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In Vivo Antitumor Efficacy of CW252053, A Folate-based Thymidylate Synthase Inhibitor

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Previous studies have demonstrated that CW252053, a quinazoline antifolate, exhibits potent inhibitory activity against thymidylate synthase (TS) as well as cytotoxic activity against tumor cell lines *in vitro*. In this study, we evaluated the *in vivo* antitumor efficacy of CW252053 in the mouse tumor model. Female $B6D2F_1$ mice were injected with LY3.7.2C TK-/- (thymidine kinase deficient mouse lymphoma) cells into the gastrocnemius muscle. Then, CW252053 was administered twice daily by intraperitoneal injection for 10 days, and tumor growth was monitored daily by leg diameter measurement. All animals in the vehicle, 5-FU, and low dose (30 mg/kg) CW252053 treated groups died between days 12 and 23 because of the tumor burden. In contrast, dosing with 60 mg/kg of CW252053 produced a cure rate against tumor growth of 37.5% and a survival rate of 50%. Even more significantly, a higher dose of CW252053 (120 mg/kg) elicited both a 100% cure rate and a 100% survival rate at the termination of the study, confirming that this compound has very potent *in vivo* antitumor activity against tumor growth. During the experimental period of this study no signs of toxicity were observed even at the high CW252053 dosage rate of 120 mg/kg.

Key words: CW252053, Thymidylate synthase (TS) inhibitor, *in vivo* antitumor efficacy, LY3.7.2C TK-/-

INTRODUCTION

Thymidylate synthase (TS) is a critical enzyme for the *de novo* synthesis of deoxythymidine-5'-monophosphate (dTMP, thymidylate) from deoxyuridine-5'-monophosphate (dUMP), and it plays an essential role in DNA synthesis and repair (Johnston *et al.*, 1993; Takemura *et al.*, 1997; Walton *et al.*, 1996). The maximal activity of cellular TS occurs during the S phase of the cell cycle and is 20-fold higher in rapidly proliferating cells than in nondividing cells (Johnston *et al.*, 1991). Thus, the inhibition of TS leads to a "thymineless cell death" particularly in cancer cells where proliferation is very rapid, and this fact has made TS an attractive target in the development of anticancer chemotherapeutic agents for many years (Jackman *et al.*, 1994). 5,10-Methylenetetrahydrofolate is involved in the catalytic action of TS as a cofactor which forms a ternary complex with TS and dUMP, and this cofactor is oxidized to dihydrofolate with the concomitant reductive methylation of dUMP to dTMP. For this purpose many classical antifolates, best exemplified by raltitrexed (ZD1694, Tomudex) (Jones *et al.*, 1981; Stephens *et al.*, 1993), have been reported as antitumor agents. But some drawbacks were discovered such as drug resistance resulting from defective cell transport due to mutation of the cancer cell itself and such as toxicity to the host resulting from the unnecessarily long retention inside the normal cell. These complications were reported to occur due to the presence of the glutamate component of the antifolates (Appelt *et al.*, 1991), and in order to overcome these complications, we modified the glutamic acid component of folate analogues into phenylglycines, converting these analogues into nonclassical lipophilic inhibitors of TS.

Recently, we reported that one of the quinazoline compounds, CW252053 (Fig. 1), functions as a nonclassical antifolate inhibitor of human and *E. coli* TS, and further that this compound showed cell growth inhibition in submicromolar concentration against several tumor

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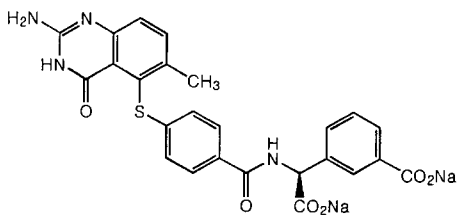

Fig. 1. Structure of CW252053.

cell lines of murine and human origin *in vitro* (Baek *et al.*, 1998). To gain further verification of this antitumor efficacy, evaluation of this compound *in vivo* in mice was performed.

Evaluation of the *in vivo* efficacy of TS inhibitors is, however, problematic in mice because of the high level of thymidine in plasma (~1 μM) (Webber *et al.*, 1996). Therefore, unlike in humans, thymidylate can be alternatively synthesized in mice from thymidine by thymidine kinase (TK) in the salvage pathway, a process which results in decreased cytotoxic effects of TS inhibitors. To overcome this salvage problem, the TK-deficient (TK-/-) mutant cell line of murine lymphoma was used for both *in vitro* cytotoxicity and *in vivo* antitumor screening of folate analogue TS inhibitors (Stephens *et al.*, 1993).

MATERIALS AND METHODS

Test compounds

CW252053 was synthesized as described in the literature (Baek *et al.*, 1998), and is freely soluble in water. 5-Fluorouracil (5-FU) was purchased from Sigma.

Cell lines

The LY3.7.2C TK-/- (thymidine kinase deficient mouse lymphoma) cell line was derived from a mouse lymphoma cell line. The cells were maintained in Fishers medium with 10% fetal bovine serum (Gibco Inc., Grand Island, NY) and grown in 75 cm^2 plastic culture flasks (Falcon Labware, Oxnard, CA).

Mice

Four week old female B6D2F_1 mice (purchased from Charles River Japan) were housed, one treatment group (consisting 8 mice) per sawdust containing cage, in a regular 12-h light/dark cycle. Food and water was supplied *ad libitum*. Animals were acclimated for 1 week after arrival and were then used for experimental study of antitumor activity.

In vivo antitumor activity

The *in vivo* antitumor activity of CW252053 was measured according to Stephenss method (Stephens *et al.*, 1993) with some modifications. In brief, B6D2F_1 mice were injected with 5×10^6 cultured LY3.7.2C TK-/- cells into the gastrocnemius muscle of the right hind leg. The day of tumor inoculation was designated as day 0, with drug treatment being initiated on day 1 through day 10. CW252053 (30, 60, 120 mg/kg) and 5-FU (10 mg/kg) were dissolved in saline and administered twice daily by *i.p.* injection. The dosage of 5-FU, the reference drug, was determined after a preliminary study in tumor-bearing mice showed that a dose of 10 mg/kg was maximally tolerable when the mice were given 5-FU twice daily for 10 consecutive days. The vehicle control group was treated with saline alone. The diameters of the inoculated right leg and the untreated left leg were measured using digital calipers (CD-15B, Mitutoyo Corp., Japan) and the animals were sacrificed on day 35. The animals were

Table I. Changes of tumor size after implantation of LY3.7.2.C TK-/- lymphoma. The tumor was inoculated into the gastrocnemius muscle of the right hind leg on day 0 with 5×10^6 cells per mouse. Test compounds were administered for 10 days twice daily by *i.p.* injection. Tumor size is represented as the ratio of the diameters of right to left legs in group mean percentages

Group	Dose (mg/kg)	% ratio of leg diameters[a]										
		Day 0	Day 2	Day 4	Day 7	Day 10	Day 14	Day 18	Day 22	Day 25	Day 31	Day 35
Vehicle	0	101 ± 2 (n=8)	100 ± 1 (n=8)	159 ± 15 (n=8)	381 ± 31 (n=8)	521 ± 23 (n=8)	713 ± 31 (n=6)	670 (n=1)	-[b]	-[b]	-[b]	-[b]
5-FU	10	101 ± 1 (n=8)	101 ± 1 (n=8)	118 ± 11* (n=8)	259 ± 29** (n=8)	377 ± 43** (n=8)	551 ± 48* (n=8)	713 ± 57 (n=6)	797 (n=1)	-[b]	-[b]	-[b]
CW 252053	30	100 ± 1 (n=8)	101 ± 1 (n=8)	121 ± 7** (n=8)	277 ± 27** (n=8)	374 ± 22** (n=8)	581 ± 64* (n=8)	678 ± 56 (n=6)	689 (n=1)	-[b]	-[b]	-[b]
	60	101 ± 1 (n=8)	100 ± 1 (n=8)	100 ± 2** (n=8)	100 ± 1** (n=8)	114 ± 35** (n=8)	172 ± 93** (n=8)	244 ± 152 (n=8)	322 ± 218 (n=8)	353 ± 241 (n=8)	254 ± 229 (n=5)	219 ± 100 (n=4)
	120	101 ± 2 (n=8)	100 ± 1 (n=8)	100 ± 1** (n=8)	100 ± 1** (n=8)	99 ± 1** (n=8)	100 ± 1** (n=8)	100 ± 1 (n=8)	100 ± 1 (n=8)	100 ± 1 (n=8)	100 ± 1 (n=8)	100 ± 1 (n=8)

[a]Data represent mean ± SD from the animals alive (numbered in parentheses) at the time of measurement.
[b]No animals remaining alive.
*,**Significantly different from the vehicle group at $p<0.01$ and 0.001, respectively.

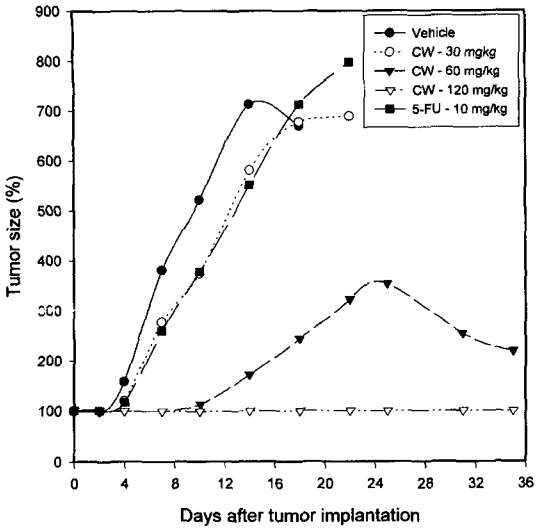

Fig. 2. Changes of tumor size in tumor-bearing B6D2F$_1$ mice.

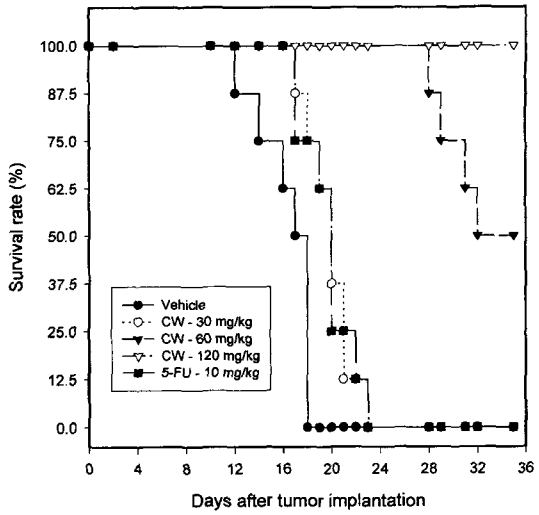

Fig. 3. Survival rates after implantation of LY3.7.2C TK-/- mouse lymphoma. The tumor was inoculated into the gastrocnemius muscle of the right hind leg on day 0 with 5 $\times 10^6$ cells per mouse. Test compounds were administered from day 1 to day 10, twice daily by *i.p.* injection.

considered to be cured if the diameter of the inoculated right leg on day 35 was less than or equal to that of the left leg.

Statistics

All results are presented as mean values from 8 mice groups and were compared using Students unpaired *t*-test.

RESULTS

The LY3.7.2C TK-/- cell line formed a solid tumor mass when implanted *i.m.* into the B6D2F$_1$ mice. Changes of tumor size for all groups are presented in Table I and Fig. 2. The tumor mass was visible on day 3 and thereafter grew rapidly in the vehicle control group. The tumor size in the 10 mg/kg of 5-FU treated group and in the 30 mg/kg of CW252053 treated group increased at a similar rate, albeit a lower one than in the vehicle control group. The increase in tumor size was significantly reduced in the 60 mg/kg of CW252053 treated group, and was completely suppressed in the 120 mg/kg of CW252053 treated group for the duration of the study. All animals in the vehicle, 5-FU, and low dose (30 mg/kg) CW252053 treated groups died between days 12 and 23 as a result of the tumor burden. In contrast, half of the test animals survived in the 60 mg/kg of CW252053 treated group, and all in the 120 mg/kg of CW252053 treated group, at the termination of study. The results of these survival rates are shown in Fig. 3, while the effects of CW252053 and 5-FU on tumor growth in B6D2F$_1$ mice implanted with LY3.7.2C TK-/- tumor cells are summarized in Table II.

Table II. Antitumor activity of CW252053 on B6D2F$_1$ mice implanted with LY3.7.2C TK-/- mouse lymphoma. The tumor was inoculated *i.m.* on day 0 with 5 $\times 10^6$ cells per mouse. Test compounds were treated twice daily by *i.p.* injection from day 1 to day 10. Observation was continued until day 35.

Group	Dose (mg/kg)	Survival Rate	Incidence of cure[a]	Survival time (days)[b]	ILS (%)[c]	Maximum weight loss (%)[d]
Vehicle	0	0/8(0%)	0/8(0%)	16.4 ± 2.3	-	ND[e]
5-FU	10	0/8(0%)	0/8(0%)	19.8 ± 2.2	21	ND[e]
CW252053	30	0/8(0%)	0/8(0%)	20.0 ± 1.9	22	1.0
	60	4/8(50%)	3/8(37.5%)	> 32.5	> 98	0.6
	120	8/8(100%)	8/8(100%)	> 35.0	> 113	1.9

[a]Cure is defined as occurring when the diameter of the right leg of the tumor-implanted mouse on day 35 was equal to or less than that of the left leg.
[b]Survival time is represented as mean ± SD.
[c]ILS indicates the increase in life span compared to the vehicle control group.
[d]Group mean maximum weight loss.
[e]ND, body weight is not decreased.

Although treatment with 10 mg/kg of 5-FU and 30 mg/kg of CW252053 did not produce a cure, it did slightly increase the life span. Dosing with 60 mg/kg of CW 252053 produced a 37.5% cure rate and reduced tumor size significantly. Dose with 120 mg/kg of CW252053 cured all tested animals and completely inhibited tumor growth until experiment termination on day 35. These results indicate that CW252053 has much better antitumor activity against tumor growth than 5-FU. Furthermore, even the high dose CW252053 administered in this study was not found to be toxic to the animals over the 35 day period of the study, judged on the basis of a transient body weight loss of less than 2% and an absence of any other signs of toxicity.

DISCUSSION

Rodent tumor models are typically refractory to TS inhibitor due to a high plasma level of thymidine (approximately 10-fold greater than that in humans) which is high enough to allow cancer cells to circumvent TS inhibition through a salvage process by thymidine kinase in the cells (Webber *et al.*, 1996). Thus, TK-deficient (TK-/-) mutant cell lines need to be used for this purpose. In this study, we investigated the antitumor efficacy of CW252053, a quinazoline-based antifolate TS inhibitor, against a solid tumor model transplanted with the LY3.7.2C TK-/- cell line. The results of this study show that the CW252053 compound exhibits excellent antitumor activity against the solid tumor model. In addition to its potent efficacy, CW252053 was well tolerated up to a dose of 120 mg/kg (twice daily for 10 days) without any adverse effects being observed over a 35 day period. We suggest that this drug represents an alternative antifolate antitumor agent to 5-FU.

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