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Growth inhibition of human tumor xenografts in nude mice by treatment with the antitumor agent 4,6-benzylidene-d_1-D-glucose (P-1013)

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4,6-Benzylidene-d_1-D-glucose, P-1013, a deuterated benzaldehyde derivative which acts as a reversible protein synthesis inhibitor *in vitro*, was evaluated for antitumor effects in two human tumor xenografts implanced s.c. in nude mice. The drug, dissolved in isotonic saline, was given p.o. daily for several weeks. For evaluation of drug efficacy, mean tumor volume growth curves were generated and tumor volume doubling time (TD) as well as per cent change in tumor size for the treated tumors compared to control (T/C) were calculated. P-1013 at 90 mg/kg for 49 days was effective against SK-OV-3 human ovarian carcinoma (T/C 47% at day 40 and 50% increase in TD). P-1013 at 90 mg/kg also suppressed growth of PANC-1 human pancreatic carcinoma in two experiments (T/C 44 and 50%, 20 and 40% increase in TD, respectively). There was no indication of systemic toxicity in mice receiving P-1013. Histological examinations of each tumor showed that P-1013 treatment of pancreatic xenografts reduced tumor volume without inducing greater necrosis than that comparable to respective control tumors. For the ovarian xenograft, the histological examination indicated a higher fraction of tumors with more than 50% necrotic tissue in two of the P-1013-treated groups compared with the control group (Fisher exact test, $p = 0.12$). It is possible that P-1013, in addition to inhibiting the rate of tumor volume growth, also induces tumor necrotization in the ovarian xenograft.

Key words: Benzylidene-glucose, deuterated benzaldehyde derivative, growth inhibition, nude mice, xenografts.

Introduction

Almost all drugs developed during the past four decades displaying activity against cancer also cause toxic side effects which limit the dose that can be administered and therefore limit the curative potential of all these drugs.[1] Although substantial progress has been made in chemotherapeutic drug design and implementation, there is clearly a need for developing new drugs for cancer therapy with novel mechanisms of action and low toxicities. Such a group of new drugs may be the benzaldehyde derivatives, which have received considerable attention due to their anticancer effects and low systemic toxicity.

Benzaldehyde is the anticancer principle isolated from figs used traditionally in the treatment of cancer.[2] Benzaldehyde and its derivatives, benzylidene-glucose (BG) and sodium benzylidene ascorbate, induce reversible inhibition of protein synthesis in human cells in culture.[3–5] This, in turn, gives rise to a reduction in cell cycle progression and subsequently cell inactivation,[5,6] and could be the primary mechanism for the antitumor effect. Several derivatives of benzaldehyde have been shown to induce antitumor effects both in animals[2,7] and humans,[8–12] although some studies show small effects.[13,14] In clinical trials with benzaldehyde derivatives no side effects were found in normal tissues.[8–13] A possible explanation for this is as follows. Since cancer cells are not able to respond properly to growth regulatory signals, they will not respond to the up-regulatory signals from the normal tissue exposed to protein synthesis inhibitors. Normal cells will compensate by increasing the rate of protein synthesis, while cancer cells will continue with the reduced rate of protein synthesis. If the treatment continues long enough, the shortage of vital proteins may become life-threatening to the cancer cells while normal cells are left unharmed.[15] This hypothesis presents a possibility to develop new cancer-specific chemical agents. However, little is known about the degree of effect induced by such chemicals on the tumor.

For benzaldehyde and sodium benzylidene ascorbate it has been reported that exchanging the hydrogen in the formyl group with a deuterium in-

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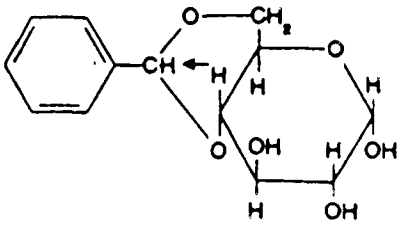

4,6-BENZYLIDENE-D-GLUCOSE (BG)

Figure 1. The chemical structure of 4,6-benzylidene-D-glucose (BG). The α isomer is shown, although the compound exists as both benzylidene-α- and benzylidene-β-glucose, when in aqueous solution. The hydrogen in the acetal group, marked with an arrow, is exchanged with a deuterium in P-1013.

duced increased effects on protein synthesis inhibition as well as an increase in cytotoxicity.[5,16] Since the exchange of hydrogen with deuterium increases the cellular effects of the benzaldehyde derivatives, it may also improve their tumor inhibiting properties, thus resulting in more powerful drugs.[17] The deuterated analog to sodium benzylidene ascorbate [^{2}H]zilascorb, has been shown to induce tumor necrosis in human tumor xenografts of the malignant melanoma and the ovarian carcinoma type,[15] and induce growth inhibition on prostatic carcinoma xenografts in nude mice.[18] In a phase I clinical trial [^{2}H]zilascorb induced no fever and no bone marrow-, nephro- or neurotoxicity.[19]

The purpose of this preclinical study was to evaluate the *in vivo* efficacy of the deuterated analog to the clinically tested benzaldehyde derivative 4,6-benzylidene-D-glucose (BG),[8–10,13] denoted P-1013 (Figure 1). P-1013 was tested in the treatment of two human cancer xenografts and the effect was compared with that of BG. The antitumor activity was measured by tumor volume growth curves and by histological evaluation of each tumor.

Materials and methods

Animals

Female, athymic mice (*nu nu*-BALB cABom) supplied from Bomholt Gård (Ry, Denmark) or female, athymic mice (of the *nu nu*-BALB c type) bred at the animal house at The Institute for Cancer Research, The Norwegian Radium Hospital (Montebello, Norway) were used in the experiments. The animals were housed in plastic cages, with sterilized wood chips bedding. The cages were kept in air-conditioned rooms with controlled temperature (22–24°C) and humidity (>50%), and with a 12 h light/dark cycle. The animals were given a commercial pelleted maintenance diet (RM3; SDS, Witham, Essex, UK) and tap water *ad libitum* with an autowatering system. All studies were conducted in conformity with the laws and regulations controlling experiments in live animals in Norway.

Drugs

BG and P-1013 (4,6-benzylidene-D-glucose undeuterated and deuterated, respectively), Figure 1, were provided by Norsk Hydro Research Centre (Porsgrunn, Norway). BG and P-1013 contain 0.1% benzaldehyde and, when in aqueous solution, two anomers (benzylidene-α- and benzylidene-β-glucose) are present at a ratio of 24.5 and 75.5%, respectively. The degree of deuteration in the acetal group of P-1013 was 99%. P-1013 is prepared from deuterated benzaldehyde dimethylacetate and D-glucono-δ-lactone as described earlier.[17]

Tumor lines

The PANC-1 cell line, derived from a pancreaticoduodenectomy specimen removed from a patient,[20] and the SK-OV-3 line, established upon cultivation of cells from the ascitic fluid of a patient with an ovarian tumor,[21] were purchased from the American Tissue Culture Collection (Rockville, MD) and cultivated shortly *in vitro* before being implanted into nude mice. The tumor lines were passaged as s.c. implants in nude mice.

Tumor transplantation, measurement and treatment

Mice were 8–9 weeks of age at the time of tumor implantation. Small tumor pieces were implanted s.c. on the left flank of the animals. Tumor growth was followed by measuring two perpendicular diameters using calipers and tumor volume was estimated by the formula: volume = (length × width^2)/2. Animals with growing tumors (tumor volumes 25–110 mm^3) were randomly assigned to drug-treated or control groups, such that there were 8–10 animals/group, with the average tumor size among the groups being approximately equal (about 50–75 mm^3). During treatment, tumor volumes were measured every 2–3 days and weight was recorded once a week. Drug treatment was given daily by p.o. administration of 0.2 ml of a solution of drug dissolved in isotonic saline. The control group was administered 0.2 ml isotonic saline at the same in-

terval as the treated groups. Oral administration was carried out by gastric intubation with a blunt steel canula. Animals were routinely sacrificed when the longest tumor diameter reached 20 mm. Treatment within one group was continued until the first animal had to be sacrificed due to this criteria. Of the 112 mice totally included in the experiments, nine had to be sacrificed due to illness caused by other reasons than P-1013 treatment, such as eye catarrh, eczema and rectum prolapse. These mice were excluded from the analysis.

Evaluation of response

Treatment efficacy was assessed by three evaluation criteria used in parallel.

Firstly, by tumor volume growth curves. The tumor sizes were standardized in the different groups by obtaining relative tumor volume (RV) calculated by the formula RV = V_x/V_1, where V_x is the tumor volume at day x and V_1 is the initial volume at the start of treatment (day 1). An exponential curve was fitted to the relative tumor volume growth data for each individual animal and the interval during which each tumor increased to twice its volume, tumor volume doubling time (TD), was determined from the fitted curve ($\log_e 2/k$, where k is the estimated rate constant for the process). For each group of treated or control tumors the mean time to double was calculated. The mean volume with standard errors for each treatment group were plotted as a function of time to obtain the tumor growth curves. Significant differences were determined using Student's t-test (two groups) or ANOVA following Bonferroni's t-test (several groups).

Secondly, the per cent change in tumor size (T/C) was calculated according to the formula:

$$\%T/C = \frac{\text{mean relative tumor volume of treated mice}}{\text{mean relative tumor volume of control mice}} \times 100$$

Thirdly, each separate tumor was evaluated histologically. All tumors were examined macroscopically and measured before two to four representative sections were taken from each tumor and processed for light microscopy. The specimens were fixed in 5% buffered formalin, dehydrated and embedded in paraffin. Sections (6–8 μm thick) cut from the paraffin blocks were stained with hematoxylin and eosin, and used for light microscopical evaluation. The light microscopic features were compared with the observations made at the time of macroscopic examination and a semi- quantitative evaluation of the degree of tumor necrosis compared to vital tumor tissue was made. Both the macroscopic and the microscopic examinations were done blindly.

Results

Tumor volume growth curves for the SK-OV-3 ovarian carcinoma and the PANC- 1 pancreatic carcinoma xenograft grown in nude mice are shown in Figure 2. From the growth curves it can be seen that P-1013, following p.o. administration on a daily schedule, demonstrated growth inhibitory effects in both the PANC-1 and SK-OV-3 xenograft, measured by an increase in the tumor volume doubling time (TD) and a T/C < 50% (Table 1). The dose–response experiment with the SK-OV-3 xenograft

Table 1. TD and T/C values in human tumor xenografts grown in nude mice, treated daily with BG or P-1013 at doses indicated

Type of tumor	Drug	Dose (mg/kg day)	TD ± SE	T/C (%)	Treatment days
SK-OV- 3	control		13 ± 1		40
	P-1013	30	16 ± 3	76	49
	P-1013	90	19 ± 2[b]	47[c]	49
	P-1013	200	15 ± 2	100	40
PANC-1	control		21 ± 2		82
(p5)[a]	P-1013	90	25 ± 3	44[c]	82
PANC-1	control		13 ± 1		64
(p12)[a]	P-1013	90	18 ± 2[b]	50[c]	64
	BG	90	15 ± 1	73	64

[a] Tumor passage number.
[b] Indicates significant differences between treated group and control group.
[c] Indicates antitumor activity (T/C < 50).

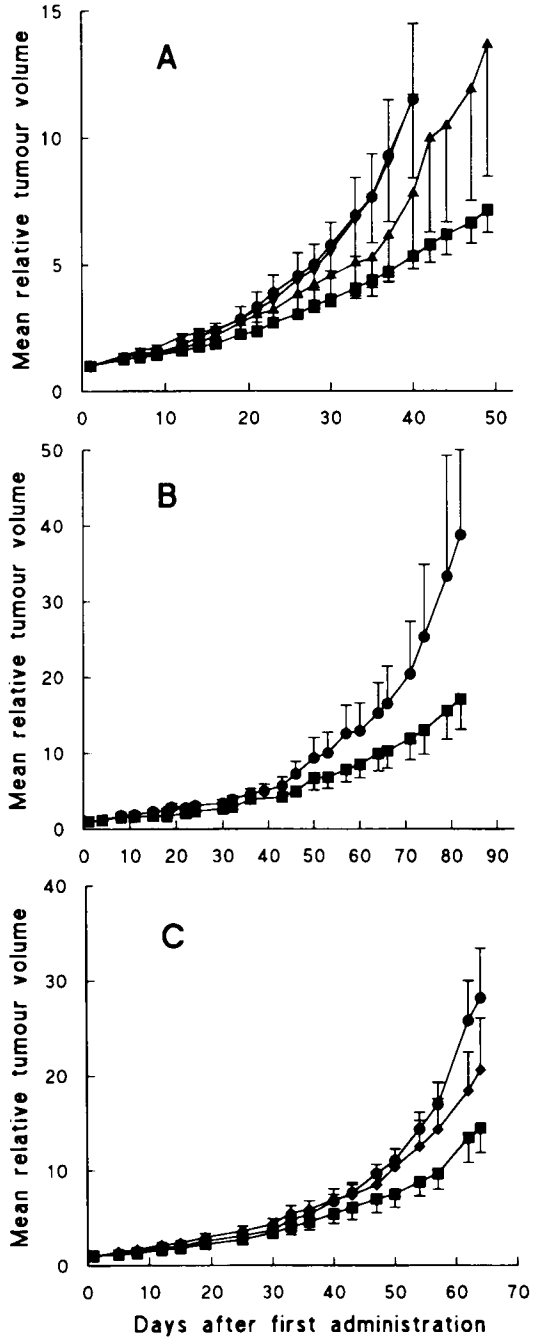

Figure 2. Mean tumor volume growth curves of tumor lines SK-OV-3 ovarian carcinoma xenograft (A) and PANC-1 pancreatic carcinoma xenograft passage 5 (B) and passage 12 (C). Mice were treated daily p.o. with 30 mg/kg P-1013 (▲), 90 mg/kg P-1013 (■), 200 mg/kg P-1013 (▼) (coincides with the growth curve for the control group) and 90 mg/kg BG (◆). ●, Control group received 0.9% NaCl. Each point represents the mean tumor volume of seven to 10 mice related to tumor volume at day 1. Vertical bars represent standard error.

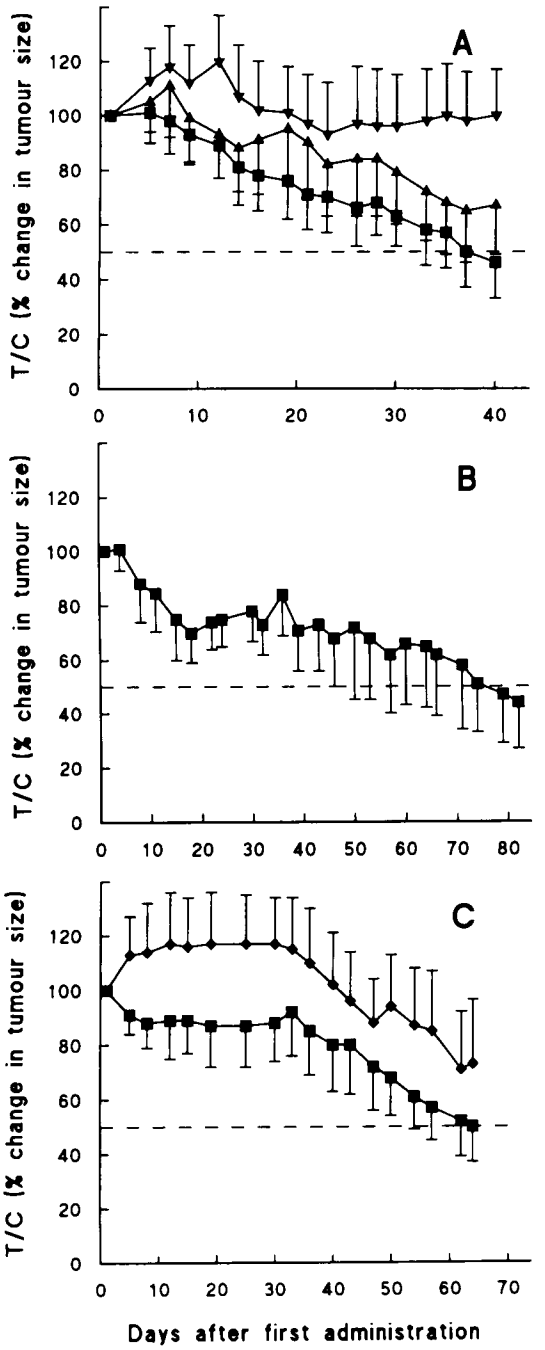

Figure 3. The percent change in tumor size of the treated groups related to the control group (T/C) plotted as a function of time for the SK-OV-3 ovarian carcinoma xenograft (A) and the PANC-1 pancreatic carcinoma xenograft passage 5 (B) and passage 12 (C).▲, T/C for group receiving 30 mg/kg P-1013; ■, T/C for the groups receiving 90 mg/kg P-1013; ▼, T/C for the group receiving 200 mg/kg P-1013; ◆, T/C for the group receiving 90 mg/kg BG. Each point represents the mean of seven to 10 mice. Vertical bars represent standard error. Stippled line indicates T/C = 50%.

Table 2. Histological evaluation of the fractional amount of necrotic tissue in the tumors after treatment

Tumor type	Drug	Dose (mg/kg day)	Number of animals[a]				
			1+	2+	3+	4+	Total
SK-OV-3	control		0	5	3	0	8
	P-1013	30	1	0	4	2	7
	P-1013	90	0	1	6	0	7
	P-1013	200	1	2	3	2	8
PANC-1	control		1	5	2	0	8
(p5)[b]	P-1013	90	0	8	2	0	10
PANC-1	control		3	4	1	0	8
(p12)[b]	P-1013	90	0	5	3	0	8
	BG	90	0	3	4	0	8

[a] A semi-quantitative evaluation of necrosis compared to vital tumor tissue was made as follows 1+ = 0–25% necrotic tumor volume; 2+ = 25–50% necrotic tumor volume; 3+ = 50–75% necrotic tumor volume; 4+ = 75–100% necrotic tumor volume.
[b] Tumor passage number.

Table 3. Mean body weight (g) of animals treated by daily oral administration starting at day 1

Treatment	Time after first injection (days)				
	0	14	28	42	64
Control	25.9 ± 0.8	25.3 ± 0.7	26.0 ± 0.8	26.6 ± 0.9	26.8 ± 0.9
P-1013	25.3 ± 0.4	25.0 ± 0.4	25.4 ± 0.4	25.8 ± 0.5	26.0 ± 0.4
BG	25.7 ± 0.6	25.1 ± 0.7	25.8 ± 0.9	27.5 ± 0.8	27.6 ± 0.7

(Figure 2A) showed that the greatest inhibitory effect was found in mice receiving 90 mg/kg P-1013. In Figure 2(C) the undeuterated analog to P-1013, BG, is also included to compare the effects of the two drugs. It seems that P-1013 induces greater growth inhibitory effects than BG. Data obtained from the growth curves are shown in Table 1 and Figure 3. As can be seen in Figure 3, the %T/C decreases continuously with time.

We have repeated the experiment with the PANC-1 xenograft to see if the effect of P-1013 is reproducible. There are seven tumor passages between the first and the second experiments. As can be seen in Table 1, the tumor volume doubling times are different in the two experiments, but the antitumor effect induced by P-1013 is demonstrated in both experiments.

The extent of necrosis in each tumor was evaluated in order to determine whether the reduced tumor volume growth was due to only growth inhibitory effects or to cell inactivation. An overview of the extent of necrosis estimated in the experiments is given in Table 2. For the SK-OV-3 xenograft there is a tendency of a higher fraction of necrotic tissue in the tumors treated with 30 and 90 mg/kg P-1013 than in the control tumors. A statistical testing of the difference between the control group and these two dosing groups, concerning tumors with more than 50% necrotic tissue, gives $p = 0.12$ (Fisher exact test). P-1013 or BG treatment did not lead to an significant increase in the degree of necrosis in the PANC-1 xenografts, although there was a tendency of a higher fraction of necrotic tissue in the treated tumors compared with control tumors for the 12th passage of the PANC-1 xenograft. The morphological appearance of some typical SK-OV-3 tumors are shown in Figure 4. In all the P-1013-treated tumors a characteristic vital peripheral rim can be seen with more or less central necrotic areas. The morphological appearance of the viable tumor cells in the P-1013-treated tumors cannot be distinguished from the viable tumor cells in the placebo-treated tumors.

During treatment, animal body weight was recorded once a week. In Table 3 a representative experiment (PANC-1, passage 12) showing the development of the body weight during the treatment period is shown. No untoward effects giving rise to a body weight reduction were seen even after 64 days of daily p.o. administration of P-1013.

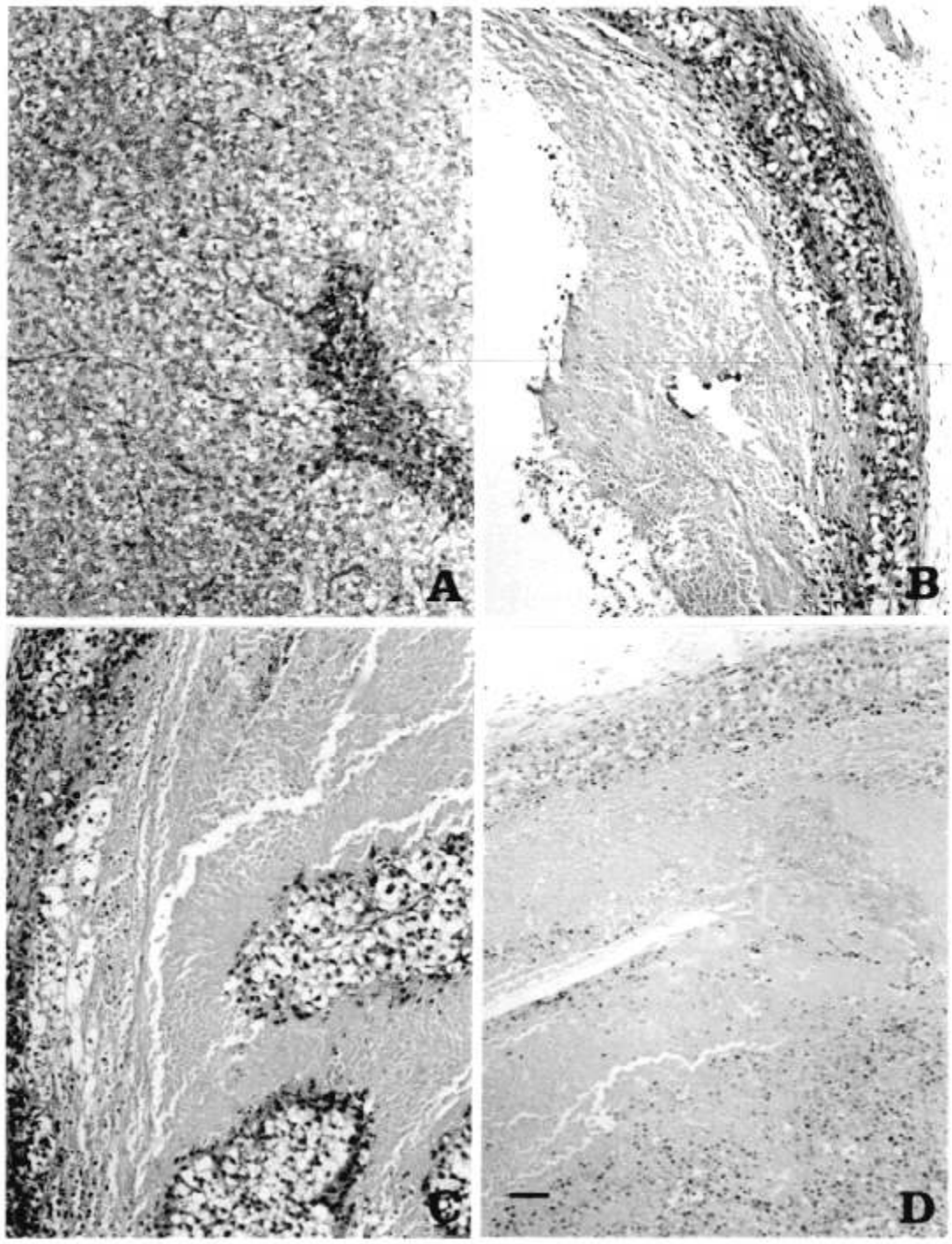

Figure 4. Morphological appearance of typical SK-OV-3 tumors from each of the four groups of animals. (A) Placebo group. Tumor volume: 816 cm^3. Large vital tumor areas. (B) P-1013 30 mg/kg. Tumor volume: 30 cm^3. Small tumor with extensive necrosis. Only a peripheral rim with vital tumor tissue is present. (C) P-1013 90 mg/kg. Tumor volume: 91 cm^3. Relatively small tumor with extensive necrosis. A peripheral rim and also foci with vital tumor tissue is present. (D) P-1013 200 mg/kg. Tumor volume: 91 cm^3. Relatively small tumor with extensive necrosis and a thin rim of vital tumor tissue in the periphery. (Hematoxylin and eosin. Bar = 54 μm.)

Discussion

The LD_{50} of BG in mice is more than 3000 mg/kg by oral administration.[9] In clinical trials daily doses on the order of 40 mg/kg have been given.[8,9,13] A daily dose of 85 mg/kg BG given to rats was therapeutically effective.[7] On the basis of this a dose range of 30–200 mg/kg P-1013 was chosen for the experiment with the SK-OV-3 xenograft. From Figures 2 and 3 and Table 1 it can be seen that a dose of 90 mg/kg P-1013 had optimal effectiveness. Doses of 30 and 200 mg/kg were less effective. Consequently, a p.o. dose of 90 mg/kg was selected for the PANC-1 experiments.

The human tumor xenograft grown in immunodeficient mice is a widely used model for drug testing. According to Golding *et al.*,[22] a criteria used by NCI for positive tumor response to therapy is any treatment group in which the tumor volume is reduced to less than 42% relative to the control group (T/C) at any time during a specific range of days after the last treatment day. The lowest T/C value observed is regarded as the optimal one. Boven *et al.*[23] uses T/C = 50% as a criteria for response. The use of the T/C ratio reflects the way in which established chemotherapeutics exert their effect, i.e. by initially reducing the tumor volume beyond its pretreatment value, but sooner or later the tumor starts to regrow and the T/C ratio increases.

As seen in Figure 3, P-1013 and BG exert their effects in a different manner. We observe that the T/C ratio decreases continuously with time. The lowest T/C ratio is always obtained at the last day when mice in both the control group and the treated groups are alive. From Figure 3 there is reason to believe that the T/C ratio would decrease further upon continued treatment. The endpoint for treatment was based upon humanitarian reasons, the tumor volume of some animals growing too large. Table 1 shows that treating the SK-OV-3 zenograft for 40 days with 90 mg/kg P-1-13 resulted in T/C = 47%. For the PANC-1 xenograft a P-1013 treatment (90 mg/kg) for 82 days resulted in T/C = 44% and the same treatment for 64 days resulted in T/C = 50%. Since P-1013 treatment gives T/C ratios at the last day of treatment comparable to the T/C ratios suggested as criteria for response, and a decreasing T/C with time, we believe that our results indicate that P-1013, given at this daily dose, has an antitumor effect against the SK-OV-3 and the PANC-1 xenografts.

A significant difference was found in the doubling time, TD, for the SK- OV-3 ovarian carcinoma xenograft treated daily with P-1013 (90 mg/kg) compared with the control ($p = 0.016$) (Table 1). This treatment led to a 50% increase in TD, the TD increased from 13 to 19 days. P-1013 treatment with 30 mg/kg daily resulted in 23% increase in TD and 200 mg/kg resulted in 15% increase in TD. Significant difference was also found in TD for the PANC-1 pancreatic carcinoma passage 12 treated with 90 mg/kg P-1013 ($p = 0.042$). Here a 38% increase in TD was obtained. In the experiment with PANC-1 passage 5 no significant difference were found in TD, an increase in TD of only 16% was obtained, although a low T/C ratio (44%) suggests antitumor activity (Table 1).

In most experiments, treatment was terminated in all groups on the same day, i.e. on that day when the tumor burden in the control group reached the upper limit. However, in the experiment with SK-OV-3 we continued treatment in the group receiving 30 mg/kg P-1013 after the control group had been terminated due to heavy tumor burden. The treatment was continued until the tumor burden in the P-1013-treated group reached the same upper limit (Figure 2). This led to an 'increase in life-span' of 23% compared with the control group. The treatment of the group receiving 90 mg/kg P-1013 was also continued as long as for the group receiving 30 mg/kg P-1013.

The histological examinations (Table 2 and Figure 4) show that for the SK-OV-3 ovarian carcinoma xenograft treated with 30 and 90 mg/kg P-1013 there is an indication of a higher fraction of treated tumors with more than 50% necrotic tissue than the control tumors, although it is not significant (p=0.12, Fisher exact test). This finding should be considered in light of the observations that the tumors treated with 90 mg/kg P-1013 have a smaller volume than the placebo-treated tumors at the time of fixation, thus probably underestimating the drug-induced necrotization. We therefore believe that P-1013 induces necrotization in the SK-OV-3 xenograft. The histological examinations of the PANC-1 passage 5 xenografts do not indicate a higher fractional amount of necrosis in the P-1013 tumors than in the control tumors. In the experiment with PANC-1 passage 12 there is a tendency of a higher fraction of treated tumors with more than 50% necrotic tissue compared with the control tumors, especially for the BG treated tumors. However, the differences are not statistically significant.

Both animal experiments and clinical trials indicate that the treatment time needed with benzaldehyde derivatives could well be several months.[15] For instance, the effect of P-1013 on the PANC-1 xenograft is not seen until the tumors have been

treated for 30–40 days (Figure 2). We believe that the mild protein synthesis inhibition induced by the benzaldehyde derivatives might need to be imposed on the cells for a considerable time before lack of vital proteins may become life threatening. In a study where no effect of BG on *in vivo* growth of two human tumor xenografts was reported, the treatment was continued for only 30 days.[14] One possibility is, of course, that BG induces no growth inhibitory effects in these two tumor xenografts. Another possibility is that the treatment time was too short to detect an eventual effect.

P-1013 appears to have an unusual dose-response. In the experiment with the SK-OV-3 xenograft (Figure 2A) we observe a growth inhibitory effect upon daily treatment with P-1013 at a dose of 30 mg/kg, a greater inhibition with 90 mg/kg and no inhibition with 200 mg/kg. There is no current explanation for this anomaly.

One expected consequence of exchanging a hydrogen with a deuterium is that the compound becomes more stable, due to the higher zero point energy of the C–D bond compared with the C–H bond. The greatest effect is expected in reactions where the C–D bond is broken, referred to as a primary kinetic isotope effect. Secondary isotope effects refer to reactions where cleavage of the deuterium bond is not directly involved in the rate-determining step and are normally much smaller than the primary kinetic isotope effect.

A deuterium isotope effect has been observed in rats, but not in dogs and mice, in *in vivo* pharmacokinetic studies on BG and P-1013. The observed effect is in the first step of P-1013 metabolism and does not involve a cleavage of the C–D bond, therefore it is probably a secondary isotope effect. Complete metabolism of P-1013 apparently involves a step where the C– D bond is cleaved, thereby inducing a primary isotope effect.[24] The growth inhibitory effects of P-1013 and BG are compared on PANC-1 xenografts passage 12 grown in nude mice. Figure 2(C) shows that P-1013 seems to induce a greater growth inhibitory effect than BG given daily at the same dose, which may reflect a deuterium isotope effect. The T/C ratio for BG after treatment for 64 days is 73%, while for P-1013 it is 50%. The difference in TD for the P-1013 treated group compared with the control group is statistically significant, while this is not the case for BG. Thus, the results indicate that P-1013 induces stronger biological effects than BG *in vivo*.

Our results show that P-1013 induces antitumor effects with a mechanism different from those of the established chemotherapeutic drugs, in that the T/C value decreases continuously with time without any tumor regrowth. Generally, drug testing with different types of xenotransplanted tumors has shown that the response of xenografts obtained in immunodeficient mice is comparable to that in clinical practice.[25–28] We observe that P-1013 induces growth inhibitory effects in both the SK-OV-3 ovarian carcinoma xenograft and the PANC-1 pancreatic carcinoma xenograft without inducing systemic toxicity. In the present experiments, no signs of any side effects were seen in the animals. No difference in the body weights between drug treated and control animals were observed (Table 3). We believe that a growth inhibition, if it is induced in the tumor only, may give a significant contribution in the treatment of many cancer patients. Pancreatic carcinoma is notorious for its relative resistance to available treatment. At best, conventional therapy offers a few months prolongation of life and some palliation of symptoms. Based on our current results, P-1013 should be further tested against pancreatic adenocarcinoma, ovarian carcinoma and other human cancer types as a drug without significant clinical toxicity. Since oral administration is possible,[29] the drug is well suited for long-term treatments.

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