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Note

Calcium pterin as an antitumor agent

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Abstract

A series of in vivo studies are reported that provide evidence for an immunologically mediated mechanism for the antitumor response from a calcium pterin (CaPterin) suspension. Strong antitumor efficacy was demonstrated in fully immunocompetent female C3H/HeN-MTV+ mice (retired breeders) presenting spontaneous mammary gland adenocarcinomas. Comparison of results obtained by testing CaPterin in either nude or SCID mice (severely compromised immunodeficient) implanted with MDA-MB-231 human cancer cells showed a significant antitumor response in the nudes and no response in the SCIDs. This comparison argues for B-cell immunological involvement in the mechanism of CaPterin antitumor activity since nude mice possess B-cell capability while SCID mice do not. This comparison also indicates that there is no measurable direct cancer cell toxicity from the CaPterin. Results showing no CaPterin antitumor efficacy against EMT6 tumor cells implanted in Balb/c mice also suggest an antitumor mechanism involving B-cells, since transforming growth factor beta (TGF-beta), produced by EMT6 cells, is known to cause B-cell apoptosis. Taken together, these results, along with those of other researchers, indicate that CaPterin's antitumor mechanism involves antibody-dependent cellular cytotoxicity (ADCC) mediated, for example, by natural killer (NK) cells, interlukin-2, and CaPterin.

Keywords: Pterins; Xanthopterin; Isoxanthopterin; Drug screening assays; Antitumor; Xenograft model; Antitumor models; Immunotherapy

1. Introduction

Soon after it became available synthetically, xanthopterin (2-amino-4,6(3*H*,5*H*)-pteridinedione) was found to inhibit sarcoma growth in female mice (Lewisohn et al., 1944). Interestingly, this antitumor effect was counteracted by leucopterin (2-amino-4,6,7(3*H*,5*H*,8*H*)-pteridinetrione). Some time later, isoxanthopterin (2-amino-4,7(3*H*,8*H*)-pteridinedione) was also shown to inhibit tumor growth (Kokolis et al., 1972). The National Cancer Institute subsequently tested xanthopterin and isoxanthopterin, as well as pterin (2-amino-4(3*H*)-pteridinone), for their tumor-growth inhibitory properties, achieving inconsistent results. Some tumor-animal model systems, such as random-bred albino rats intramuscularly injected with Walker Carcinoma 256, demonstrated limited anticancer activity for xanthopterin and isoxanthopterin but the response was found to be non-significant in other rodent strains. Pterin also demonstrated a degree of antitumor efficacy in Swiss mice injected subcutaneously with Sarcoma 180 but the effect was not reproducible (Drug Evaluation Branch,

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NCI, 1957–1977). More recently, a 2:1 (w/w) xanthopterin/isoxanthopterin suspension in Mammalian Ringer's demonstrated strong antitumor efficacy against spontaneous mammary gland adenocarcinomas in female C3H/HeOuJ mice (Moheno, 1996).

The investigator hypothesized that the use of immunocompetent mouse strains producing spontaneous tumors, rather than immunocompromised, induced tumor-animal systems, are more appropriate experimental models for testing the antineoplastic efficacy of pterins since these compounds have demonstrated immunomodulatory activity (Ziegler et al., 1983a,b; Ziegler, 1990). The testing of antineoplastic agents in spontaneous mammary gland adenocarcinoma producing mice, such as the C3H/HeN-MTV+ strain, has played a central role in the development of drugs efficacious in humans (Gould, 1995). Cytoxan, 5-FU, and Adriamycin were tested in spontaneous mammary gland tumors (Yagi et al., 1987). Chronic low-dose radiation-caloric restriction (Kharazi et al., 1994), chronic indomethacin and intermittent interleukin-2 (IL-2) therapy (Lala and Parhar, 1993), an oxalatoplatinum complex of *trans*-1-dach (1-OHP) (Bourut et al., 1989) and immunization against human choriogonadotropin (Acevedo et al., 1987) have all been tested successfully in spontaneous mammary gland adenocarcinoma tumor-producing mice.

Based upon the above findings, and DNA modeling considerations, the investigator decided to test the antitumor efficacy of a calcium pterin suspension (CaPterin) (Fig. 1) in female C3H/HeN-MTV+ mice. These immunocompetent mice reliably produce (>90%) palpable and relatively uniform spontaneous mammary gland adenocarcinoma tumors, rendering them methodologically suitable for comparison to three immunocompromised mouse-tumor systems:

(1) MDA-MB-231 human mammary tumor xenografts in athymic nude mice (which lack T-cell immunity);

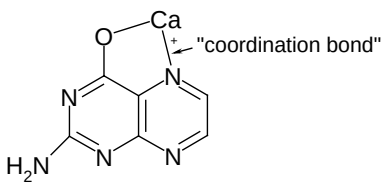

Fig. 1. Proposed structure of calcium pterin.

(2) EMT6 mammary tumor allografts in Balb/c mice (which have attenuated B-cell immunity). EMT6 tumor cells produce transforming growth factor beta (TGF-beta), which is an immunosuppressive cytokine that contributes to the immunological escape of tumor cells (McAdam et al., 1994; McEarchern et al., 1999; Park et al., 1997). Balb/c mice with EMT6 allographs lack full B-cell functioning due to B-cell apoptosis, caused by one TGF-beta, Activin A (Shav-Tal and Zipori, 2002); and
(3) MDA-MB-231 human mammary tumor xenografts in SCID mice (which lack both B-cell and T-cell immunity).

2. Materials and methods

2.1. Materials

CaPterin suspension was prepared by first making two pterin (obtained from Schircks Laboratories, Jona, Switzerland) suspensions, A and B. Suspension A was 5 mM pterin in distilled water. Suspension B was 5 mM pterin and 5 mM $CaCl_2$ in distilled water, made by first dissolving the appropriate amount of $CaCl_2{\cdot}2H_2O$, followed by the addition of pterin powder. Suspension A was then mixed with suspension B in a ratio of 3 parts A to 1 part B. The resultant CaPterin suspension was 5 mM pterin and 1.25 mM $CaCl_2$. Initial observations indicated that this final suspension more effectively reversed tumor growth than suspension B.

2.2. In vivo testing

2.2.1. C3H/HeN-MTV+ female mice with spontaneous mammary gland adenocarcinomas

Six- to eight-month-old C3H/HeN-MTV+ female mice, retired breeders with a high propensity (~90%) to develop mammary gland adenocarcinomas within a few weeks after their arrival, were received from the National Cancer Institute. As each mouse developed a palpable tumor, it was assigned alternately to either the Test or Control groups. The mice in the Test group received 3/16 ml of the CaPterin suspension (7 mg/kg/day) by oral gavage for 7 days. Using a Vernier caliper, daily tumor sizes (average of largest

and smallest diameters) of all palpable tumors were measured and recorded. Tumor volumes (V) were determined by $V = 4/3\pi(\text{average diameter}/2)^3$.

2.2.2. MDA-MB-231 human mammary tumor xenografts in nude mice

Athymic nude (nu/nu) female mice, age 3–4 weeks, were purchased from Harlan Sprague–Dawley, Inc. (Indianapolis, IN, USA). 5×10^6 MDA-MB-231 human breast cancer cells were injected subcutaneously into the right leg of the female immunodeficient mice. When the tumors reached a mean diameter of 3–5 mm, the mice were divided into two groups, each consisting of eight members, and the treatment begun. The mice were treated by oral gavage once daily for 14 days with either 3/16 ml of the vehicle control (deionized H_2O) or with 3/16 ml of the CaPterin suspension (7 mg/kg/day).

Tumor dimensions and body weights were measured two to three times weekly. Vernier calipers were used to measure tumors in three planes, and tumor volume (V) was calculated as follows: $V = \pi(xyz)/6$, where x, y, and z were the tumor measurements minus skin thickness. The mean tumor volume was calculated at each time point. For each group, the mean of the ratio V/V_0 was plotted as a function of time after treatment. Treatment toxicity was assessed from reductions in body weight during and after treatment.

2.2.3. Mammary EMT6 allografts implanted in female Balb/c mice

Balb/c female mice, age 3–4 weeks, were purchased from Simonsen Laboratories (Gilroy, CA, USA). The experimental animals were each implanted subcutaneously in the right flank with 2×10^7 EMT6 mouse mammary tumor cells. Tumor dimensions and body weights were measured two to three times weekly. Vernier calipers were used to measure tumors in three planes, and tumor volume (V) was calculated as follows: $V = \pi(xyz)/6$, where x, y, and z were the tumor measurements minus skin thickness.

When the tumor volumes reached a predetermined size (a mean tumor volume of 25–150 mm^3), mice were randomly sorted into two treatment groups of eight mice each. The mice were each treated once daily for 15 consecutive days with either an oral injection of vehicle control (3/16 ml deionized H_2O) or 7 mg/kg/day of test article (3/16 ml CaPterin suspension). The mean tumor volume was calculated at each time point. For Control and Test groups, the mean tumor volume was plotted as a function of time after treatment. Treatment toxicity was assessed from reductions in body weight during and after treatment.

2.2.4. MDA-MB-231 human breast tumor xenographs in SCID mice

The antitumor activity of the oral CaPterin suspension was subsequently tested in SCID mice (severely compromised immunodeficient) bearing the human breast tumor cell line MDA-MB-231. Thirty-two SCID mice were inoculated with scraped MDA-MB-231 human breast cancer cells in matrigel using a subcutaneous flank injection. The mice were randomly assigned eight mice to each of the following treatment groups: Control (distilled water); 13 mg/kg CaPterin; 20 mg/kg CaPterin; and 26 mg/kg CaPterin. Administration of either CaPterin or the vehicle by oral gavage was from Monday through Friday for 75 days. Subcutaneous tumors were measured twice weekly and volumes estimated according to the formula: $V = [(\text{width})^2 \times \text{length}]/2$. Mice were weighed before the beginning of the experiment and weekly thereafter to check for signs of toxicity.

3. Results

Fig. 2 shows the daily relative mean tumor volumes of the Control and Test C3H/HeN-MTV+ mice, which were given CaPterin suspension daily for 7 days. As can be seen from Fig. 2, the treatment strongly regressed tumor growth relative to controls. The ratio of Test tumor volumes to Control tumor volumes (T/C) at Day 7 was 0.1 (or 10%). The National Cancer Institute considers a $T/C \leq 42\%$ to be a significant level of antitumor activity. A T/C value $<10\%$ is indicative of a high degree of antitumor activity. The increased relative tumor volume variance associated with the larger tumors seen in the Controls is due to inherent tumor growth characteristics that increasingly diverge as the tumors grow.

Fig. 3 shows the growth curves of the Test and Control tumors in the nude mice implanted with MDA-MB-231 human breast cancer xenographs. At Day

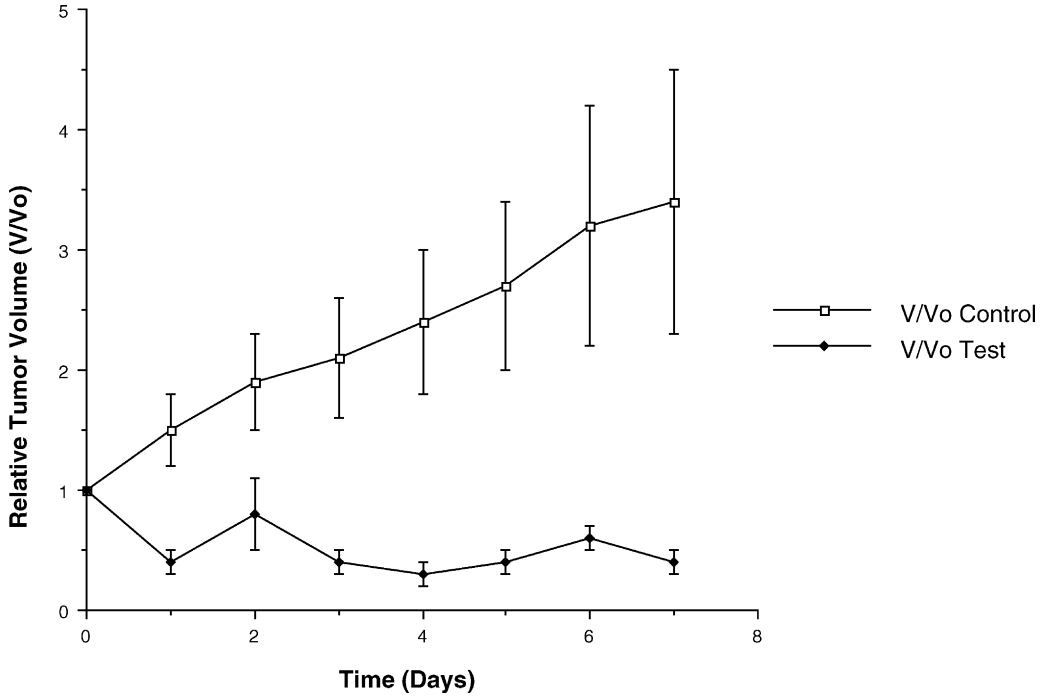

Fig. 2. Mean growth response as relative tumor volume of spontaneous mammary gland adenocarcinoma tumors in C3H/HeN-MTV+ mice treated with CaPterin. When tumors reached a palpable size (Day 0), Test animals were treated daily with oral doses of CaPterin. Key: upper line, control; lower line, treated; V_0, mean initial volume. Error bars represent the standard error of the mean for measurement of groups of animals; Test and Control groups each consist of four members.

14 the *T*/*C* ratio was 0.41 (or 41%), within the NCI range for significant antitumor activity ($T/C \leq 42\%$). Throughout the course of treatment no evidence of toxicity, as measured by weight change, was found in any of the experimental nude mice.

The CaPterin suspension treatment produced no significant effect on tumor growth in the Balb/c mice with EMT6 allographs (Fig. 4). Here again, no measurable animal toxicity, as determined by decreased body weight, was found.

The CaPterin suspension showed no antitumor efficacy in the SCID mice (Fig. 5). A least squares regression statistical analysis of these tumor volume data indicated that there was no significant difference in either tumor growth rate or tumor latency between any of the three treatments—13 mg/kg CaPterin, 20 mg/kg CaPterin, and 26 mg/kg CaPterin—and Controls. Again, as shown in Fig. 6, the experimental SCID mice demonstrated no measurable toxicity over the 75 days of CaPterin suspension administration.

4. Discussion

Comparison of the results from the C3H/HeN-MTV+ spontaneous mammary tumor system to those from the nude mouse human breast cancer xenograft system shows that even though the spontaneous tumors responded more dramatically to the CaPterin than the implanted tumors, the human breast cancer tumors in the nude mice nevertheless responded significantly. The difference in degree of response (90% versus 59% tumor growth inhibition) was initially considered likely due to (1) the presence of one or more mediating immune system components in the C3H/HeN-MTV+ mice not present in the nude mice, and/or (2) an inherently greater cytotoxic sensitivity

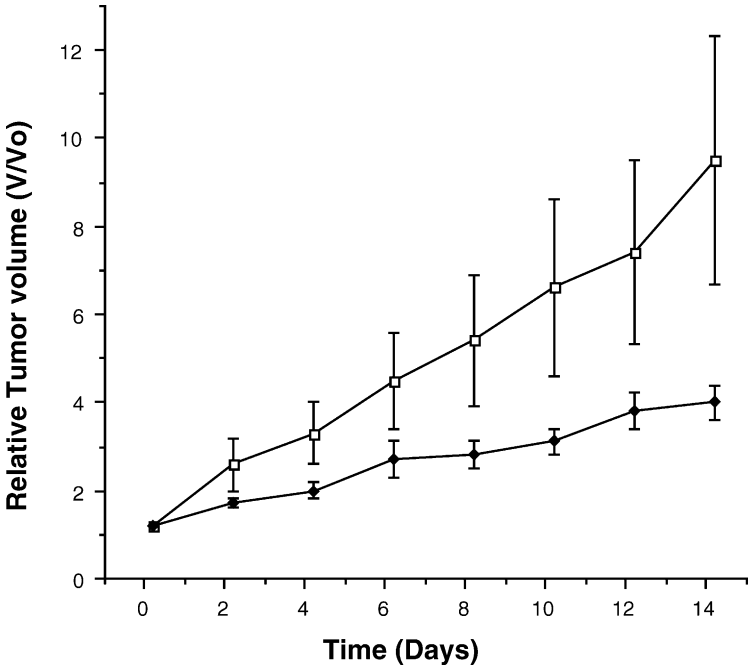

Fig. 3. Mean growth response as relative tumor volume of MDA-MB-231 human breast xenografts in nude mice treated with CaPterin. When tumors reached 3–5 mm in diameter (Day 0), Test animals were treated daily with oral doses of CaPterin. Key: upper line, control; lower line, treated; V_o, mean initial volume. Error bars represent the standard error of the mean for measurement of groups of animals consisting of eight members each.

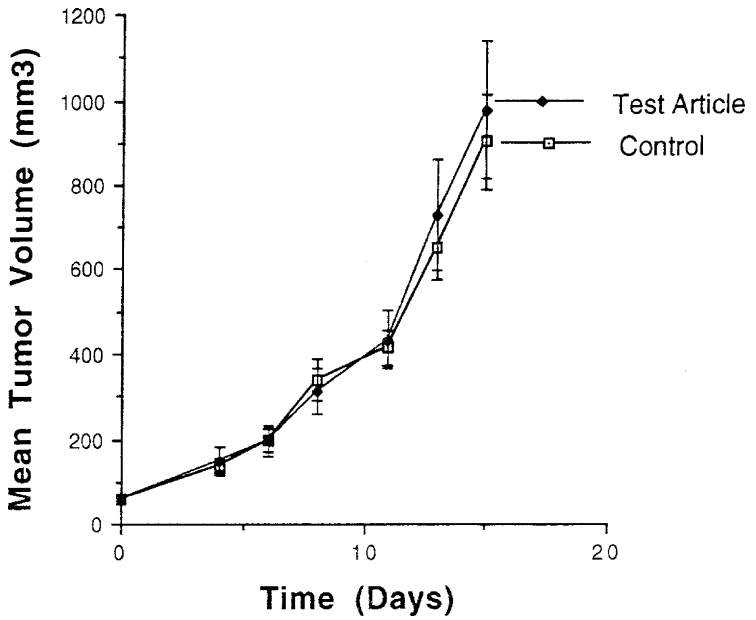

Fig. 4. Mean growth response of EMT6 mouse mammary tumors in Balb/c mice to the test article, CaPterin suspension. When tumors reached 25–150 mm^3 in volume (Day 0), Animals were treated daily for 15 days with oral doses of 3/16 ml test article or vehicle alone (deionized water). Error bars represent the standard error of the mean for measurement of groups of animals consisting of eight members each.

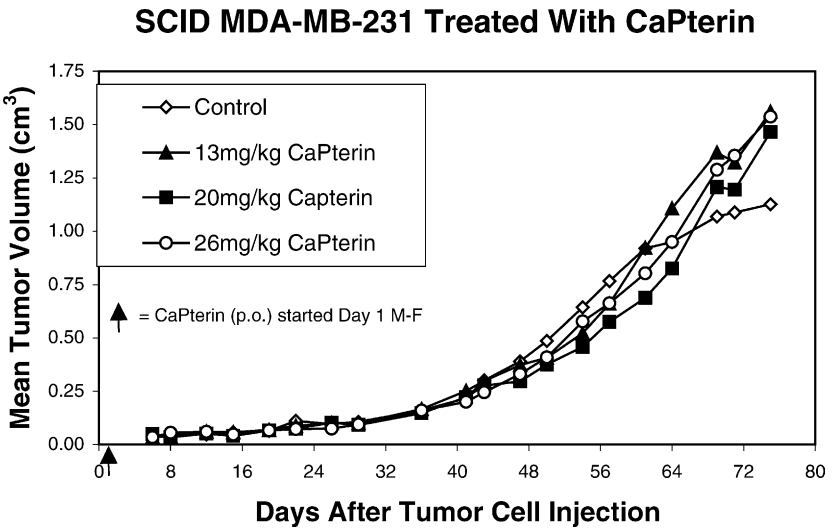

Fig. 5. Thirty-two SCID mice, implanted with MDA-MB-231 human breast cancer cells, were randomly assigned to the following Monday through Friday oral dosage groups: water (control), 13, 20, and 26 mg/kg CaPterin. Subcutaneous tumors were measured twice weekly.

on the part of the spontaneous adenocarcinoma cells to CaPterin as compared to the MDA-MB-231 human breast tumor cells implanted in the nude mice.

The first explanation is supported by comparing the results obtained from testing CaPterin in the immunocompromised SCID mice to those results obtained with the nude mice, in which both strains were implanted with MDA-MB-231 tumor cells. Direct tumor cell toxicity by CaPterin is not supported because there was no tumor sensitivity found with

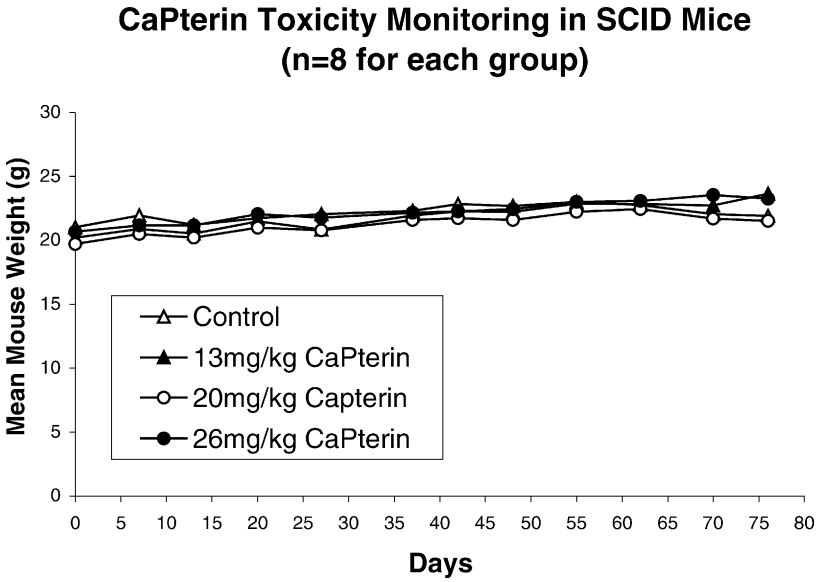

Fig. 6. Thirty-two SCID mice, implanted with MDA-MB-231 human breast cancer cells, were randomly assigned to the following Monday through Friday oral dosage groups: water (control), 13, 20, and 26 mg/kg CaPterin. Mice were weighed before the beginning of the experiment and weekly thereafter to check for signs of toxicity.

the SCID mice as was observed with the nudes. Lack of direct tumor cell toxicity by CaPterin was also determined in an NCI screen of 60 human tumor cell lines in vitro in the concentration range of 2.5×10^{-4} to 2.5 ug/ml (Drug Evaluation Branch, NCI, 2002). The unconjugated pterin, isoxanthopterin, similarly tested in vitro by NCI was found to inhibit the growth of 3 out of 60 tumor cell lines, while 6-(hydroxymethyl)tetrahydropterin showed some growth inhibition against 8 out of 60 tumor cell lines (Drug Evaluation Branch, NCI, 1994, 2003).

Unlike the SCID mice that have neither T-cell nor B-cell immune functions, nude mice retain a degree of B-cell function, indicating B-cell involvement in the antitumor mechanism of CaPterin. Furthermore, the results from the EMT6 allograph system, which expresses TGF-beta, also suggest B-cell involvement in CaPterin's antitumor mechanism since at least one TGF-beta, Activin A, inhibits B-cell proliferation (Shav-Tal and Zipori, 2002).

Antibody-dependent cellular cytotoxicity (ADCC) is a defined immunological mechanism used by many organisms to destroy cancer cells, which can be induced by natural killer (NK) cells, monocytes, and polymorphonuclear neutophils (PMNs) (Sondel and Hank, 2001). NK cells in particular can be proliferated and activated by various cytokines, such as interleukin-2 (IL-2). Research carried out and reviewed by Ziegler (1990) found that at least one pteridine, H_4Biopterin, modulates the efficiency of IL-2 high-affinity binding to two IL-2-dependent murine cytotoxic T-cell lines, CTLL-2 and CSP2.1. Similar modulating efficacy by this pterin was found with a human T-cell population, human peripheral blood mononuclear cells, and murine spleen cells, raising the likelihood of pteridine modulation of other lymphocytic cell lines as well. Future research into this question might begin by focusing on elucidating the interactions among, for example, NK cell, IL-2, and metal–pterin complexes, such as CaPterin.

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