



Effect of Orally Administered Dipterinyl Calcium Pentahydrate (DCP) on Oral Glucose Tolerance in DIO Mice

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Abstract

Calcium pterins have been shown to be significant immunotherapeutic agents in models of breast cancer, hepatitis B, and tuberculosis (*Bacillus Calmette-Guérin* mycobacteria). These compounds modulate the immuno-enzyme indoleamine 2,3-dioxygenase (IDO) and the blood levels of several identified inflammatory cytokines. Recent research into the pathology of diabetes implicates inflammatory factors in the progression of the disease, leading the authors to study its possible control by one of the calcium pterins, dipterinyl calcium pentahydrate (DCP). The investigators tested (DCP) as a novel therapeutic for Type 2 diabetes. Female DIO mice, C57BL/6J, fed a high-fat diet were administered DCP in 0.4% carboxymethylcellulose for 21 days. Blood glucose was followed during the dosing period, and an oral glucose tolerance test (OGTT) was carried out on day 21, along with measurements of plasma indoleamine 2,3-dioxygenase (IDO) metabolites (tryptophan and kynurenine), and certain cytokines and chemokines. 7 mg/(kg d) DCP reduced OGTT/AUC (area under OGTT curve) by 50% ($p < .05$). A significant multivariate regression ($p = .013$; $R^2 = .571$) of OGTT/AUC was derived from DCP dosage and plasma tryptophan. Elevated plasma tryptophan, likely from heterogeneity in diet and/or IDO activity, was found to correlate with higher OGTT/AUC diabetic measures, possibly

via inhibition of histamine degradation. In conclusion, an optimum dose of 7 mg/(kg d) DCP significantly improved the OGTT diabetic state in these female DIO mice.

Introduction

Dipterinyl calcium pentahydrate (DCP) is a new molecular entity whose structure is based upon the endogenous substance pterin, and that works through a novel immunomodulatory mechanism. DCP shows antitumor efficacy in mouse models of breast cancer¹ and antiviral activity in transgenic mice with hepatitis B virus,² as well as anti-mycobacterial activity in an *in vitro* model of tuberculosis (BCG).³ These preclinical studies also show that DCP works through a broad immunomodulatory mechanism involving a key immuno-inhibitory enzyme, indoleamine 2,3-dioxygenase (IDO) which DCP appears to modulate to a “homeostatic” level. DCP inhibits IDO in some systems,¹⁻³ and DCP promotes IDO in other systems³ and in the DIO model as reported here. DCP also increases the plasma cytokines IL-12 and IL-4,⁴ and chemokine GM-CSF,² while decreasing IL-6⁴ and MCP-1.² DCP potentiated monocyte antimycobacterial activity by induction of the C-C chemokine MIP-1 β , and inducible nitric oxide synthase 2.³

Emerging literature^{5,6} suggests that inflammation, as evaluated by high inflammatory cytokines levels and other inflammatory markers, may represent a basic cause and consequence of obesity, type 2 diabetes, and comorbidities. Modulation of the levels of anti-inflammatory and pro-inflammatory cytokines might be an important strategy of therapeutic intervention in the treatment of type 2 diabetes and metabolic disease. We hypothesized that oral administration of DCP can improve glucose tolerance by normalizing the levels of pro-inflammatory cytokines which are elevated in obesity and type 2 diabetes. Effects on glucose tolerance and other measures *in vivo* were determined in mice fed a high-fat diet to induce obesity and insulin resistance. DCP was found to significantly improve oral glucose tolerance (OGTT) as determined by 2-hour area under curve (AUC) comparisons.

Methods

24 female DIO mice, C57BL/6J⁷ (000664, B6; Jackson Labs, Sacramento, CA) were used because they were presumed to have higher levels of indoleamine 2,3-dioxygenase (IDO) than males based upon an earlier study.² The mice were fed *ad libitum* a high-fat diet (60 kcal % fat, Research Diets Inc. D12492i) starting at six weeks of age until 18 weeks. For 21 days afterwards, the test article, dipterinyl calcium pentahydrate (DCP) in 0.4% carboxymethylcellulose, was administered daily by oral gavage at 0, 7, 21, or 63 mg/(kg d) to four groups of six mice each. During the DCP gavaging period three mice were lost due to gavaging trauma: one control and two from the 63 mg/(kg d) group. Two of the collected plasma samples collected after DCP dosing were inadvertently not labeled and therefore excluded from the analyses: one from the 21 mg/(kg d) group and one from the 63 mg/(kg d).

Blood glucose measurements were taken from fasted animals twice weekly as follows:

- 1) Animals were fasted for 6 hours prior to blood collections.
- 2) Blood (approximately 10 to 20 μ L) was collected via tail snip, and subsequent bleeds by removing the scab. Additional bleeds were done by snipping the tail and then removing the scab, until 2 mm of the tail tip was removed. Additional bleeds were then done via tail pricks (*i.e.*, sticking the vein or artery with a needle) from all animals beginning just prior to group assignment. Blood glucose was measured using the One Touch Ultra 2 Blood Glucose Monitoring System (LifeScan, a Johnson & Johnson company).
- 3) During the 21 day DCP dosing period, blood was collected 2 hours post-dose on days 1, 3, 5, 9, 10, 13, 17, and 21.
- 4) On Day 21 after DCP administration, an oral glucose tolerance test (OGTT) was carried out measuring pre-challenge blood glucose, and 30, 60, 90, and 120 minutes post-challenge with 2 g/kg glucose.

Plasma samples were collected by cardiac puncture on Day 21 after DCP administration. The plasma IDO metabolites, tryptophan and kynurenine, were measured by high-performance liquid chromatography (HPLC)⁸ and the following cytokines and chemokines; GM-CSF, IFN γ , IL-1 α , IL-1 β , IL-4, IL-6, IL-10, IL-12(p40), IL-12(p70), IL-13, MCP-1, RANTES, and TNF α were measured by EMD Millipore (St. Charles, MO) using the Mouse Cytokine / Chemokine

Magnetic Bead Panel Kit (96-Well Plate Assay #MICYTOMAG-70K) (Luminex Corporation). Reported precisions for the overnight protocol for these cytokines and chemokines are: Intra-assay %CV \leq 4.9, Inter-assay %CV \leq 12.4.

Plasma insulin and other glucose metabolism regulator levels were not determined for this pilot study.

Standard and repeated measures analyses of variance (ANOVAs), and stepwise regression of the data were carried out using the SPSS Graduate Pack 15.0 (2006) and IBM SPSS Statistics v.19 for Windows (2010). The Test of Homogeneity of Variances was carried out to determine the appropriate Contrast Tests to be used in the Oneway ANOVAs. Statistical probability less than .05 ($p < .05$) was used to establish significance.

Results

During the 21-day DCP dosing period there was no significant difference in blood glucose levels or body weights among the four treatment groups of 0, 7, 21, or 63 mg/(kg d) DCP as determined by repeated measures ANOVA (Analysis of Variance) (Figures 1a and 1b). The mice appear to be hyperglycemic on the high-fat diet and not strikingly obese.

ANVOAs for the OGTT/AUC, plasma IDO metabolite, and cytokine/chemokine measures are given in Table 1 showing a significant 50% OGTT/AUC decrease for the mice treated with 7 mg/(kg d) DCP ($p < .05$).

Four variables were identified by stepwise regression to yield the following OGTT/AUC linear regression (*eq. 1*) ($R^2 = .571$; $p = .013$; see Table 2):

$$\text{OGTT/AUC} = 0.009 \text{ DCP}^3 + 31.178 \text{ DCP}^2 - 574.513 \text{ DCP} + 29.828 \text{ Trp} + 1935.382$$

where DCP = DCP dosage in mg/(kg d), and Trp = μM plasma tryptophan. From this equation it can be predicted that lowered plasma tryptophan levels are expected to improve (*i.e.*, decrease) OGTT/AUC measures.

The DCP dose corresponding to the minimum (least diabetic) OGTT/AUC values can be determined by setting the first derivative of eq. 1 equal to 0:

$$0 = d(\text{OGTT/AUC})/d(\text{DCP}) = 0.027 \text{ DCP}^2 + 62.356 \text{ DCP} - 574.513$$

which can be solved for DCP by using the quadratic formula:

$$\text{DCP} = \frac{-62.356 \pm [(62.356)^2 - 4(0.027)(-574.513)]^{1/2}}{2(0.027)} = \frac{-62.356 \pm 62.852}{0.054}$$

= **9.19 mg/(kg d) DCP**

This DCP dosage calculated from the regression equation corresponds very well with the experimentally derived value of 7 mg/(kg d) from Table 1 for the anti-diabetic dosage giving the minimum OGTT/AUC.

Discussion

From the Table 1 values for Trp (tryptophan), Kyn (kynurenine), and the calculated Kyn/Trp ratio, it appears that IDO activity is not involved with the anti-diabetic OGTT/AUC minimum at 7 mg/(kg d) DCP. Interestingly, the plasma IL-6 pattern from Table 1, though non-significant, resembles the significant ($p < .05$) OGTT/ AUC pattern in that the lowest values for IL-6 and OGTT/AUC both correspond to a DCP dose of 7 mg/(kg d). Investigation into the role of IL-6 in diabetes Type 1 and Type 2, ⁶ and its role in the immune system generally in Type 2 diabetes ⁵ has been called for by previous researchers.

Of particular interest is the finding that plasma tryptophan enters into the significant ($p = .013$) multivariate regression predicting the OGTT/AUC response (Table 2). FDA researchers ⁹ have determined that elevated tryptophan levels can lead to increased formation of formate and indolyl metabolites, several of which inhibit the degradation of histamine, potentially leading to eosinophilia-myalgia syndrome (EMS) and diabetes. ¹⁰ In this regard, 98% of surveyed EMS patients from the US reported having used L-tryptophan-containing products prior to the onset of illness at a median dosage of 1,500 mg/day. ¹¹ This human dose of 1,500 mg / 70 kg = 21 mg/kg corresponds to a mouse dosage of 264 mg/kg based upon body surface area allometrics. $264 \text{ mg/kg} \times [\text{mmol Trp} / 204 \text{ mg Trp}] \times [\text{kg} / 0.7 \text{ l}] = 1.85 \text{ mM Trp}$, the estimated corresponding mouse plasma/tissue level increase, substantially increases the DIO mouse plasma Trp levels beyond those shown in Table 1.

In conclusion, we find that:

- 1) DCP significantly ($p < .05$) improves oral glucose tolerance at 7 mg/(kg d) in female DIO mice.

- 2) A significant ($p = .013$) multiple regression, in terms of DCP³, DCP², and DCP dosages, and plasma tryptophan levels, can be constructed, which explains 57% of the OGTT/AUC variance in this measure of glucose tolerance in the DIO mice.

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Conflict of Interest

All the authors conducted the research. Phillip Moheno and Svetlana E. Nikoulina performed the data analysis and wrote the manuscript. Phillip Moheno holds stock, and all authors hold stock options, in SanRx Pharmaceuticals, Inc., which has been assigned patent rights to DCP.

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Table 1

One-way Analyses of Variance (ANOVAs) of Oral Glucose Tolerance Test Area-Under-Curve (OGTT/AUC); plasma Indoleamine 2,3-dioxygenase (IDO) metabolites tryptophan (Trp), kynurenine (Kyn), and calculated Trp/Kyn ratio; and certain cytokines/chemokines

Mean ± SEM N = 19	0 mg/(kg d) DCP n = 5	7 mg/(kg d) DCP n = 6	21 mg/(kg d) DCP n = 5	63 mg/(kg d) DCP n = 3
OGTT/AUC* (mg min)/dl	5218 ± 905	2632 [†] ± 517	4085 ± 600	5868 ± 831
0min-glu mg/dl	170 ± 15	172 ± 20	183 ± 11	162 ± 17
30min-glu**** mg/dl	233 ± 11	207 ± 11	277 [†] ± 19	312 ^{†††} ± 7.8
60min-glu mg/dl	200 ± 18	201 ± 17	211 ± 15	211 ± 7.5
90min-glu** mg/dl	241 ± 15	178 ^{††} ± 15	163 ^{††} ± 7.7	192 [†] ± 18
120min-glu mg/dl	162 ± 13	159 ± 12	162 ± 6.3	183 ± 15
Trp μM	110 ± 14	110 ± 9.0	119 ± 12	122 ± 5.0
Kyn** μM	.90 ± .14	.96 ± .10	1.00 ± .19	1.84 ^{††} ± .25
Kyn/Trp* μM/mM	8.34 ± 1.0	8.92 ± .87	8.52 ± 1.3	15.13 [†] ± 2.0
GM-CSF pg/ml	< 160	< 160	< 160	192 ± 32
IFNγ pg/ml	< 6.4	< 6.4	< 6.4	15.7 ± 9.3
IL-1α pg/ml	467 ± 258 (4)	496 ± 170	329 ± 225	348 ± 197
IL-1β pg/ml	34.7 ± 2.7 (4)	< 32.0	41.6 ± 8.5	36.7 ± 4.7
IL-4 pg/ml	7.76 ± 1.4	< 6.4	8.34 ± 1.9	< 6.4
IL-6 pg/ml	59.2 ± 8.8 (4)	24.7 ± 9.6	43.2 ± 8.2 (4)	56.2 ± 36.6
IL-10 pg/ml	< 32.0	49.0 ± 17.0	32.8 ± .76	< 32.0
IL-12(p40) pg/ml	32.5 ± .52 (4)	< 32.0	< 32.0 (4)	< 32.0
IL-12(p70) pg/ml	69.0 ± 31.5	37.8 ± 5.8	123.6 ± 80.5	72.9 ± 23.8
IL-13 pg/ml	197 ± 23.1	< 160	< 160	765 ± 604.7
MCP-1 pg/ml	70.8 ± 26.1	46.0 ± 14.0	49.6 ± 13.3	60.3 ± 22.2
RANTES pg/ml	36.6 ± 3.0 (4)	46.6 ± 13.0	33.1 ± 1.1 (3)	49.5 ± 17.5
TNFα pg/ml	12.0 ± 5.3	8.0 ± 1.6	10.2 ± 2.4	9.5 ± 3.1
* p < .05	[†] p < .05	Actual cell n's, when different, are given in parentheses.		
** p < .01	^{††} p < .01			
*** p < .005	^{†††} p < .005			
**** p = .001				
For all DCP dosages tested	For contrast tests versus 0 mg/(kg d) DCP			

Table 2

Multivariate Linear Regression of Oral Glucose Tolerance Test Area-Under-Curve (OGTT/AUC)

Trp, Kyn, Kyn/Trp, GM-CSF, IFN γ , IL-1 α , IL-1 β , IL-4, IL-6, IL-10, IL-12(p40), IL-12(p70), IL-13, MCP-1, RANTES, and TNF α were tested by Stepwise Regression as predictors of OGTT/AUC. The selected variables were DCP³, DCP², DCP, and Trp (μ M), where DCP is dosage in mg/(kg d). R² = .571; p = .013 for the regression:

$$\text{OGTT/AUC} = 0.009 \text{ DCP}^3 + 31.178 \text{ DCP}^2 - 574.513 \text{ DCP} + 29.828 \text{ Trp} + 1935.382$$

Coefficients^a

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	1935.382	1717.742		1.127	.279
DCP ³	.009	.003	17.143	2.650	.019
DCP ²	31.178	11.113	23.797	2.806	.014
DCP	-574.513	190.252	-6.604	-3.020	.009
Trp	29.828	14.533	.369	2.052	.059

a. Dependent Variable: OGTT/AUC

Figure 1a

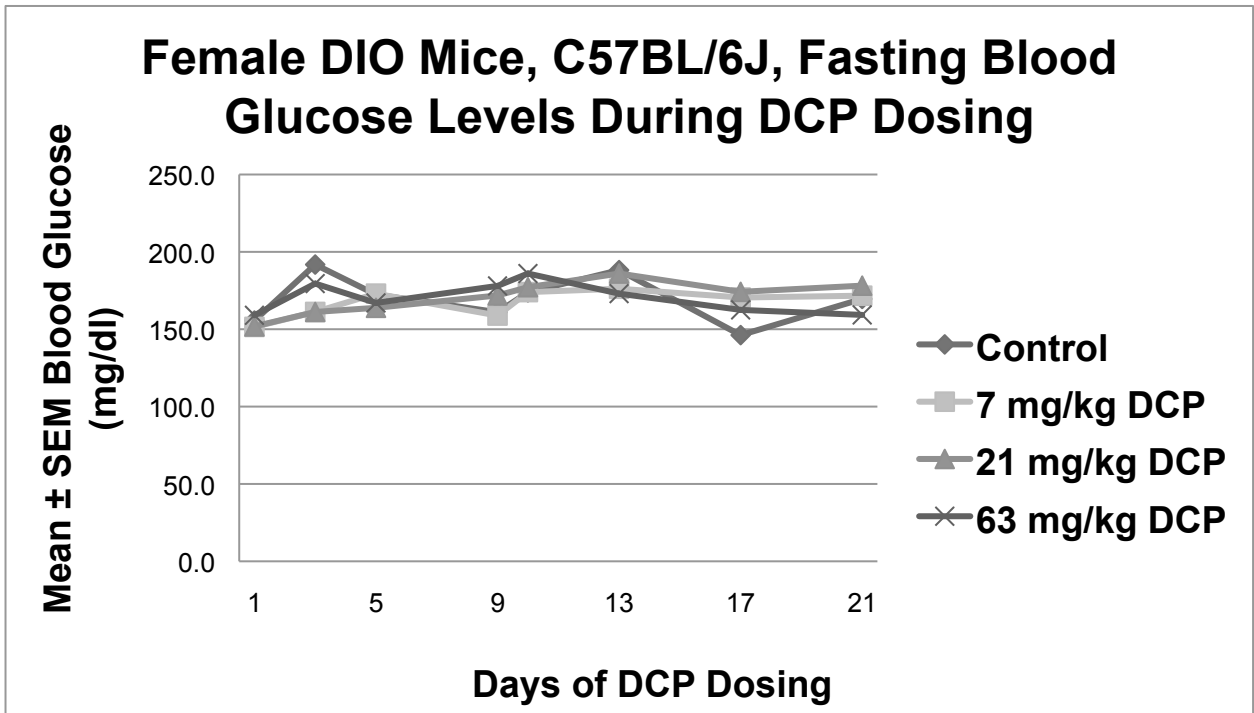


Figure 1b

