in these tumors. Many patients with neuroendocrine-derived tumors have very high levels of creatine kinase B in tumor and plasma [4].

Cyclocreatine, an analog of creatine, is an efficient substrate for creatine kinase both in vitro and in vivo [1, 11, 13, 14, 23]. It is phosphorylized by creatine kinase. It generates a new synthetic phosphagen, cyclocreatine phosphate, which partially replaces creatine phosphate. This new synthetic phosphagen is turned over 160-fold less efficiently and, hence, the rate of ATP generation through the creatine/creatine kinase system is significantly reduced when cyclocreatine phosphate is the substrate. Recent studies have shown that cyclocreatine given as 1% or more of the diet can slow the growth of several rodent solid tumors and human tumors grown as xenografts [11, 13].

The current studies were undertaken to examine the effects of cyclocreatine used alone and in combination with other chemotherapeutic agents in vitro and in vivo.

Materials and methods

Drugs

Cyclocreatine was obtained from AMIRA, Inc. (Cambridge, Mass.). *cis*-Diamminedichloroplatinum(II)(CDDP), melphalan (L-PAM), cyclophosphamide (CTX), 5-fluorouracil (5-FU), and Adriamycin were purchased from Sigma Chemical Co. (St. Louis, Mo.). 4-Hydroperoxycyclophosphamide (4-HC) was kindly provided as a gift by Dr. J. Pohl (Asta Medica, Frankfurt am Main, Germany). Carmustine (BCNU) was obtained from the Dana-Farber Cancer Institute pharmacy.

Cell line

The SW2 human small-cell lung-carcinoma cell line was initiated from pleural fluid obtained from a patient with small-cell carcinoma [7, 8]. The creatine kinase activity in SW2 cells has been determined to be 4.6 U/mg protein at 37°C (V. Khandekar, personal communication). These cells grow exponentially in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; Sterile Systems, Logan, Utah) and antibiotics as enlarging spheroids with a doubling time of 2–4 days, eventually reaching a plateau by day 30. The spheroids were dispersed to make a single-cell suspension for drug exposure. Colonies were grown in soft agar, and the plating efficiency of this cell line was 10%–15%.

Survival curves

SW2 cells in exponential growth were exposed to concentrations of cyclocreatine ranging from 0.5 to 5 m*M* for 24 h or were exposed to 0.5 m*M* of cyclocreatine for 24 h with exposure to various concentrations of BCNU, CDDP, 4-HC, or L-PAM during the 5th h of cyclocreatine treatment. After exposure to the agents, the cells were washed three times with phosphate-buffered 0.9% saline, then plated in duplicate at three dilutions in 0.5% soft agar for colony formation as described above. Results were expressed as the surviving fraction of treated cells as compared with vehicle-treated control cells [18, 19, 22].

Additivity analysis of survival curves

Isobolograms (envelopes) were generated for the special case in which the dose of one agent is held constant [6]. Dose-response curves for each agent alone were generated first. Combinations producing an effect that fell within the envelope boundaries were considered additive; those displaced to the left were denoted greater than additive (i.e., supra-additive), whereas those displaced to the right were considered less than additive (i.e., subadditive) [3, 17]. This general approach can be extrapolated to the special case in which the level of an agent is held constant. Under these conditions, an isobologram can be derived that plots the expected effect (modes I and II) for any level of the variable agent combined with the fixed agent [20]. Experimentally, this approach is simple and readily facilitates the determination of additive and nonadditive combinations. To facilitate isobologram analyses, a flexible, interactive computer program was written. The program first deduces the best-fitting dose-response curves using dose (or log dose) and effect, log effect, probit-percentage of effect, or logit-percentage of effect relationships. For cell-survival, dose-response curves, correlations of greater than or equal to 0.96 have been obtained. The program then calculates an isobologram at a constant level of the selected agent.

Tumor

The rat mammary adenocarcinoma 13762 is a carcinogen-induced (DMBA) tumor of the female Fischer 344 rat [16]. The tumor can metastasize to the lung and abdominal organs. This tumor is composed of epithelial tissue in folds and acini. Many acini are present with a high percentage of mitoses. The acini are usually double- or triple-cell-layered and are cuboidal, columnar, or pseudocolumnar. Growth is invasive. The creatine kinase activity in the 13762 tumor has been determined to be 0.26 U/mg protein at 37°C (V. Khandekar, personal communication). Tumor cells (2×10^6) prepared from a brei of stock tumors were implanted s.c. in a hind leg of 8- to 10-week-old rats on day 0. The tumor grows to a volume of 1 cm^3 in about 28 days when implanted s.c. in the leg.

Tumor growth delay

Cyclocreatine (0.5 or 1 g/kg) was given i.p. or i.v. via the tail vein beginning on day 4, 7, or 11 and was given daily for 5, 7, or 14 days or for two courses of 5 days.

For drug combination studies, cyclocreatine (1 g/kg) was given i.v. beginning on day 3 or 5 after tumor cell implantation and treatment was continued daily through day 11 or 13. The other antitumor agents were given i.p. beginning on day 7. CDDP (8 mg/kg) was given as a single dose on day 7; cyclophosphamide (100 mg/kg) was injected on days 7, 9, and 11; and Adriamycin (1.75 mg/kg) and 5-Fu (30 mg/kg) were given daily on days 7–11.

The progress of each tumor was measured thrice weekly until it reached a volume of 500 mm^3. Tumor-growth delay was calculated as the days taken by each individual tumor to reach a volume of 500 mm^3 as compared with the untreated controls. Each treatment group had 4 animals and the experiment was repeated 3 times. Days of tumor-growth delay are presented as the mean value ± SE for the treatment group as compared with the control.

Results

The structure of cyclocreatine is shown in Fig. 1. Cyclocreatine concentrations of up to 5 m*M* (24 h) were only slightly cytotoxic to human SW2 small-cell lung carcinoma cells (Fig. 2). It is likely in vivo that tissue levels of cyclocreatine of 0.5–1 m*M* can be achieved; therefore, for combination studies in cell culture a concentration of 0.5 m*M* cyclocreatine was used.

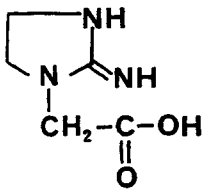

Fig. 1 The structure of cyclocreatine (2-imino-1-imidazolidineacetic acid)

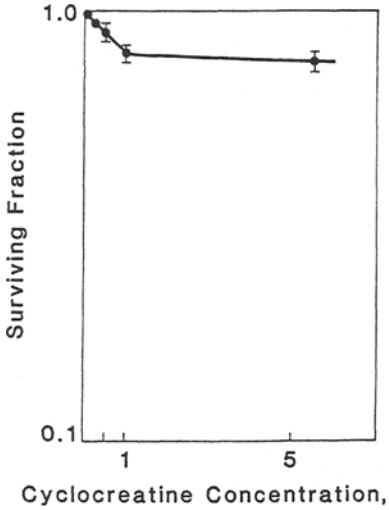

Fig. 2 Survival of exponentially growing human SW2 small-cell lung carcinoma cells exposed to various concentrations of cyclocreatine for 24 h. The results are presented as the mean values ± SEM for 3 independent experiments

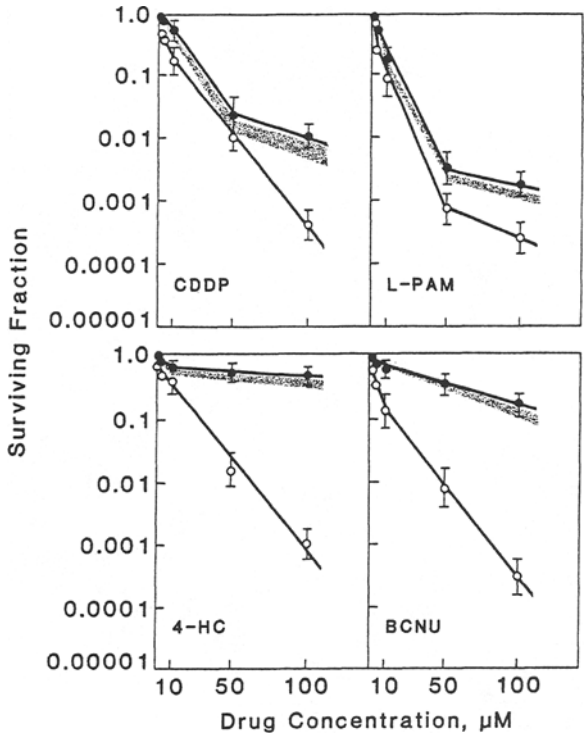

Fig. 3 Survival of exponentially growing human SW2 small-cell lung carcinoma cells exposed to cyclocreatine (0.5 m*M*, 24 h) with exposure to various concentrations of CDDP, L-PAM, 4-HC or BCNU for 1 h. during the 5th h of cyclocreatine exposure (○). *Solid circles* indicate the survival value obtained for each of the antitumor alkylating agents alone (1 h), and *shaded areas* represent the envelopes of additivity as determined by isobologram analysis. The results are presented as the mean values ± SEM for 3 independent experiments

Survival curves generated for exponentially growing SW2 cells after 1 h of exposure to each of four antitumor alkylating agents are shown in Fig. 3. The cyclocreatine/antitumor alkylating agent regimen consisted of a 24-h exposure of the cells to 0.5 m*M* cyclocreatine with a 1-h exposure to an antitumor alkylating agent during the 5th h such cyclocreatine was present prior to, during, and after antitumor alkylating agent treatment. The combination of cyclocreatine and CDDP resulted in additive to greater-than-additive killing of SW2 cells, with the greatest synergy occurring at high concentrations of CDDP. The combination of cyclocreatine and L-PAM also produced additive to greater-than-additive killing of SW2 cells. The level of synergy attained with the cyclocreatine/L-PAM combination increased with increasing L-PAM concentrations.

The SW2 cells are relatively resistant to the cytotoxic effects of 4-HC, such that 100 μ*M* 4-HC (1 h) kills about 50% of the cells. The combination of cyclocreatine and 4-HC produced markedly greater-than-additive tumor cell killing such that at 50 μ*M* 4-HC about 1.5-log more cells were killed and at 100 μ*M* 4-HC 2.5-log more cells were killed than would be expected for additive cytotoxicity of the agents. SW2 cells are moderately sensitive to BCNU; 1 log (or 90%) of SW2 cells were killed by exposure to about 140 μ*M* BCNU for 1 h. The combination of cyclocreatine and BCNU resulted in markedly greater-than-additive killing over the BCNU concentration range examined. With a concentration of 50 μ*M* BCNU along with cyclocreatine there was about 1.5-log greater killing of SW2 cells than expected for additivity, and with 100 μ*M* BCNU along with cyclocreatine there was about 2.5-log greater killing of SW2 cells than expected for additivity of the two drugs.

Cyclocreatine given as 1% or more of the diet has demonstrated growth-inhibitory effects in the 13762 rat mammary carcinoma [13]; therefore, this tumor was chosen for in vivo studies. Cyclocreatine was given to 13762 tumor-bearing female Fisher 344 rats by i.p. injection or by i.v. injection via the tail vein (Table 1). Several schedules of daily cyclocreatine administration were studied beginning at 4, 7, or 11 days after tumor cell implantation, thus allowing treatment of tumors of various volumes (various tumor cell burdens). Cyclocreatine (0.5 or 1 g/kg) produced measurable growth delay of the 13762 tumor on each of the treatment schedules tested. There was no weight loss in the animals treated with cyclocreatine on any of the regimens.

Table 1 Growth delay of the rat 13762 mammary carcinoma produced by daily cyclocreatine given i.p. or i.v.[a]

Cyclocreatine dose, g/kg	Administration route	Schedule, days	Initial tumor volume, mm^3	Tumor-growth delay[b], days
0.5	i.p.	4–17	25	3.0 ± 0.4
0.5	i.p.	11–17	100	1.8 ± 0.3
1.0	i.p.	4–17	25	4.8 ± 0.6*
1.0	i.p.	11–17	100	1.6 ± 0.4
0.5	i.v.	4–8	25	4.8 ± 0.6
0.5	i.v.	7–11	50	3.3 ± 0.4
0.5	i.v.	11–15	100	1.8 ± 0.3
1.0	i.v.	4–8	25	7.6 ± 1.0*
1.0	i.v.	7–11	50	3.8 ± 0.6
1.0	i.v.	11–15	100	1.5 ± 0.3

* Significantly different from the control group as determined by the Dunn multiple-comparisons test: $P < 0.01$

[a] Data are presented as the mean values of 4 animals/group, and each experiment was done 3 times ($n = 12$)

[b] Tumor-growth delay is the difference in the number of days required for treated tumors to reach a volume of 500 mm^3 as compared with untreated controls. Control tumors reached a volume of 500 mm^3 in 18.0 ± 1.5 days

Table 2 Growth delay of the rat 13762 mammary carcinoma produced by cyclocreatine given i.v.[a]

Initial Tumor cyclocreatine dose, g/kg	Schedule, days	Tumor volume, mm^3	Growth delay[b] days
0.5	4–8	25	4.8 ± 0.6
0.5	7–11	50	3.3 ± 0.4
1.0	4–8	25	7.6 ± 1.0*
1.0	7–11	50	3.8 ± 0.6
0.5	4–8; 14–18	25	6.4 ± 1.0*
0.5	7–11; 14–18	50	6.7 ± 1.1*
1.0	4–8; 14–18	25	10.1 ± 1.2**
1.0	7–11; 14–18	50	8.3 ± 1.2**

* Significantly different from the control group as determined by the Dunn multiple-comparisons test;* $P < 0.01$: ** $P < 0.001$

[a] Data are presented as the mean values for 2 experiments with 4 animals/group; therefore, $n = 8$

[b] Tumor-growth delay is the difference in the number of days required for treated tumors to reach a volume of 500 mm^3 as compared with untreated controls. Control tumors reached a volume of 500 mm^3 in 18.0 ± 1.5 days

Cyclocreatine was more effective when injected at a dose of 1 g/kg than when given at 0.5 g/kg. The drug was also more effective when given as an i.v. injection than when given as an i.p. injection beginning at the same tumor volume, even though the i.v. schedule involved daily injections for 5 days as compared with daily injections for 14 days for the i.p. schedule (Table 1). As would be expected for most antitumor treatments, cyclocreatine (1 g/kg) was most effective when the tumor burden (tumor volume) was smaller, producing a maximal tumor-growth delay of 7.6 days when given i.v. daily for 5 days beginning on day 4 after tumor cell implantation.

When treatment with cyclocreatine (0.5 or 1 g/kg) given i.v. was extended to two 5-day courses with a 5-day interval between them, an increase in tumor-growth delay was observed as compared with a single 5-day course of the drug (Table 2). The effect of cyclocreatine dose was marked in that the administration of two courses at a dose of 0.5 g/kg was less effective than that of a single course at 1 g/kg when treatment was initiated on day 4 ($P < 0.01$).

For initial combination treatments, cyclocreatine (1 g/kg) was given beginning 2 days before the standard anticancer agents and treatment was continued daily over the course of those therapies for a total of seven injections (Table 3). Each of the standard anticancer drugs was given by i.p. injection at a standard dose and on a standard schedule for the particular drug. CDDP (8 mg/kg) given as a single dose produced a

Table 3 Growth delay of the rat 13762 mammary carcinoma produced by anticancer drugs in the presence or absence of cyclocreatine given i.v.[a]

Treatment group	Tumor-growth delay, days[b]		
	Drug alone	+ Cyclo	
		7 × 1 g/kg	10 × 1 g/kg
Cyclocreatine (1 g/kg)[c]	–	2.6 ± 0.4	4.1 ± 0.5
CDDP (8 mg/kg), day 7	7.2 ± 0.6	11.0 ± 1.1	14.0 ± 1.2**
Adriamycin (1.75 mg/kg), days 7–11	5.3 ± 0.5	6.5 ± 0.7	12.5 ± 1.1**
Cyclophosphamide (100 mg/kg), days 7, 9, 11	9.3 ± 0.7	10.7 ± 1.0	16.2 ± 1.3**
5-Fluorouracil (30 mg/kg), days 7–11	5.5 ± 0.5	7.7 ± 0.9	11.8 ± 1.0**

* Significantly different from the drug-alone group as determined by the Dunn multiple-comparisons test; * $P < 0.01$: ** $P < 0.001$

[a] Data are presented as the mean values for 1 experiment with 4 animals/group and the experiment was done twice; therefore, $n = 8$.

[b] Tumor-growth delay is the difference in the number of days required for treated tumors to reach a volume of 500 mm^3 as compared with untreated controls. Control tumors reached a volume of 500 mm^3 in 18.0 ± 1.5 days

[c] Cyclocreatine (1 g/kg) was given i.v. on days 5–11 or days 4–13 after tumor cell implantation. All other drugs were given i.p. on the schedules shown

tumor-growth delay of about 7 days, which was increased to 11 days by the combination of this drug with cyclocreatine. Adriamycin (1.75 mg/kg) given daily for 5 days produced a tumor-growth delay of about 5 days, which was increased to 6.5 days by the addition of cyclocreatine. Cyclophosphamide (100 mg/kg) was given on alternate days for three injections beginning on day 7. Cyclophosphamide produced about 9 days of tumor-growth delay in the 13762 tumor, which was increased to about 11 days by combination treatment with cyclocreatine. 5-FU (30 mg/kg) was given daily for 5 days, resulting in 5.5 days of tumor-growth delay, whereas the combination of 5-Fu and cyclocreatine resulted in 7.7 days of tumor-growth delay.

In a second combination therapy study, treatment with cyclocreatine (1g/kg) was initiated 3 days prior to administration of the standard antitumor agents and was carried on through day 13 after tumor cell implantation. This 10-day treatment with cyclocreatine produced about 4 days of tumor-growth delay in animals bearing the 13762 mammary carcinoma (Table 3). The addition of 10 days of cyclocreatine administration to treatment with the standard anticancer agents produced an increase in tumor-growth delay as compared with therapy with the drugs alone or the drugs with the 7-day cyclocreatine regimen. The 10-day course of cyclocreatine plus CDDP resulted in a 2-fold increase in tumor-growth delay as compared with CDDP alone, in a 2.4-fold increase in tumor-growth delay as compared with Adriamycin alone, in a 1.7-fold increase in tumor-growth delay as compared with cyclophosphamide alone, and in a 2-fold increase in tumor-growth delay as compared with 5-Fu alone. There was no additional weight loss in animals treated with the cyclocreatine/cytotoxic drug combinations as compared with the cytotoxic drugs alone.

Discussion

There is a search for new anticancer drugs with unique mechanisms of action. Cyclocreatine is an example of such an agent. Although the mechanism by which cyclocreatine is cytotoxic or growth-inhibitory toward tumor cells in cultures or tumors in vivo has not been explained, it is known that cyclocreatine phosphate is a very poor substrate for creatine kinase [1, 23]. The accumulation of cyclocreatine phosphate in cells may lead to an imbalance of high-energy phosphate and, consequently, of energy and energy-requiring processes within cells [4, 24]. Creatine levels may also play a direct role in the growth of some tumors [2, 14]. The rationale for the use of cyclocreatine in combination with other anticancer agents would include possible inhibition of energy-requiring repair processes [21], energy-requiring efflux pumps [9], and/or phosphate-requiring intracellular signal transduction.

The human SW2 small-cell lung carcinoma cell line has very high creatine kinase activity levels. Although cyclocreatine at concentrations of up to 5 m*M* was only slightly cytotoxic toward SW2 cells, exposure of the cells to an essentially noncytotoxic concentration of cyclocreatine along with each of four antitumor alkylating agents produced greater-than-additive cytotoxicity toward the cells. This finding is consistent with the notion that cyclocreatine may affect cellular metabolism in such a way that the cells are less capable of responding to the stress caused by exposure to the bifunctional alkylating agents.

In vivo, cyclocreatine and other creatine analogs have traditionally been given in the diet over very long periods without evidence of toxicity. In the current study, cyclocreatine was given by either i.p. or i.v. injection. Cyclocreatine was an effective antitumor

agent with as few as five injections, although longer courses of administration produced longer durations of tumor-growth delay. Administration of cyclocreatine at doses of 0.5 and 1 g/kg on the various schedules studied resulted in tumor-growth delays that were comparable with those achieved with standard regimens of currently clinically used anticancer drugs, including CDDP, Adriamycin, cyclophosphamide, and 5-Fu (Tables 2, 3). Cyclocreatine given in combination with each of the currently used anticancer drugs resulted in longer tumor-growth delays without evidence of increased toxicity than those achieved with any of the anticancer drugs alone. The standard anticancer agents studied included two antitumor alkylating agents (CDDP and cyclophosphamide), an antitumor antibiotic (Adriamycin), and an antimetabolite (5-Fu). With each standard anticancer drug, the longer course (10 versus 7 days) of cyclocreatine administration was a more effective addition to the therapeutic regimen than was the shorter course of cyclocreatine.

The results presented herein indicate that cyclocreatine has the potential to be a useful addition to combination chemotherapy regimens.

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