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Antitumor effects of ketoconazole and trifluoperazine in murine T-cell lymphomas

S. Naftalovich[1], E. Yefenof[2], and Y. Eilam[1]

The Departments of [1] Bacteriology and [2] Immunology,
The Hebrew University-Hadassah Medical School, Jerusalem, Israel

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Summary. In vitro and in vivo antitumor effects of ketoconazole (KTZ), trifluoperazine (TFP), and combinations of both drugs were examined in cell lines established from radiation leukemia virus (RadLV)-induced T-cell lymphomas. KTZ inhibited [^{3}H]-thymidine incorporation in the tumor cells in vitro; 50% inhibition of DNA synthesis was observed at concentrations of 4–7 μg/ml. [^{3}H]-thymidine uptake in bone-marrow and spleen cells prepared from healthy mice was also inhibited by KTZ, but 50% inhibition was observed only at a concentration of 50 μg/ml. Stimulation of spleen cells with concanavalin A led to an increase in their sensitivity to the inhibition of DNA synthesis by KTZ. The tumor-cell lines varied in their sensitivity to the inhibition of DNA synthesis by TFP, and the effects of TFP on DNA synthesis in bone-marrow and spleen cells were similar to those observed in the tumor cells. Synergistic, additive, or less than additive effects of the drug combinations on the inhibition of DNA synthesis in vitro were observed both in tumor cells and in bone-marrow cells. In vivo experiments were conducted on groups of C57BL/6 (B6) mice that were inoculated s.c. with tumor cells and then treated with i.p. injections of KTZ, TFP or both. Control groups were injected with phosphate-buffered saline (PBS). Each of the drugs alone as well as their combinations caused a significant delay in the appearance of palpable tumors, a decrease in tumor size, and a marked prolongation of survival. The concentrations of the drugs used in in vivo experiments did not affect the WBC counts in the peripheral blood of healthy mice. KTZ is currently used for the treatment of prostatic cancer because of its inhibitory effect on testosterone biosynthesis. The results of the present study indicate the hormone-independent chemotherapeutic potential of KTZ, TFP, and combinations of the two drugs.

Offprint requests to: Y. Eilam, Dept. of Bacteriology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

Introduction

Ketoconazole (KTZ) is an oral antifungal drug that shows a broad specificity [4]. In fungi, KTZ inhibits the cytochrome P-450-dependent conversion of lanosterol to ergosterol, which is the principle sterol in fungal cell membranes [37]. In mammals, it has been shown to inhibit testicular, adrenal, and ovarian steroid biosynthesis [12, 23]. This activity is most probably mediated by inhibition of the cytochrome P-450-dependent enzyme C17,20-lyase, which cleaves the side chain from the steroid nucleus [27]. This effect has led to the use of KTZ in the treatment of prostatic cancer [30, 36, 40] and Cushing's syndrome [10]. Cytotoxicity has recently been reported for KTZ in several malignant cell lines in vitro [33], but its effects on normal control cells have not been tested.

Based on the central role of the Ca^{2+}-calmodulin complex in the regulation of cell proliferation [27, 32], anticalmodulin drugs such as calmidazolium, chlorpromazine, and trifluoperazine (TFP) have been purported to have chemotherapeutic potential against cancer cells [21]. Indeed, several studies have indicated that anticalmodulin drugs are toxic to various tumor cells [19, 24, 39] and are selectively toxic to cycling vs noncycling cells in vitro [5]. In contrast, the growth of malignant epidermal keratinocytes in culture was not inhibited by TFP at doses that inhibited the growth of normal epidermal keratinocytes [17]. The selective inhibition of normal vs tumor cells was attributed to the higher content of calmodulin in the latter [7]. However, TFP and other anticalmodulin drugs have been found to reverse the resistance of malignancies to antineoplastic drugs and to potentiate the effects of chemotherapeutic drugs (for review see [35]). Recently, phase I/II clinical trials combining TFP with conventional anticancer chemotherapy have been carried out; positive responses were noted in several patients who had previously exhibited resistance to chemotherapy [28]. These results encouraged us to examine the effects of KTZ, TFP, and combinations of both drugs on in vitro and in vivo growth in the murine T-cell lymphoma model as compared with their effects on normal bone-marrow and spleen cells.

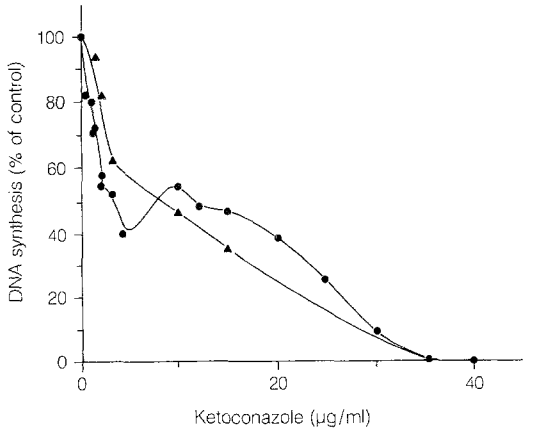

Fig. 1. Effect of KTZ on DNA synthesis in vitro in cell lines established from RadLV-induced lymphomas. Cells of the lines C41 (●) and YAB1 (▲) were incubated in a 96-well microtiter plate (10^5 cells/well) in RPMI-1640 medium containing 10% FCS and antibiotics in the absence or presence of different concentration of KTZ. The incubation was continued for 24 h at 37° C in a humidified atmosphere of 5% CO_2 and 95% air. DNA synthesis activity was determined from the uptake of [^{3}H]-thymidine (1 μCi/well) over 6 h. Thymidine uptake in the presence of KTZ was calculated as a percentage of the thymidine uptake in the drug-free controls

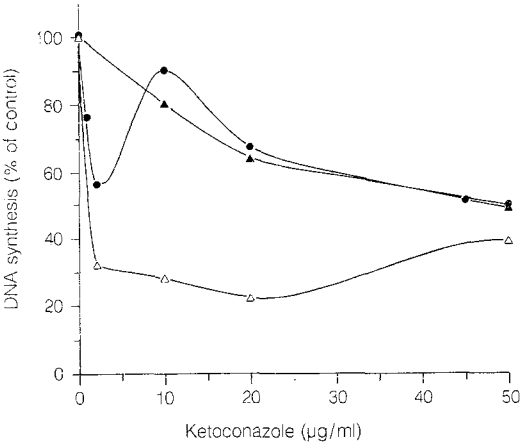

Fig. 2. Effect of KTZ on DNA synthesis in vitro in bone-marrow and spleen cells derived from healthy mice. Experiments were conducted as described in Fig. 1, but bone-marrow cells (●) and spleen cells (▲) prepared from healthy mice were examined. Spleen cells were also examined after stimulation with Con A (5 μg/ml) for 12 h prior to the addition of [^{3}H]-thymidine (△). The results are expressed as a percentage of the thymidine incorporation in the corresponding drug-free controls

The results suggest that KTZ, TFP, and combinations of these two drugs may be considered to be potential anticancer chemotherapeutic drugs.

Materials and methods

Animals. C57BL/6/(B6) female mice obtained from the animal facilities of the Hebrew University-Hadassah Medical Center were used.

Cells. C41, SR2, YAB1, and YAB9 cell lines were established from radiation leukemia virus (RadLV)-induced thymic lymphomas as described elsewhere [2]. The lines were maintained in RPMI-1640 medium containing 10% fetal calf serum (FCS), 100 IU penicillin/ml, and 100 μg streptomycin/ml. Suspensions of spleen and bone-marrow cells were prepared from normal B6 mice aged 6–8 weeks.

Drug-induced cytotoxicity in vitro. Triplicate cultures of spleen, bone-marrow, or RAdLV-transformed cells were incubated in a 96-well microtiter plate (10^5 cells/well) with various concentrations of KTZ, TFP, or combinations thereof for 24–36 h at 37° C in a humidified atmosphere of 5% CO_2 and 95% air. The cultures were pulsed with [^{3}H]-thymidine (1 μCi/well) for 6 h and then harvested on fiberglass filters using a multiple-sample harvester (Titertek). The filters were air-dried and the incorporation of [^{3}H]-thymidine was measured by scintillation counting. [^{3}H]-Thymidine incorporation in the presence of the drugs was calculated as a percentage of the [^{3}H]-thymidine incorporated in the drug-free controls. The values shown in Figs. 1–5 represent the means of triplicate determinations; each experiment was repeated at least three times, resulting in essentially similar findings.

Drug-induced antitumor effects in vivo. Lymphoma cells were inoculated s.c. into B6 mice (10^5 cells/mouse). TFP and KTZ were injected i.p. at the indicated doses starting at 2 days after tumor-cell inoculation. The drugs were given daily for cycles of 5 days followed by 2-day intervals until the end of the experiments. The drug concentrations used were selected on the basis of preliminary experiments. The dose of each drug given in the drug-combination experiment was half that used for single-agent administration. This protocol enabled the evaluation of responses to the drug combination in comparison with responses to each individual drug. Control mice were injected with phosphate-buffered saline (PBS). The mice were examined daily for both the appearance and the size of palpable tumors and for survival.

Drug toxicity in vivo. To evaluate the toxic effect of TFP and KTZ on hematopoiesis, mice treated with the drugs were bled once a week and the WBC count in the peripheral blood was determined using gentian violet staining.

Results

In vitro inhibition of [^{3}H]-thymidine incorporation

Previous results in our laboratory have indicated that [^{3}H]-thymidine incorporation is a sensitive and reliable assay for the inhibition of lymphocyte growth [41]. Marked inhibition of DNA synthesis by KTZ as measured by [^{3}H]-thymidine incorporation was observed in the four malignant cell lines examined. At a concentration of 35 μg/ml, KTZ induced 100% inhibition in all cell lines investigated. Figure 1 shows the dose-response curves for the inhibitory effect of KTZ in two malignant cell lines (C41 and YAB1). Biphasic dose-response curves were obtained; a rapid decrease in thymidine incorporation was observed at lower concentration ranges, reaching 50% inhibition at concentrations of 4 and 7 μg/ml in cell lines C41 and YAB1, respectively. At higher KTZ concentrations, the increase in drug concentration led to a slower decrease in DNA synthesis activity. The sensitivity of normal cells to the inhibition of DNA synthesis by KTZ was much lower than that observed in malignant cells; both the spleen cells and the bone-marrow cells maintained 50% of DNA synthesis on exposure to KTZ at 50 μg/ml (Fig. 2). Stimulation of the spleen cells with concanavalin A (Con A) increased their sensitivity to KTZ; in Con A-stimulated cells, exposure to KTZ at 3 μg/ml inhibited 70% of DNA synthesis, but the

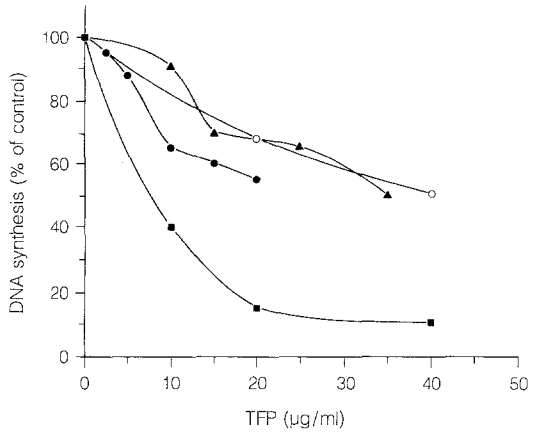

Fig. 3. Effect of TFP on DNA synthesis in vitro in cell lines derived from RAdLV-induced lymphomas. Experiments were conducted as described in Fig. 1, but different concentrations of TFP instead of KTZ were added to the cells. Lines C41 (●), SR2 (▲), YAB9 (○) and YAB1 (■) were examined

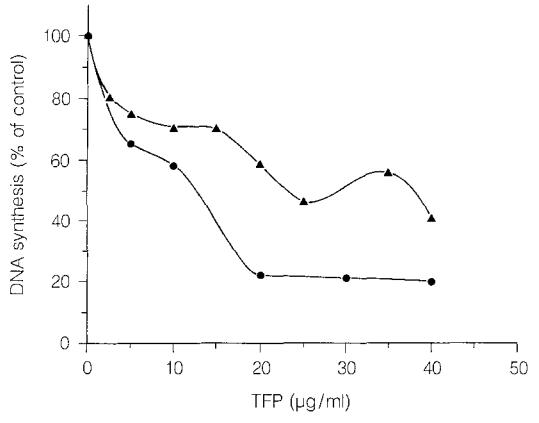

Fig. 4. Effect of TFP on DNA synthesis in vitro in bone-marrow cells and spleen cells. Experiments were conducted as described in Fig. 2, but different concentrations of TFP instead of KTZ were added to the cells. Spleen cells (●) and bone-marrow cells (▲) prepared from healthy mice were examined

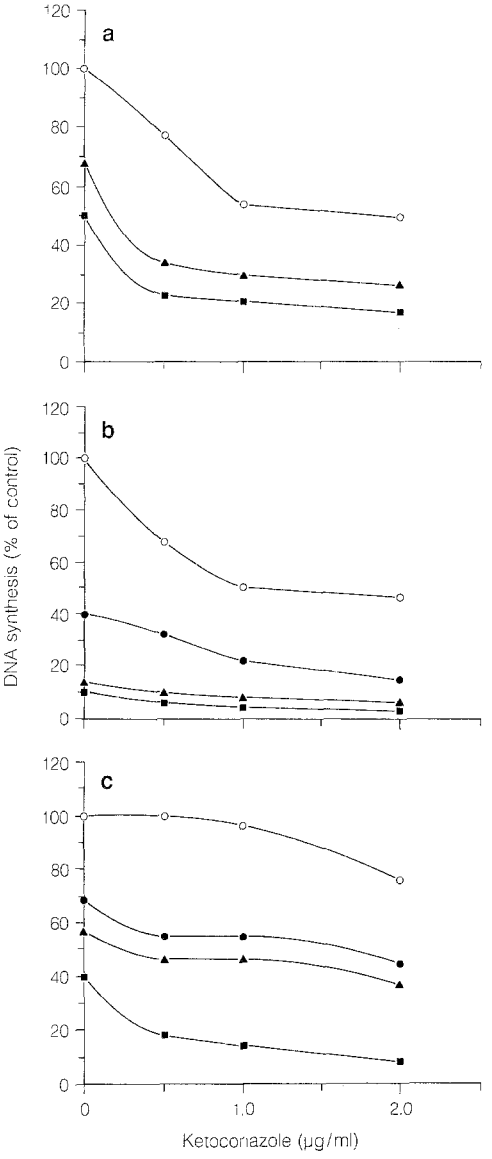

Fig. 5a–c. Effects of combinations of TFP and KTZ on DNA synthesis in malignant and normal cells. Experiments were conducted as described in Figs. 1 and 2, but different concentrations of KTZ alone (○) or in combination with TFP at 10 μg/ml (●), 20 μg/ml (▲), or 40 μg/ml (■) were added to the cells. Lines YAB9 (**a**) and YAB1 (**b**) as well as normal bone-marrow cells (**c**) were tested. The results are expressed as a percentage of the [^{3}H]-thymidine uptake in the corresponding drug-free controls

residual 30% of DNA synthesis activity was not affected by KTZ at concentrations of up to 50 μg/ml.

The dose-response curves for inhibition of DNA synthesis by TFP showed a significant variability among the malignant cell lines examined (Fig. 3). Whereas YAB1 cells were most sensitive to the inhibitory effect of TFP [concentration for 50% inhibition (IC_{50}) = 8 μg/ml], cell lines SR2 and YAB9 reached 50% inhibition only after exposure to TFP at 40 μg/ml. Stronger inhibitory effects were observed in normal spleen cells (IC_{50} = 13 μg/ml] and in bone-marrow cells (Fig. 4).

The combined effects of TFP and low concentrations of KTZ on the inhibition of DNA synthesis were examined in two malignant cell lines (YAB1, which showed the highest sensitivity to TFP, and YAB9, which displayed the lowest) as well as in bone-marrow cells. Inhibition of DNA synthesis (expressed as a percentage of the control value) by the drug combination was compared with that exerted by each of the individual drugs at the same concentration that was used for the drug-combination treatment (all measurements were done in the same experiment). A synergistic, additive, or less than additive effect was indicated when the inhibition by the drug combination was respectively higher than, equal to, or lower than the sum of the inhibition values achieved by the individual drugs. In YAB9 cells, the inhibitory effect of KTZ at 0.5 μg/ml was synergistically enhanced by the addition of TFP; at higher concentrations, the inhibitory effect was less than additive (Fig. 5a, Table 1). In YAB1 cells, exposure to TFP at 10 μg/ml caused 60% inhibition; the combined effect of TFP at 10 μg/ml with KTZ at 0.5, 1, or 2 μg/ml was less than additive (Fig. 5b, Table 1). Bone-marrow cells displayed lower sensitivity to inhibition by KTZ; a concentra-

Table 1. Effects of combinations of TFP and KTZ on DNA synthesis in tumor-cell lines and in normal bone-marrow cells

Type of cells	KTZ (μg/ml)	TFP (μ/ml)	Inhibition of DNA synthesis (%)
YAB9	0.5	–	22±2
	–	20	32±2
	0.5	20	67±3[a]
	2		53±3
	2	20	76±3[b]
YAB1	0.5	–	31±1
	–	10	60±2
	0.5	10	69±3[b]
Bone marrow	0.5	–	0±1
	–	10	31±2
	0.5	10	47±2[a]
		40	60±3
	0.5	40	83±3[a]

Data represent mean values ± SD ($n = 6$)

[a] Synergistic inhibition of DNA synthesis: the inhibition by the drug combination is significantly higher than the sum of inhibition values achieved by the individual drugs ($P \leq 0.05$; Student's t-test)

[b] Less than additive inhibition of DNA synthesis: the inhibition by the drug combination is significantly lower that the sum of inhibitions values achieved by the individual drugs ($P \leq 0.05$; student's t-test)

Table 2. Effects of KTZ and TFP on hematopoiesis in normal mice

Drug	Days after the initiation of treatment	Number of white blood cells/ml blood (mean $\times 10^{-5} \pm$ SD)
PBS	0	55.5± 5.4
	13	66.1±15
	21	61.7±12.7
KTZ	0	60.6± 3.2
	13	59.3± 9.3
	21	60.6± 2.9
TFP	0	61 ± 1.63
	13	57.8±10.8
	21	56.5±12.9
TFP + KTZ	0	41.3± 7.5
	13	44.1±13.9
	21	54.2±12.6

Groups of B6 mice were injected i.p. with TFP (2.9 μg/g body weight), KTZ (4 μg/g body weight) and a combination of the two (1.45 and 2 μg/g body weight for TFP and KTZ, respectively) in cycles of 5 daily injections followed by a 2-day interval. The mice were bled once a week and the concentrations of WBC in the peripheral blood were determined. Control animals received PBS only

tion of 0.5 μg/ml did not cause any reduction in DNA synthesis, whereas 2 μg/ml caused 20% inhibition in marrow cells as compared with approximately 50% inhibition in YAB1 and YAB9 cells. The addition of TFP caused a synergistic inhibitory effect, and the dose-response curve for inhibition of DNA synthesis became similar to that obtained for YAB9 cells (Fig. 5c, Table 1).

Effects of the drugs on tumor growth in mice and on mouse survival

Groups of B6 mice were inoculated s.c. with YAB1 cells (10^5 cells/mouse) and treated with injections of TFP (2.9 μg/g body weight), KTZ (4 μg/g body weight), or a combination thereof (1.45 and 2 μg/g body weight for TFP and KTZ, respectively). Control groups were injected with PBS. A schedule involving a weekly cycle of 5 daily injections followed by a 2-day interval was adopted. Drug injection started at 2 days after tumor-cell inoculation and continued until the end of the experiment. The mice were examined daily for the appearance of palpable tumors and the diameters of any lesions found were measured. Only 67% of the TFP-treated mice had developed tumors by the end of the experiment (42 days after tumor-cell inoculation). In this group, tumors were first visible on day 22 after injection as compared with day 12 in the control animals and day 15 in KTZ-treated mice as well as those that received combination treatment. Whereas 100% of the mice treated with KTZ developed tumors by day 22 (as compared with day 17 in the control group), only 85% of those given the drug combination had developed tumors by the end of the experiment (day 42, Fig. 6a). The mean diameter of the tumors was markedly reduced in all treated mice (Fig. 6b) and survival was significantly prolonged. By day 31, all of the control animals had died, whereas by day 38, we observed 100% survival among the TFP-treated mice and 40% survival among those given KTZ and those that received the drug combination. Full mortality was observed in KTZ-treated mice only on day 42, at which time 33% of the TFP-treated animals and 15% of those given the drug combination continued to survive and were free of tumors (Fig. 6c).

Drug toxicity

Daily injections of TFP, KTZ, and the drug combination into healthy mice at the concentrations used in the experiment depicted in Fig. 6 did not cause a decrease in the WBC count (Table 2).

Discussion

The results of the present study demonstrate that KTZ displays selective in vitro toxicity to malignant rather than normal cells. Whereas concentrations of between 4 and 7 μg/ml induced 50% inhibition of DNA synthesis in malignant cells, the spleen and bone-marrow cells maintained 50% of their DNA synthesis activity on exposure to KTZ at 50 μg/ml. Interestingly, mitogenic stimulation of the spleen cells by Con-A caused an increase in their sensitivity to KTZ up to the range observed in malignant cells.

KTZ is a widely used broad-spectrum antifungal drug. Its antifungal activity is based on inhibition of the conversion of lanosterol to ergosterol [37]. Occurrence of gynecomastia in KTZ-treated patients [11] led to investigations

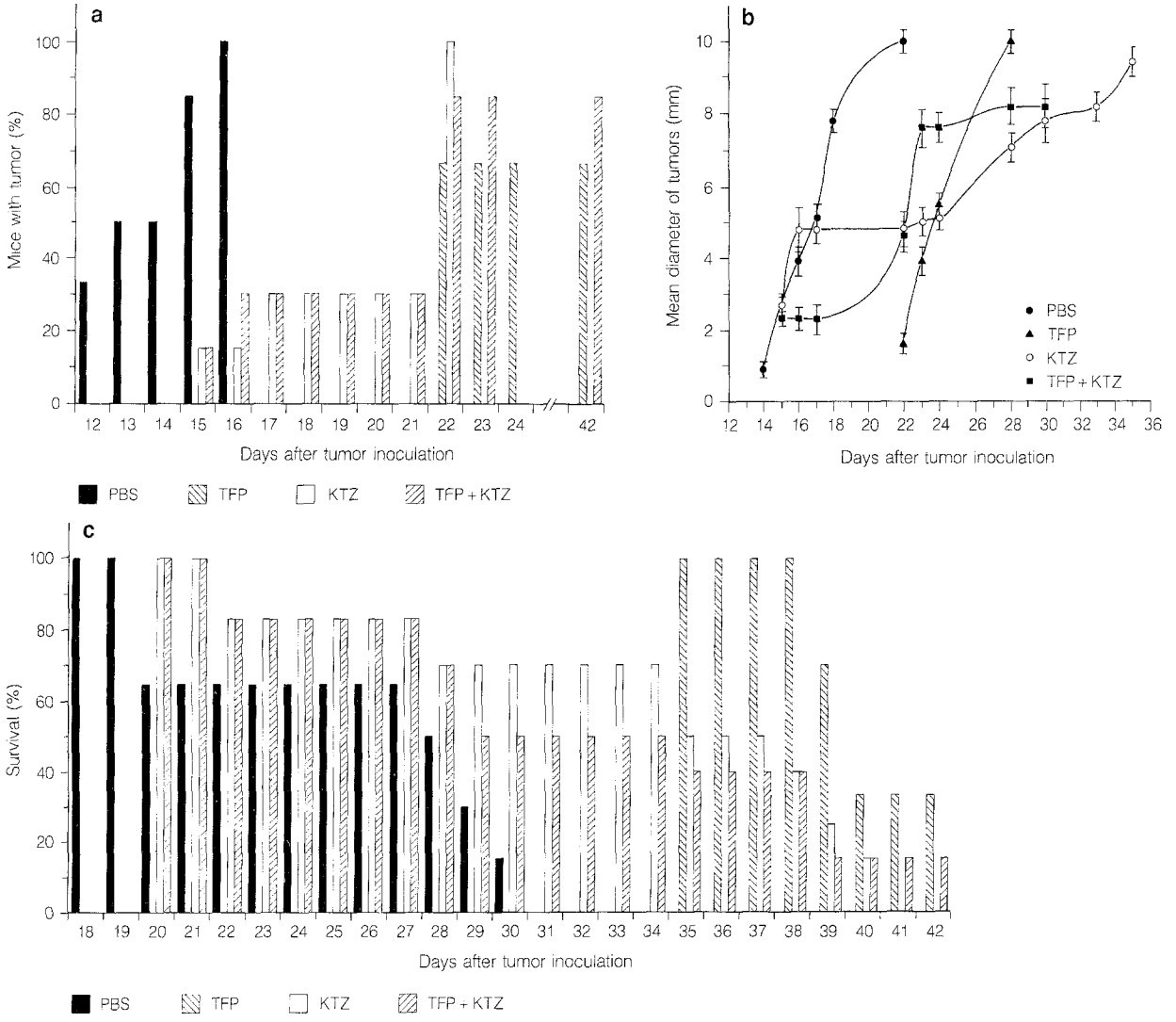

Fig. 6 a–c. Effects of injections of TFP, KTZ and combinations of the two drugs on the appearance and size of tumors and on the survival of mice inoculated with tumor cells. B6 mice were injected s. c. with cells of the line YAB1 (10^5 cells/mouse). TFP (2.9 μg/g body weight), KTZ (4 μg/g body weight) and combinations of the two (1.45 and 2 μg/g body weight for TFP and KTZ, respectively) were injected i. p. beginning on day 2 following tumor inoculation. The drugs were given in cycles of 5 daily injections followed by a 2-day interval until the end of the experiment; groups of 6–8 mice were subjected to each treatment. The control group was injected with PBS. **a** Percentage of mice exhibiting palpable tumors in each group. **b** Mean diameter of the tumors; data represent the mean ± SE (n = number of mice bearing a tumor at the time of measurement). **c** Percentage of survival in each group

that confirmed the inhibition of testosterone biosynthesis by the drug [12, 23, 26, 37]. Other investigators then took advantage of this effect in chemotherapy of prostatic cancer using high-dose KTZ [30, 36, 40]. The positive therapeutic results obtained using KTZ were attributed to a reduction in serum testosterone levels, since this type of cancer had previously been treated in part by castration, which also produced a decrease in serum testosterone levels.

Some indications for hormone-independent growth inhibition by KTZ have recently been reported. A cytotoxic effect for KTZ along with an IC_{90} value of ≤ 10 μg/ml was found in six of eight malignant cell lines of human origin in an in vitro study [33]. Growth inhibition by KTZ of a human lymphoblastic leukemia cell line along with an IC_{50} value of 25 μM has been found in vitro [18]. KTZ showed antimetastatic activity in mice injected with murine melanoma cells, with no effect being observed on survival or primary tumor size [29].

The mechanism underlying the selective inhibition exerted by KTZ is not yet clear. Inhibition of steroid synthesis has been suggested to be the mechanism of action of KTZ in the treatment of hormone-dependent tumors such as prostate cancer [23, 26], male metastatic breast cancer [14], metastatic adrenal carcinoma [9] and postmenopausal breast cancer [20]. Hormone-independent inhibition of neoplastic cell growth such as that demonstrated in the present study may possibly be attributable to inhibition of cholesterol biosynthesis [22]. Actively dividing cells are expected to be more sensitive than nondividing cells to the inhibition of cholesterol biosynthesis, which leads to altered membrane composition and permeability. Although the low sensitivity of non-stimulated spleen cells to KTZ found in the present study may possibly be interpreted on

this ground, it is not clear why the actively dividing bone-marrow cells displayed low sensitivity to the drug. This low sensitivity was manifested in vivo by the lack of effect of high doses of KTZ on the WBC count in the peripheral blood, indicating that the drug did not inhibit hematopoiesis. The selective growth-inhibitory effect of KTZ on malignant cells has potential chemotherapeutic value.

YAB1 is a highly metastatic lymphoma that grows progressively when inoculated into syngeneic mice; s.c. inoculation of 10^5 cells results in the appearance of a palpable tumor within 10–15 days. The cells then infiltrate the spleen and lymph nodes and produce lesions in the lungs. All of the infected mice die within 1 month. In the small-scale in vivo experiment performed in the present study, high-dose KTZ treatment resulted in a significant delay in tumor appearance and prolongation of the survival of mice that had been inoculated with YAB1 tumor cells. Pronounced extension of survival and a delay in tumor appearance were also induced in tumor-inoculated mice by treatment with TFP and with the combination of TFP and KTZ.

The phenothiazines, a group of drugs to which TFP belongs, are widely used for their antipsychotic, tranquilizing, and antiemetic effects [1, 38, 41]; they are also strong anticalmodulin agents [25, 31]. It has been established that calmodulin is required for cell-cycle progression during mitosis [27, 32] and that calmodulin antagonists block cell-cycle progression in vitro at both the G1/S and the G2/M boundaries [8, 13, 34]. Therefore, the use of anticalmodulin drugs in cancer chemotherapy was suggested [5, 21]. Inhibition of neoplastic cell growth by anticalmodulin agents has been reported [19, 24, 39]. However, since malignant cells contain elevated calmodulin levels [7], normal cells may be more sensitive to inhibition by calmodulin antagonists than are malignant cells [17]. The results reported in the present study showed variability in the sensitivity to growth inhibition by TFP among the malignant cell lines. DNA synthesis in vitro was inhibited by the same range of TFP concentrations in the normal cells as in the malignant cells. On the other hand, in vivo experiments on tumor-inoculated mice resulted in a marked delay in the appearance of tumors and in prolonged survival; 30% of the mice treated with the higher dose of TFP had not developed tumors by day 42 after tumor inoculation. No toxic effect on hematopoiesis was associated with these doses. These results may indicate the chemotherapeutic potential of TFP.

TFP has been widely used in combination with other chemotherapeutic agents to potentiate the efficacy of chemotherapy and to reverse drug resistance [6, 16, 35]. The mechanism underlying the drug's activity is under investigation [15]. In previous studies in our laboratory on the chemotherapy of fungal diseases, we have found synergistic effects between TFP and KTZ on inhibition of the growth of several pathogenic yeasts [3]. These results led us to investigate the effects of this drug combination on malignant cell lines. Growth-inhibitory effects varying from less than additive to synergistic were obtained in vitro in the different cell lines. However, DNA synthesis in bone-marrow cells was synergistically inhibited by the drug combination, and the resulting dose-response curves were similar to those obtained for the malignant cells. In vivo experiments showed that the combination of TFP and KTZ exerted a more pronounced effect than did KTZ alone on the prolongation of mouse survival without producing toxicity. On the other hand, KTZ diminished the efficacy of TFP treatment in in vivo experiments. The mechanism underlying this effect is not yet understood.

The small scale of the present in vivo experiments does not enable us to draw definitive conclusions as to the chemotherapeutic value of these drugs. However, our preliminary data should encourage further research on their potential value in both single-agent and combination chemotherapy.

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