Drug Designing: Open Access
ISSN: 2169-0138

Moheno, Drug Des 2017, 6:1
DOI: 10.4172/2169-0138.1000140

Review Article Open Access

A Review of the Development of Calcium Pterins and (250:1 Mol:Mol) Calcium Folate for the Immunotherapy of Certain Diseases

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Abstract

The development of a class of immunotherapeutics, the calcium pterins, beginning with Ca Pterin·2Cl (Ca-Pterin) and culminating with calcium pterin 6-carboxylate chelate (CP6CC) for several immunologically-related preclinical and clinical indications is reviewed here. A preliminary analysis of their immuno-mechanisms of action is discussed. The preclinical murine models first tested with CaPterin and dipterinyl calcium pentahydrate (DCP), a dimerized version of CaPterin, were four murine breast tumor models. These four models included: C3H/HeN-MTV+ female mice with spontaneous mammary gland adenocarcinomas; Mammary EMT6 allografts implanted in non-immune female Balb/c mice; MDA-MB-231 human breast tumor xenographs in SCID mice; and MDA-MB-231 human breast tumor xenographs in athymic nude mice. An analysis of the differing tumor responses in these breast tumor models led to the determination that B-cell antibody-based antitumor mechanisms were involved.

A transgenic hepatitis B murine model and a diabetes-induced obese (DIO) type 2 diabetes murine model were also tested with DCP, giving positive results. Further testing giving positive results with DCP in an *in vitro* tuberculosis model is also reviewed.

The investigator hypothesized from a review of the pterin chemical literature that (250:1 mol:mol) calcium folate (designated Ca_{250} folate·498Cl or CaFolate) can serve as an FDA-cleared immunotherapeutic supplement and generate CaPterin, However, mass spectrometry revealed that instead calcium pterin 6-carxylate chelate (CP6CC) was formed *in situ*. Through an observational study in humans, CP6CC derived in this way demonstrated clinical evidence of therapeutic efficacy against colds/flu, osteoarthritis, several cancer cases, type 2 diabetes and other indications, at relative dosages of 0.007 mg/kg·day, approximately 1,000-50,000 times less than those found efficacious with CaPterin and DCP in the preclinical studies. Clinical trials with CaFolate are currently in progress with osteoarthritis, and further trials with the active ingredient CP6CC and CaFolate, are planned for cancer and colds/flu.

Keywords: Immunotherapeutic; Calcium pterin; (250:1 mol:mol) Calcium folate; Osteoarthritis; Colds/flu; Cancer; Type 2 diabetes

Introduction

Since the determination that oral calcium CaPterin (Figure 1A) is efficacious against tumor development in murine breast cancer models and a modulator of the immuno-regulatory enzyme indoleamine 2,3-dioxygenase (IDO), further investigation into its mechanisms of action has been of crucial interest to investigators [1-4]. Two of these studies [3,4] also focused on the anti-tumor properties of oral dimeric DCP (Figure 1B), which forms CaPterin at pH<4 (gastric conditions). This chelation dissociates under gastric pH conditions and likely re-chelates in the higher pH conditions of the duodenum. DCP was also shown to: i) inhibit hepatitis B virus replication in transgenic mice [5]; ii) improve glucose tolerance in DIO mice, a type 2 diabetes model [6]; and iii) inhibit intracellular mycobacterial growth (*Mycobacterium bovis* and *Mycobacterium tuberculosis)* in human monocytes [7].

In an effort to more easily deliver therapeutic CaPterin in humans, a review of the scientific literature revealed several studies that identified pterin 6-carboxylate (P6C) [8-12] and pterin (Pte) [8] as acid-oxidation products of folic acid (FA) under various conditions. Using Ca^{+2} ions, which have both Lewis acid and oxidative properties, from $CaCl_2·2H_2O$, Moheno [13] prepared calcium pterin 6-carboxylate chelate from FA+250 M excess $CaCl_2·2H_2O$, Ca_{250} folate·498Cl (CaFolate) *in situ* (Figure 2). CaFolate supplements given to human supplement users resulted in a series of remarkable reported therapeutic efficacies at relative dosages 1,000-50,000 times less than those found with CaPterin and DCP [13]. Clinical trials in osteoarthritis are in progress with CaFolate and its putative active ingredient, determined by mass spectrometry to be calcium pterin 6-carboxylate chelate (CP6CC).

Review of Methods and Results

Suspensions of CaPterin, pterin, and dipterinyl calcium pentahydrate (DCP) were prepared as described in [1,3]. CaPterin crystals were grown for X-ray crystallographic analysis as described by Moheno et al. [3] and gave the structure given in Figure 1A. DCP crystals were grown as described by Moheno et al. [3] and gave the structure given in Figure 1B.

In vitro human PBMC studies of indoleamine 2,3-dioxygenase (IDO) inhibition

The purpose of these *in vitro* studies [2,3] was to measure the IC_{50} values of IDO in PBMCs for i) CaPterin, $CaCl_2$, and pterin to determine measurable synergistic effects between Ca^{+2} and pterin, and ii) to compare these values with those of DCP for the assessment of relative IDO inhibitory strength. IC_{50} (μM) values for IDO inhibition by CaPterin, DCP, $CaCl_2$ and pterin were determined *in vitro* with human PBMCs (both PHA-stimulated and unstimulated) by measuring kynurenine/tryptophan ratios as described by Winkler [2] and Moheno et al. [3].

In supernatants of unstimulated PBMC average concentrations of tryptophan and kynurenine were mean ± S.E.M.: 17.7 ± 1.0 and 4.1 ± 0.6 μmol/l, kyn/trp was 251 ± 42.7 μmol/mmol. In the unstimulated PBMC, kyn/trp was lower in cells treated with 200 mg/ml concentration ($p<0.05$; Friedman test). The IC_{50} values for IDO inhibition determined

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Received December 11, 2016; **Accepted** January 09, 2017; **Published** January 15, 2017

Citation: Moheno PBB (2017) A Review of the Development of Calcium Pterins and (250:1 Mol:Mol) Calcium Folate for the Immunotherapy of Certain Diseases. Drug Des 6: 140. doi: 10.4172/2169-0138.1000140

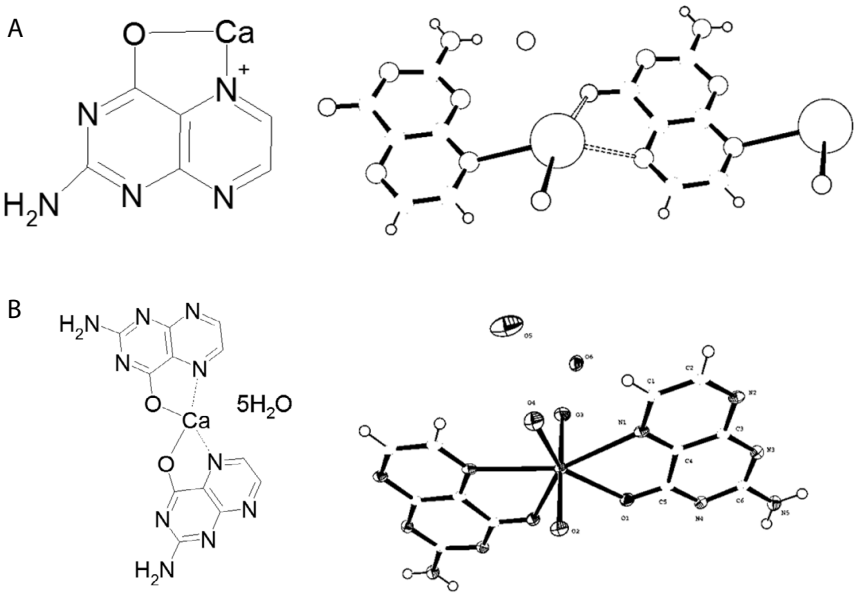

Figure 1A: Calcium pterin chelate; X-ray crystallographic structure (right). **B)** Dipterinyl calcium pentahydrate (DCP); X-ray crystallographic structure (right).

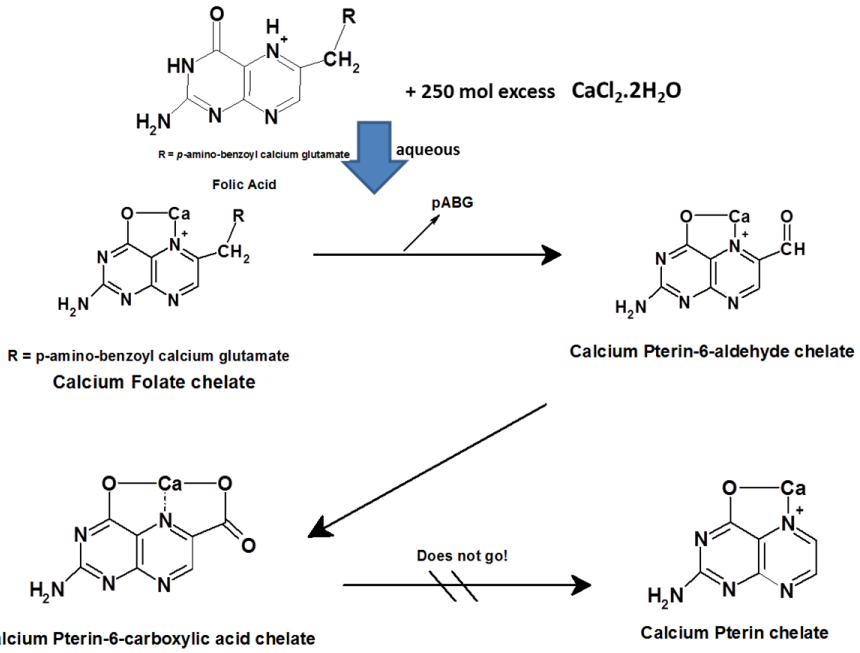

Figure 2: Acid-oxidation of folic acid yields a series of pterins [8] shown here as Ca^{+2} chelates. Calcium folate in water yields calcium pterin-6-carboxylate chelate, as determined by mass spectrometry (updated from Figure 1 of reference [13]).

in vitro with human PBMCs, both unstimulated and PHA-stimulated, found that both CaPterin and DCP show significantly greater *in vitro* IDO inhibition than either calcium or pterin tested alone. At the concentrations tested, no toxicity could be observed by the trypan blue exclusion method.

In vivo breast cancer models

C3H/HeN-MTV+ female mice with spontaneous mammary gland adenocarcinomas [1] - Initial determination of CaPterin efficacy.

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. Daily tumor sizes of all palpable tumors were measured. The ratio of Test tumor volumes to Control tumor volumes (*T*/*C*) at Day 7 was 0.1 (or 10%) at 7 mg/kg·day.

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.

Mammary EMT6 allografts implanted in female non-immune Balb/c mice [1]

Non-immune Balb/c female mice (see reference [14]), 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, and tumor volumes were calculated. When the tumor volumes reached a predetermined size (a mean tumor volume of 25-150 mm^3) the mice were randomly sorted into two treatment groups of eight each. The mice were also 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). Treatment toxicity was assessed from reductions in body weight during and after treatment. The CaPterin suspension treatment produced no significant effect on tumor growth in the non-immune Balb/c mice with EMT6 allographs.

MDA-MB-231 human breast tumor xenographs in severely combined immunodeficient (SCID) mice [1]

The antitumor activity of the oral CaPterin suspension was subsequently tested in SCID mice, characterized by disrupted development of both T and B cells, 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. Mice were weighed before the beginning of the experiment and weekly thereafter to check for signs of toxicity.

The CaPterin suspension showed no antitumor efficacy in the SCID mice among any of the three daily treatments: 13 mg/kg CaPterin; 20 mg/kg CaPterin; 26 mg/kg CaPterin; and Controls. The experimental SCID mice demonstrated no measurable toxicity over 75 days of CaPterin suspension administration.

MDA-MB-231 human breast tumor xenographs in athymic nude mice

Three experiments were performed in this model with greatly reduced numbers of T cells. 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 groups and the treatment begun [1,3,4]. In three experiments, the mice were given the following protocols.

The aim of the first experiment [1] was to establish the efficacy of CaPterin in MDA-MB-231/nudes. The mice were treated once daily for 14 days with either 3/16 ml of the vehicle control (deionized H2O) or with 3/16 ml of the CaPterin suspension (7 mg/kg·day). At Day 14 the T/C ratio for the 7 mg/kg·day CaPterin suspension was 0.41 (or 41%), within the NCI range for significant antitumor activity ($T/C \leq 42\%$).

The aims of the second experiment [3] were to determine a dose-response curve for the CaPterin suspension in the MDA-MB-231/nudes, to compare the antitumor activity of this suspension to pterin alone (pterin control), and to test the effect of CaPterin mega-dosing at 100 mg/(kg day). The four treatment groups were: CaPterin (7 mg/(kg day); pterin (21 mg/(kg day)); CaPterin (21 mg/(kg day)); and sterile water control. In the second experiment, the treatment effects upon the concentration of various plasma cytokines and IDO activity were also determined. The second experiment showed that (1:4 mol/mol) CaPterin at 21 mg/kg·day significantly inhibits MDA-MB-231 human breast tumor growth in nude mice, giving a 41% T/C ratio (mean treatment tumor volume to mean control tumor volume) after 60 days. CaPterin at 7 mg/kg·day and pterin at 21 mg/kg·day did not show significant tumor inhibition in the mice.

The aims of the third experiment [4] were three-fold: 1) to test the antitumor effect of the increased $[Ca^{+2}]$ with a (1:2, mol/mol) Ca $pterin_2 \cdot 2Cl$ suspension compared to the (1:4, mol/mol) Ca $pterin_4 \cdot 2Cl$ suspension; 2) to evaluate the antitumor efficacy of DCP at two concentrations, 23 and 69 mg/(kg·day); and 3) to evaluate the antitumor activity of the CaPterin to calcium chloride alone ($CaCl_2$ control). In this experiment, the treatment effects upon the concentration of various plasma cytokines were determined. The experiment showed a 30% T/C after 43 days for CaPterin; (1:2 mol/mol) CaPterin (T/C=19%); DCP at 23 (T/C=21%) and 69 (T/C=39%) mg/kg·day; and $CaCl_2 \cdot 2H_2O$ (T/C=25%) were all found to be significantly efficacious. The calcium chloride efficacious finding was hypothesized to be due to its interaction with the dietary folic acid in the chow.

From these experiments with CaPterin [3] and DCP [4], dose-response relationships were derived:

1) 60 day rel. Tumor size=21-0.6 (1:4 mol/mol) CaPterin (mg/kg·day) dose; (p=0.005)

2) 42 day rel. Tumor size=minimizes @ 23 DCP (mg/kg·day) dose

Pterin at 21 mg/(kg day) was found to have no antitumor activity. There was no observed toxicity, as determined by body weight changes, among any of the mice in either the first, second or third experiments. Similarly, there was no observed toxicity (appreciable weight loss) among any of the mice mega-dosed by oral gavage with 100 mg/(kg day) CaPterin for up to 31 days. The greatest weight loss among the mega-dosed group was with one mouse that lost 8.1% (2.1 g) of body weight after 32 days, which included a loss of 0.4 cm^3 of tumor mass during this period.

Summary analysis of efficacy difference in four breast tumor models tested with CaPterin

An analysis of the differing tumor responses in the four breast tumor models led to the determination that B-cell antibody-based antitumor mechanisms were involved. This conclusion is based upon the findings that in the two models showing efficacy, C3H/HeN-MTV+ female mice with spontaneous mammary gland adenocarcinomas and MDA-MB-231 human breast tumor xenographs in athymic nude mice, B cell functions are present. In contrast, in the two models not showing efficacy, Mammary EMT6 allografts implanted in female non-immune Balb/c mice and MDA-MB-231 human breast tumor xenographs in severely combined immunodeficient (SCID) mice, both lack functioning B cell components of the immune system. Since the B cells are closely linked to the production of antibody responses, antibody complement mechanisms are likely involved.

Plasma cytokine levels and IDO activity [3] determined in athymic nude mice

Blood was collected from animals via cardiac puncture at termination (after 70-98 days of treatment) and processed to EDTA plasma for analysis in the second and third experiments above, with the MDA-MB-231 nude mice [3,4]. Cytokine levels for IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IFN-γ and TNF-α for the mice were determined by ELISA assay. As appropriate, time course statistical analyses based upon repeated measures ANOVA (analysis of variance) models and standard ANOVA models for group effects, were used (StatView SE+Graphics, v 1.03).

Univariate ANOVAs determined that none of the cytokine and IDO metabolite plasma concentrations were significantly different by Bonferroni criteria across treatment groups: 0, 7 and 21 mg/kg·day CaPterin. The large variances for some of the group measures indicated that substantial variability was associated with these plasma levels. While no significant rank-order or linear correlations to CaPterin dosage and Day 60 relative tumor volumes by these plasma measures were found, a multivariate statistical analysis of the data derived through stepwise regression analysis, found a significant underlying pattern of cytokine and IDO metabolite effects were attributable to CaPterin dosing. The resultant statistically significant (p<0.047) ACIP (Antitumor Cytokine/IDO Pattern) model derived was:

3) CaPterin dose (mg/kg·day)=10.5 - 0.096 [IL-6 pg/ml]+0.31 [IL-10 pg/ml] - 3.16 [IFNγ pg/ml] + 7.89 [kyn μM]; (p=0.047)

Substituting equation 3 into equation (1) gives:

4) 60 day rel. Tumor size=21 - 0.6 (10.5 - 0.096 [IL-6 pg/ml] + 0.31 [IL-10 pg/ml] - 3.16 [IFNγ pg/ml] + 7.89 [kyn μM])

Simplifying,

5) 60 day rel. Tumor size=15 + 0.058 [IL-6 pg/ml] - 0.19 [IL-10 pg/ml] + 1.9 [IFNγ pg/ml] - 4.7 [kyn μM]

Equations (3) and (5) show that, globally, CaPterin dosing decreases IL-6 and IFNγ, and increases IL-10 and kynurenine plasma levels, while decreasing tumor size.

For DCP the measured cytokines included: IL-1b, IL-2, IL-4, IL-6, IL-10, IL-12, IFN-g and TNF-a. The major findings from the analysis of these data are that the overall relative tumor volumes caused by DCP treatments correlated significantly with decreased plasma IL-6, increased IL-4 concentrations and increased IL-12 levels. Therefore, DCP caused these cytokine changes in concert with tumor volume decreases.

In vivo hepatitis B study [5]

In this efficacy and mechanism of action study, DCP was administered per os, once daily for 14 days to 10 male and 10 female hepatitis B virus (HBV) transgenic mice at 23, 7.3 and 2.3 mg/kg·day. Multivariate stepwise regression and MANOVA analyses, by gender and treatment, of liver HBV DNA and RNA measures, liver core and serum HBe antigen assays, serum cytokine/chemokine profiles, and IDO metabolite measurements were performed.

DCP caused a significant dose-response reduction of log liver HBV DNA as measured by PCR in the female HBV mice in an *In vivo* hepatitis B study [5]. The gender-dependence of the anti-HBV DNA activity found in this study was explained by the DCP Effects Model (DCPEM) (p=0.001) [5] which includes three serum biomarker changes caused by DCP and correlated with i) HBV DNA reductions, ii) decreased MCP-1; iii) decreased Kyn/Trp (an estimation of IDO activity); and iv) increased GM-CSF. The following regression, Equation (4), shows this quantitatively:

6) [DCP dose (mg/kg·day)]=26.31 - 4.06 log HBV DNA- 0.022 [MCP-1 rel.pg/ml] - 0.56 [kyn/trp μM/mM] + 0.070 [GM-CSF rel.pg/ml]; (p=0.001)

In vitro mycobacterium-infected monocytes, a tuberculosis model [7]

In this study, DCP was tested for the ability to mediate inhibition of intracellular mycobacteria within human monocytes. DCP treatment of infected monocytes resulted in significant reduction in viability of intracellular *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* but not extracellular *M. bovis* BCG. The antimicrobial activity of DCP was comparable to that of pyrazinamide (PZA), one of the first-line anti-tuberculosis drugs currently used. DCP potentiated monocyte antimycobacterial activity by induction of the cysteine-cysteine (C-C) chemokine macrophage inflammatory protein 1β (MIP-1β) and inducible nitric oxide synthase 2. Addition of human anti-MIP-1β neutralizing antibody or a specific inhibitor of the L-arginase-nitric oxide pathway (N^G-monomethyl L-arginine [L-NMMA] monoacetate) reversed the inhibitory effects of DCP on intracellular mycobacterial growth. These findings indicate that DCP induced mycobacterial killing via MIP-1β- and nitric oxide-dependent effects. Hence, DCP acts as an immunoregulatory compound enhancing the antimycobacterial activity of human monocytes.

In vivo diabetes type 2 model [6]

Twenty-four female diet-induced obese (DIO) C57BL/6J mice were used in this study. The mice were fed *ad libitum* a high-fat diet (D12492i, 60 kcal % fat; Research Diets Inc, New Brunswick, NJ) from 6 to 18 weeks of age. For 21 days afterwards, the test article, DCP in 0.4% carboxy methyl cellulose, was administered daily by oral gavage at 0, 7, 21 or 63 mg/kg·day DCP to four groups of six mice each. Blood glucose was followed during the dosing period, and an oral glucose tolerance test (OGTT) was carried out on day 21. Measurements of plasma indoleamine 2,3-dioxygenase metabolites (tryptophan and kynurenine) and certain cytokines and chemokines were taken. In addition, plasma samples were collected by cardiac puncture on day 21 after DCP administration. The following cytokines and chemokines were assayed: GM-CSF, IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-12(p40), IL-12(p70), IL-13, MCP-1, RANTES and TNFα. Standard and repeated measures analysis of variance (ANOVA) and stepwise regression of the data were carried. 7 mg/kg·day DCP significantly reduced oral glucose tolerance (OGTT area under the curve; OGTT/AUC) by 50% (p<0.05), significantly ameliorating the OGTT diabetic state in these female diet-induced obese (DIO) mice.

Preliminary *in human* observational study of CaFolate (Ca_{250} folate·498Cl) [13]

To find a simple, convenient way to administer oral CaPterin, a search of the literature revealed the work of Lowry et al. and others [8-12] showing the following acid-oxidation sequence for folic acid: folic acid (pteroylglutamic acid) → pterin 6-aldehyde → pterin 6-carboxylate → pterin. It was speculated [13] that excess Ca^{+2} ions could be used to drive the acid-oxidation, since i) Ca^{+2} was a Lewis acid, ii) Ca^{+2} has oxidative properties, and iii) the formation of calcium chelates could occur. To test this hypothesis, 1 mg of folic acid was mixed into an aqueous solution of 75 mg $CaCl_2{\cdot}2H_2O$. The resultant

mixture was analyzed by mass spectrometry (Figure 3), showing the primary reaction product to be calcium pterin 6-carboxylate chelate (CP6CC).

Based upon NIH recommended doses for folic acid, more than 54 non-randomized subjects, aged 42-84, in varying degrees of health were directed to take, *ad libitum*, one to three capsules of the CaFolate

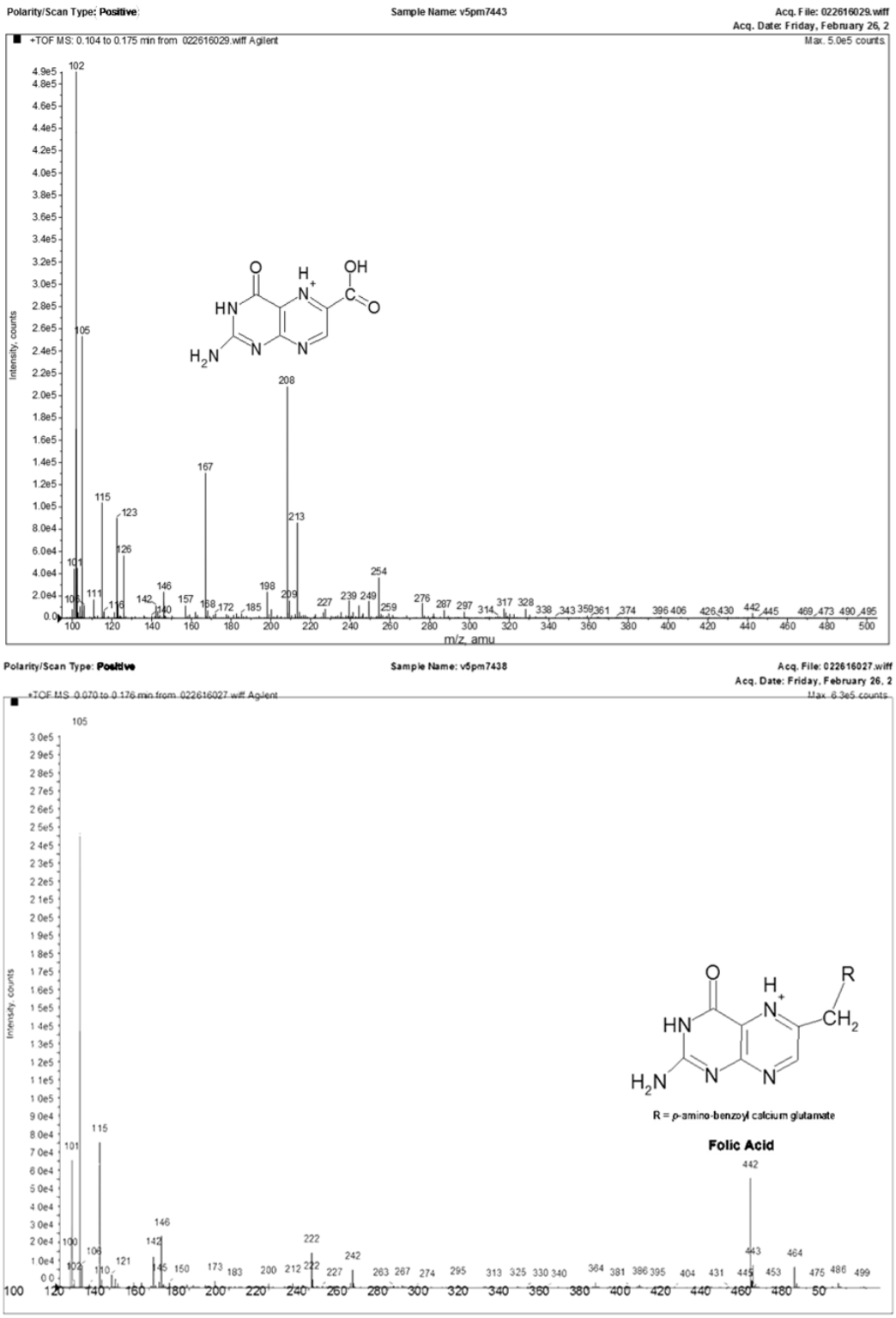

Figure 3: Mass Spec of Folic acid+excess $CaCl_2 \cdot 2H_2O$ [aq] (Top); Folic acid (Bottom). C-18 reverse-phase HPLC with dual phase elution of 1) 0.1% formic acid and 2) acetonitrile+0.1% formic acid.

food supplement daily. Each capsule contained 300 µg folic acid+75 mg $CaCl_2 \cdot 2H_2O$ and the excipients tricalcium phosphate, microcrystalline cellulose, gelatin, silicon dioxide and magnesium stearate, and was cleared by the FDA. The investigators received medical and personal reports from the study participants as to their reactions to CaFolate regarding the various ailments each subject was tracking. Case details and other personal information were not collected, all identities were coded.

Mass spectrometry analysis identified pterin 6-carboxylate as the sole pterin generated *in situ* by the CaFolate mixture. Given that the strong acid conditions of the mass spectrometry analysis striped off the chelated Ca^{+2}, it was deduced that calcium pterin 6-carboxylate chelate (CP6CC) was the reaction product (Figures 2 and 3) and the active compound. The oxidative sequence terminated at the carboxylate. The last step conversion to pterin requires stronger oxidative conditions [8] (Figure 2).

For the several therapeutic clinical efficacies listed in Table 1 found for calcium folate at dosages of 300-900 µg/day folic acid equivalents (0.002-0.007 mg/kg·day; CP6CC equivalents) [13], calcium pterin 6-carboxylate chelate was calculated to be approximately 1,000-50,000 times more potent than the original calcium pterin chelates, tested in mice at dosages of 7-100 mg/kg·day [1,3-6].

The updated data from the observational study participants yielded the following reported evidences from those taking CaFolate:

- >20/20 subjects reporting on colds or flu had none over the cold and flu seasons;
- 12/13 subjects reporting on arthritis (especially osteoarthritis) pain found amelioration;
- 8/8 subjects reporting on overall energy and stamina found these factors were increased;
- 4/5 cancer (various) remissions were determined plus 1 transient improvement in a terminal case likely having received immunosuppressive treatment(s); and
- 2/2 reporting on long-term use (several months) with type 2 diabetes showed amelioration

Reported side effects by the cohort of subjects included:

- 1 report of insomnia.
- 2 reports of inflammatory responses in two individuals with chronic inflammatory states due to Lyme disease.
- 1 report of a mild (level 2) headache, possibly due to other confounding factors.

A preliminary report of every person entering the study, and pertinent reports, are tabulated by Moheno [13]. Colds/flu and osteoarthritis dominated the positive findings (100% and 92% reported positive effects, respectively). The 4/5 cancer (various) remissions, type 2 diabetes reports and increased energy and stamina reports (N=8; 100%) are encouraging as well. The magnitudes of these percentages are compelling within the objectives of this observational study, especially with regard to colds/flu and osteoarthritis and serve to overcome many of the confounding issues (other treatments, lifestyles, etc.) present in an observational study such as this and to support moving ahead with the clinical testing of CaFolate and Ca pterin 6-COOH chelate. Osteoarthritis clinical studies are currently in progress.

Indication	High/low IDO from affliction [References]	High/low cytokines from affliction	Efficacious Dose Range (mg/kg)	Δ Th1/Th2/Th17 and IDO from affliction
In human			*CaPte6CC*†	
Colds/influenza	↑IDO [22,23]	Complex,↑Treg→↑IL-10 [31]	0.007	↑Th1,↑Th2,↑Th17, ↕Treg [31,36],↑IDO
Tuberculosis	↑IDO [24,25]	Complex,↑Treg→↑IL-10 [32]	N/A	↓Th1,↕Th2,↓Th17,↑Th22, ↑Treg [37,38],↑IDO
Cancer (various)	↑IDO [26,27]	Complex,↑IL-6 [33]	0.007	↓Th1,↑Th2,↑Th17,↑Treg [39-42],↑IDO
Osteoarthritis	↑IDO [28]	↑IL-6→↓Th17,↓Th22, ↓Tfh [34, 35]	0.002	↑Th1,↑Th2,↑Th17 [20],↑IDO
Type 2 diabetes	↑IDO [29,30]	↑IL-6→↓Th17,↓Th22, ↓Tfh [19, 34]	0.003	↓Th17,↓Th22,↓Tfh, ↑IDO [34]
Efficacy Model	Δ IDO from efficacious treatment	Δ Cytokines from efficacious treatment	Efficacious Dose Range (mg/kg)	Δ Th1/Th2/Treg and/or Δ IDO @ efficacious dosages
In vivo MDA-MBA-231 nude mice [1,3,4]	↑ kynurenine	↓IFNγ,↑IL12→↕Th1→↓IFNγ ↑IL-4→↑Th2,↑Th9 ↑Treg→↑IL-10 ↓IL-6→↓Th17,↓Th22, ↓Tfh [34]	7 - 69 CaPterin, DCP	↕Th1,↑Th2,↑Treg, ↓Th17,↓Th22,↓Tfh, ↑IDO
In vivo Hepatitis B virus transgenic mice [5]	↓ kyn/trp	↑GM-CSF,↓MCP-1	23 DCP	↓IDO
In vivo diet-induced obese (DIO) mice [6]	No Change	↓IL-6*	7 DCP	↓IL-6*
In vitro Tuberculosis *Mycobacterium* [7]	↓20-fold of IDO mRNA	↑MIP-1β,↑NO, ↑IL-12,↑GM-CSF	500 mg/kg (eqv) DCP	↓IDO
In vitro assay [2] PBMCs	↑IDO*@14 mg/kg ↓IDO @140 mg/ml	N/A	14–140 CaPterin	@14 mg/kg: ↑IDO* @140 mg/ml: ↓IDO
*** Compelling data trend; † Calcium pterin 6-carboxylate chelate**				

Table 1: Immunological effects of calcium pterins.

Discussion

The cytokine changes associated with the preclinical efficacies of CaPterin and DCP are depicted in Table 1 for the MDA-MBA-231 graphed nude mice [1,3,4]. These changes include:

- Decreased IFNγ and increased IL-12 are found, paradoxically indicating both an increase and decrease in Th1;
- Increased IL-4, indicating increases in Th2 and Th9;
- Increased IL-10 indicating an increase in Treg activity; and
- Decreased IL-6 indicating decreases in Th17, Th22 and Tfh.

IDO immune responses in these mice increased with increasing dosages [3,4]. Th1 responds in a bimodal manner in this model. Several researchers [15-17] have proposed that the IDO-kynurenine pathway serves as a negative feed-back loop for Th1 cells and plays a distinct role in up-regulating Th2 dominant immune responses. Figure 1 of Winkler et al. [2] shows that, in the case of unstimulated PBMCs, low levels of CaPterin (14 mg/kg) appear to activate IDO, while 200 μg/ml significantly decrease IDO activity. While this activation at low calcium pterin concentrations is statistically non-significant by the test used, the Figure 1 diagrammatic trend in Winkler et al. [2] for the unstimulated PBMCs, illustrates a compelling inflexion in the regulation of IDO by CaPterin from IDO activation to IDO inhibition with increasing CaPterin concentrations. This IDO activity inflexion might explain the bivalent responses of the Th1 system. With respect to the Treg system, it can be surmised from the cytokine responses to CaPterin (Equation 3, above) that CaPterin has a direct effect on Treg (IL-10) and that IL-10 has an inverse relationship to tumor size (Equation 5). Increases in IL-4, also in inverse relationship to tumor growth, indicates increases in Th2 and Th9.

Osteoarthritis, classified as a Th1 disease by Kidd [18], responds to calcium pterin 6-carboxylate chelate (CaP6CC) generated by (250:1 mol:mol) calcium folate [13] possibly by modulating Th1, Th2 and IDO.

In diet-induced obese (DIO) mice, the diabetes type 2 model studied, relatively lower DCP concentrations are efficacious without IDO effects [6] (Table 1). The compelling correlation between IL-6 decrease and OGTT decreases (increased glucose tolerance) found here is similar in nature to the results of a meta-analysis on inflammatory markers and the risk of type 2 diabetes [19] which found increased IL-6 plasma levels associated with T2D.

The regulation of IDO activity by CaPterin and DCP is complex across the systems tested (Table 1) and suggest that there are variegated IDO responses to the calcium pterins. It is also difficult to fully explain the enhanced cytokine and T-cell subsystem effects of CaPterin, DCP and CaFolate. In the clinical administration of calcium pterin 6-carboxylate chelate generated by CaFolate [13], high Th1 and Th2 [20] and high Th1/Th2 [21] in osteoarthritis are likely corrected at dosages of 300-900 μg/day CaFolate (folic acid equivalents; 0.002-0.007 mg/kg·day CP6CC). Similarly, high dosages of 900 μg/day CaFolate (0.007 mg/kg·day CP6CC) appear to correct the Table 1 T-cell patterns for colds/flu, osteoarthritis, type 2 diabetes and cancer.

Conclusion

In conclusion, more clinical studies of the immunological correlates of CP6CC dosing for the various indications tested is needed to extend the understanding the of clinical efficacies found for CaFolate and the generated calcium pterin 6-carboxylate chelate.

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