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Original Article

Single oral dose acute and subacute toxicity of a c-MET tyrosine kinase inhibitor and CDK 4/6 inhibitor combination drug therapy

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Abstract: c-MET inhibitor, crizotinib, and CDK 4/6 inhibitor, palbociclib, have been evaluated in combination as cancer treatment in vitro. Because the toxicological data for the combination of these drugs is limited, we investigated the toxicity of the crizotinib and palbociclib combination in 80 ICR (CD-1) mice (average age = ~20 weeks). Treatments were arranged as a 2 × 2 × 2 factorial and included sex (female vs. male), crizotinib (0 or 4 mg), and palbociclib (0 or 1 mg). Drugs were administered to mice by oral gavage 24 hours (n = 40) and 7 days (n = 40) prior to the collection of blood and tissue samples to determine serum chemistry, hematology, and histopathology. After dosing, each study group of mice was observed acutely (24 hrs) and subacutely (7 days) for any clinical changes associated with toxicity from the drugs. Serum chemistry, hematological effects, and selected histological tissue samples of each animal immediately after euthanasia were analyzed at the end of the study. No significant abnormalities or changes in the clinical signs, body and organ weight, or gross and histopathological evaluations were observed. Although within the normal reference range, there was an elevation in the red blood cells ($P = 0.05$) from 24-hour crizotinib- and palbociclib-treated mice (both males and females), which contrasted with the typical anemia observed in palbociclib-treated patients. Administration of the crizotinib and palbociclib combination resulted in an elevation in the ALT liver enzyme ($P = 0.05$) in the 24-hour treated group (both male and female), but the levels were within the normal ranges of the mice. Overall, serum chemistry and hematology did not reach significant abnormal levels in any of the acute- or subacute-treated groups. The results of this study confirmed that the combination of crizotinib and palbociclib at the given doses did not cause significant treatment-related toxicities in mice.

Keywords: Crizotinib, palbociclib, toxicity, TNBC, breast cancer, chemotherapy

Introduction

Currently, there are several chemotherapeutic agents and drugs available to treat a variety of cancers. Depending on the type of cancer and severity, there may be only one drug administered to patients for the treatment, but sometimes this is not enough. Over time, the body builds resistance to a drug being given, rendering the drug ineffective in treating the cancer [1, 2]. This is especially prominent after a cancer relapse [3, 4]. In addition, a high dose therapy can increase the drug's toxicity and cause more serious side effects, making it difficult for the patient to continue the treatment [5]. To help reduce the possibility of developing resistance as well as toxicity, a patient can be given a combination therapy [6]. The rationale for combination therapy is to use drugs that work by different mechanisms with minimally overlapping toxicities, thereby decreasing the likelihood that resistant cancer cells will develop [7]. Additionally, when drugs with different tumor inhibition mechanisms are combined, it may work more effectively together to kill cancer cells by inducing a synergistic cell-killing effect [7]. For instance, the combination of cyclophosphamide and doxorubicin synergisti-

cally inhibit tumor growth in breast cancer patients [8].

A cancer of particular interest is triple-negative breast cancer (TNBC) due to the lack of effective targeted therapy. A previous study reported an in vitro combination therapy, using the mesenchymal epithelial transition factor (c-MET) inhibitor crizotinib with a selective inhibitor of the cyclin-dependent kinases CDK 4 and CDK 6, palbociclib, that showed promising results for the inhibition of cancer cell proliferation [3]. An in vivo study comparing the drug combination given orally versus in micelles from an in vitro study could be very useful in developing new treatment regimens for cancer patients as the oral route tends to be an easier and more favored route of administration [9, 10]. Before these drugs can be considered for clinical use in combination, the toxicity of oral administration of the combined drugs needs to be evaluated in animal models.

Crizotinib and palbociclib are both FDA-approved drugs that induce cell cycle arrest in cancer cells [11, 12]. Crizotinib is a c-MET inhibitor traditionally used to treat non-small cell lung cancer (NSCLC) positive for anaplastic lymphoma kinase (ALK-positive) [11]. Crizotinib has demonstrated high objective response rates (~60%) and a median progression free-survival of 7 to 10 months in ALK-positive patients with NSCLC [11]. Although the results were positive, there is inevitably a toxic side effect associated with the drug. Crizotinib is known to negatively affect the liver, kidneys, and eyes, and palbociclib exerts similar toxic effects [11, 12]. One of the main side effects of palbociclib is the reduction of white blood cells, red blood cells, and platelets along with upper respiratory infection from normal dosing [12]. While toxic side effects of drugs for cancer are usually expected, they can have even greater detriment to patients who have pre-existing organ damage. Thus, it is important to know how these two drugs will interact in an in vivo toxicity study and to identify alternative treatment options.

Previously, it was shown that the combination of crizotinib and palbociclib effectively eliminated various breast cancer cells in vitro [3]. Specifically, they effectively inhibited the colony-forming ability of BT549 breast cancer cells *in vitro* compared with single drug treatment [3]. Interestingly, the two drugs combined exhibited synergistic cytotoxic effects that seemed to be higher in TNBC cells compared with luminal breast cancer cells [3]. The normal breast epithelial cells (MCF 10A) exhibited were not significantly affected by the drug combination, suggesting that these dual-drug combinations preferentially kill cancer cells over normal breast epithelial cells [3]. However, little is known about the *in vivo* toxicity of orally administered crizotinib and palbociclib in combination. In the current study, we evaluated the crizotinib and palbociclib combination in mice at the normal human equivalent dose (HED) for any acute or subacute toxicity by analysis of the blood enzymes and blood cell counts which are known to be adversely affected in patients. We hypothesize that the drug combination exhibits a higher toxicity than compared with each drug separately.

Materials and methods

Drugs

Crizotinib and Palbociclib were purchased from Selleck Chemicals (Houston, TX, USA).

Animal study

Female and male mice (ICR (CD-1®) Outbred Mice, Envigo) were used to test the effects of crizotinib (C) and palbociclib (P) alone and in combination. Mice were fed with standard rodent chow and water ad libitum and were housed 5 mice per cage on individually ventilated caging (IVC) racks. Within each sex, mice were assigned randomly to treatment with or without C and P, in a 2 × 2 × 2 factorial arrangement. Mice receiving no C or P received sodium acetate vehicle. In C-treated mice, C was dissolved in 50 mmol/L sodium acetate buffer and administered orally at 100 mg/kg (4 mg). Mice receiving P were administered P dissolved in 50 mmol/L sodium acetate buffer and administered orally at 25 mg/kg (1 mg) to all mice in the P group and combination group. Vehicle alone in the control group and drugs in treated groups were administered at 0.2 ml total dose volume to each mouse in a single dose at the beginning of the study. Two time points were evaluated after the treatment: the acute phase at 24 hours post treatment and the subacute phase at 7 days post treatment. At the end of each time point the mice were euthanized and followed by immediate blood collection and necropsy. All mice were evaluat-

ed for the body, and liver and spleen weight changes, hematology, and serum chemistry, and their tissues subjected to gross and histopathologic examination. Clinical observations were recorded once a day to assess the general health and potential clinical side effects of drugs on these animals.

All animal protocols were reviewed and approved by the IACUC committee at the University of Texas MD Anderson Cancer Center (Protocol number). Animal studies were performed as part of an AAALAC-accredited program.

Body weight changes and weight of organs

Mice body weight was measured on the day of dosing (Day 0) just before treatment, and again at 24 hours and 7 days after dosing. The weight of liver, spleen, and kidneys were measured upon necropsy and sampling of the animal's tissues.

Blood sample collection procedures

To obtain adequate blood volumes for analysis of complete blood count (CBC) and serum chemistry, terminal intracardiac puncture blood collection was performed on euthanized mice. The animals were placed in a carbon dioxide gas chamber and euthanized via carbon dioxide inhalation. Blood was collected immediately via cardiocentesis in microtubes containing ethylenediamine tetraacetic acid (EDTA) for CBC and in microtubes with serum separator for the blood serum chemistry analysis. This was performed at 24 hours and 7 days post treatment.

Blood samples were analyzed within 4 hours of collection by using a System 120 Siemens ADVIA® Hematology for cell blood counts. The blood samples in the serum microtubes for chemistry analysis were allowed to clot for at least 30 minutes and then centrifuged for analysis by Roche Integra 400+. Blood serum chemistry analysis included albumin, alkaline phosphatase (ALKP), alanine transaminase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN), creatinine, and globulin and total protein (TP).

Tissue collection and pathological analysis

After euthanasia and blood collection, mice were necropsied and all organs were examined grossly. All tissues were sampled in a solution of 10% neutral buffered formalin for 72-hour fixation. Formalin-fixed tissues were grossed following a standard protocol and then were processed and embedded in paraffin blocks. From paraffin blocks, 4-μm thick tissue sections were cut and mounted on glass slides and stained with hematoxylin and eosin (H&E) for microscopic examination and histopathological evaluation. H&E stained sections of liver, kidney, spleen, mesenteric lymph node, stomach, small intestine (duodenum, jejunum and ileum), large intestine (cecum, colon, and rectum), bone marrow of femur and sternum, and eyes were examined histopathologically by a board-certified veterinary pathologist.

Statistical analysis

Data were analyzed by ANOVA as a 2 × 2 × 2 factorial arrangement of treatments (with or without C treatment, with or without P treatment, and female or male) within a completely randomized design, with mouse as the experimental unit. Serum chemistry, hematology values and the body and organ weights were analyzed by ANOVA utilizing the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). The model included C and P treatment, sex, and the interaction. A similar, but independent, ANOVA was conducted for data collected 7 days post treatment. However, the pre-dose body weight differed ($P = 0.002$) between the treatments prior to dosing; therefore, pre-dose body weight was used as a covariate in the aforementioned model. If treatment terms were found to be significant ($P < 0.05$) or associated with a trend ($P < 0.10$), then pair-wise comparisons of least squares means were generated with the PDIFF option of SAS.

Results

Clinical observations

No abnormal clinical signs were observed during the in-life observation periods of the mice from any drug treatment group. All mice remained presumptively healthy with no signs of debilitation, pain or discomfort. One male mouse receiving C had accidental death prior to completion of the study. Necropsy and gross examination revealed no significant findings of this mouse. All other mice survived and completed the study.

Table 1. The effects of crizotinib (C), palbociclib (P), and sex (S) on body and organ weight values (g) of CD-1 mice at 24 hours

	No C				C (4 mg)					*P*-value							
	No P		P (1 mg)		No P		P (1 mg)										
Item	F[2]	M	F	M	F	M	F	M	SEM[3]	S	C	S × C	P	S × P	C × P	S × C × P
n	5	5	5	5	5	5	5	5	-	-	-	-	-	-	-	-
Pre-Dose BW[4]	48.40	50.66	47.36	49.18	54.26	54.62	46.96	52.92	3.89	0.35	0.24	0.84	0.30	0.64	0.56	0.59
Post-Dose BW	46.06	49.60	45.08	46.68	52.54	53.24	45.92	51.48	3.75	0.29	0.15	0.92	0.26	0.78	0.68	0.53
Liver	2.64	2.60	2.68	2.02	2.38	2.42	2.04	2.98	0.33	0.77	0.90	0.08	0.73	0.77	0.42	0.11
Kidney	0.92	0.78	0.50	0.68	0.50	0.68	0.48	1.24	0.24	0.17	0.98	0.20	0.98	0.20	0.13	0.71
Spleen	0.56	0.12	0.16	0.14	0.18	0.12	0.12	0.62	0.24	0.98	0.93	0.20	0.93	0.16	0.24	0.84

[1]Mice were treated with or without C and P 24 hours prior to the post-dose weighting of mice. [2]F = female, M = male. [3]Largest standard error of the mean (SEM) among treatments. [4]BW = Body Weight (g).

Table 2. The effects of crizotinib (C), palbociclib (P), and sex (S) on body and organ weight values (g) of CD-1 mice at 7 days

	No C				C (4 mg)					*P*-value							
	No P		P (1 mg)		No P		P (1 mg)										
Item	F[2]	M	F	M	F	M	F	M	SEM[3]	S	C	S × C	P	S × P	C × P	S × C × P
n	5	5	5	5	5	5	5	5	-	-	-	-	-	-	-	-
Pre-Dose BW[4]	41.22	48.38	40.94	53.04	40.48	54.38	47.48	49.02	1.98	<0.001	0.1017	0.994	0.73	0.60	0.67	0.0002
Post-Dose BW	43.20	48.35	47.56	48.57	46.11	47.64	46.32	47.16	1.06	0.02	0.86	0.14	0.09	0.06	0.06	0.27
Liver	2.04	3.08	2.10	3.14	2.05	3.08	2.23	2.72	0.20	<0.0001	0.58	0.25	0.91	0.27	0.53	0.38
Kidney	0.49	0.74	0.50	0.79	0.52	0.80	0.50	0.75	0.054	<0.0001	0.69	1.00	0.96	0.94	0.32	0.75
Spleen	0.10	0.12	0.13	0.13	0.09	0.19	0.10	0.10	0.027	0.12	0.79	0.21	0.56	0.05	0.07	0.29

[1]Mice were treated with or without C and P 7 days prior to post-dose weighing of mice. [2]F = female, M = male. [3]Largest standard error of the mean (SEM) among treatments. [4]BW = Body Weight (g).

Mice body and organ weight changes

The body weight of all mice remained stable throughout the study. This was true for both the 24-hour and 7-day time points. We noticed a minor weight change of a very small (< 5%) decline not significant enough to be considered completely influenced by C and/or P. However, we observed two 2-way interactions. There was a trend for a C × P interaction ($P = 0.06$) and a trend for a sex × P interaction ($P = 0.06$) (**Table 1**). There were no significant ($P > 0.10$) differences between organ weight in all mice in the 24-hour and 7-day time points compared with the respective control of each time point. Also, there were no significant ($P > 0.10$) differences between the males and females compared with their control groups. We noted that males weighed more than females in all groups at the 7-day time point but this is considered physiological body growth as the males typically are larger than females in general (**Table 2**).

Blood chemistry and hematological tests

At the 24-hour time point (acute phase), serum concentrations of albumin were not affected ($P > 0.19$) by a 3- or 2-way interactions involving sex, C, and P (**Table 3**). Concentrations of albumin were greater ($P = 0.004$) for mice that did not receive C compared with those that did (**Table 3**). P tended ($P = 0.07$) to influence albumin as the concentrations of albumin were greater for mice that did not receive P compared with those that did. As with albumin, alkaline phosphatase (ALKP) was not affected ($P > 0.10$) by a 3- or 2-way interaction involving sex, C, and P (**Table 3**). The concentrations of ALKP were greater ($P = 0.005$) for mice that did not receive C compared with those that did. No 3-way interaction ($P = 0.10$) involving sex, C, and P was observed for alanine transaminase (ALT). However, a C × P interaction ($P = 0.05$) influenced ALT such that the concentrations of ALT were greater than the group that did not receive any drugs. Aspartate transaminase (AST) also had no 2- or 3-way interactions ($P > 0.14$) involving sex, C, and P. Blood urea nitrogen (BUN) ($P > 0.08$), creatinine ($P > 0.13$), and total protein (TP) ($P > 0.33$) all had no 2- or 3-way interactions with sex, C, or P although BUN did show a trend of influence ($P = 0.08$) (**Table 3**). Globulin had a 3-way interaction ($P =$

Table 3. The effects of crizotinib (C), palbociclib (P), and sex (S) on serum chemistry values of CD-1 mice at 24 hours

	No C				C (4 mg)					*P*-value						
	No P		P (1 mg)		No P		P (1 mg)									
Item	F[2]	M	F	M	F	M	F	M	SEM[3]	S	C	S × C	P	S × P	C × P	S × C × P
n	5	5	5	5	5	5	5	5	-	-	-	-	-	-	-	-
Albumin	4.3	3.9	4.1	3.8	3.7	3.9	3.7	3.5	0.17	0.18	0.004	0.19	0.07	0.46	0.81	0.23
ALKP	85.8	53.4	63.8	49.2	36.5	49.2	42	33.4	11.5	0.16	0.005	0.10	0.23	0.91	0.60	0.20
ALT	186.8	67.2	77.3	41.2	55	78.4	204.5	78.4	66.1	0.08	0.76	0.71	0.92	0.64	0.05	0.10
AST	373.6	191.2	545.3	79	131.5	99.8	933	348.6	302.8	0.06	0.62	0.96	0.10	0.20	0.14	0.68
BUN	21	29.2	22.3	20.1	21.4	19.6	20.8	20.3	2.51	0.58	0.12	0.22	0.25	0.18	0.24	0.08
Creatinine	0.49	0.41	0.35	0.29	0.42	0.31	0.34	0.26	0.04	0.001	0.03	0.62	0.001	0.59	0.13	0.99
Globulin	2.1	2.8	2.3	2.3	2.2	2.2	2.1	2.5	0.15	0.03	0.21	0.43	0.80	0.42	0.31	0.02
TP	6.4	6.7	6.4	6.1	5.9	6.1	5.8	5.9	0.25	0.62	0.009	0.71	0.17	0.33	0.63	0.47

[1]Mice were treated with or without C and P 24 hours prior to the collection of a single blood sample for the measurements of blood chemistry values. [2]F = female, M = male. [3]Largest standard error of the mean (SEM) among treatments.

Table 4. The effects of crizotinib (C), palbociclib (P), and sex (S) on serum chemistry values of CD-1 mice at 7 days

	No C				C (4 mg)					*P*-value						
	No P		P (1 mg)		No P		P (1 mg)									
Item	F[2]	M	F	M	F	M	F	M	SEM[3]	S	C	S × C	P	S × P	C × P	S × C × P
n	5	5	5	5	5	5	5	5	-	-	-	-	-	-	-	-
Albumin	4.3	3.8	4.3	3.7	4.3	3.6	3.9	3.6	0.16	0.0001	0.13	0.95	0.19	0.25	0.57	0.20
ALKP	74.1	30.9	48.4	48.7	82.8	39.1	60.6	41.5	11	0.0028	0.38	0.41	0.25	0.0073	0.62	0.53
ALT	22.2	62.9	42.5	198.3	117.8	97.3	248.6	78.8	75.24	0.81	0.14	0.01	0.21	0.90	0.97	0.14
AST	116	146.1	99.2	133.2	70.8	70.8	71.5	71.5	105.4	0.09	0.10	0.007	0.17	0.14	0.11	0.22
BUN	23.6	32.1	24.1	25.5	21.7	25.7	17.5	25.8	3.86	0.06	0.10	0.79	0.23	0.73	0.82	0.28
Creatinine	0.45	0.4	0.6	0.37	0.38	0.35	0.32	0.27	0.14	0.39	0.12	0.49	0.94	0.49	0.39	0.68
Globulin	2.17	2.53	1.71	2.7	1.82	2.28	2.09	2.5	0.17	0.0001	0.28	0.20	0.55	0.12	0.04	0.15
TP	6.5	6.3	5.98	6.4	6.16	5.85	6.02	6.16	0.27	0.9	0.12	0.44	0.67	0.10	0.33	0.89

[1]Mice were treated with or without C and P 7 days prior to the collection of a single blood sample for the measurements of blood chemistry values. [2]F = female, M = male. [3]Largest standard error of the mean (SEM) among treatments.

0.02) involving sex, C and P such that a decrease in concentration was noticed compared to the group lacking C or P (**Table 3**).

At the 7-day time point (subacute phase), the blood chemistry remained generally normal. There was a difference in enzyme levels related to the main effect of sex. Males tended to have greater concentrations of AST (P = 0.09), BUN (P = 0.06), and globulin (P = 0.0001) and had decreased concentration of the ALKP (P = 0.003) and albumin (P = 0.0001) (**Table 4**). ALT (P = 0.01) and AST (P = 0.007) had 2-way interactions with sex and C in which ALT was greater in the group receiving C but females were greater than males and AST concentrations were greater in females than the group not receiving C or P (**Table 4**). There was also a 2-way interaction for ALKP involving sex × P that showed a decrease in ALKP (P = 0.0073). The 2-way interaction of C × P showed a decrease in globulin (P = 0.04) compared to the group that did not receive the drugs (**Table 4**). This time point had no 3-way interactions, and the enzyme concentrations for creatinine and total protein did not yield any interactions.

For the hematological values at the 24-hour time point (acute phase), sex seemed to affect all blood parameters. Females had a slightly higher number of red blood cells (RBC) (P = 0.01), hemoglobin (P = 0.01), and hematocrit (P = 0.04) with males only having greater concentration in platelets (P = 0.02) over females (**Table 5**). A 2-way interaction of sex and P also showed an increase in WBCs for females along with a decrease in WBCs for males compared to the groups that did not receive any drug treatment (**Table 5**). The concentrations of WBCs were increased (P = 0.05) in mice that

Table 5. The effects of crizotinib (C), palbociclib (P), and sex (S) on serum hematology values of CD-1 mice at 24 hours

	No C				C (4 mg)					*P*-value						
	No P		P (1 mg)		No P		P (1 mg)									
Item	F[2]	M	F	M	F	M	F	M	SEM[3]	S	C	S × C	P	S × P	C × P	S × C × P
n	5	5	5	5	5	5	5	5	-	-	-	-	-	-	-	-
WBC	4.9	6.9	7.1	5.8	8.2	9.3	7.4	5.5	1.31	0.95	0.05	0.61	0.22	0.04	0.06	0.91
RBC	12.3	11.6	11.9	10.8	12.1	10.4	12.9	11.9	0.77	0.01	0.69	0.55	0.54	0.85	0.05	0.53
Hemoglobin	18.9	17.8	18.8	16.3	18.4	15.6	19.9	18.1	1.28	0.01	0.97	0.69	0.40	0.90	0.05	0.40
Hematocrit	68.7	64.5	63.8	60.0	66.6	57.3	67.2	62.6	4.49	0.04	0.74	0.55	0.73	0.61	0.12	0.66
Platelet	158.5	338.8	238	439.3	267	785.3	151.3	281.8	191.3	0.02	0.45	0.52	0.29	0.38	0.06	0.33

[1]Mice were treated with or without C and P 24 hours prior to the collection of a single blood sample for the measurements of hematology values. [2]F = female, M = male. [3]Largest standard error of the mean (SEM) among treatments.

Table 6. The effects of crizotinib (C), palbociclib (P), and sex (S) on serum hematology values of CD-1 mice at 7 days

	No C				C (4 mg)					*P*-value						
	No P		P (1 mg)		No P		P (1 mg)									
Item	F[2]	M	F	M	F	M	F	M	SEM[3]	S	C	S × C	P	S × P	C × P	S × C × P
n	5	5	5	5	5	5	5	5	-	-	-	-	-	-	-	-
WBC	3.8	5.6	8.3	7.4	6.3	6.2	6.5	8.2	1.16	0.47	0.41	0.81	0.002	0.72	0.11	0.13
RBC	11.9	12.8	11.2	11.8	11.3	12.2	13.1	12.0	0.86	0.60	0.59	0.37	0.99	0.21	0.08	0.44
Hemoglobin	18.4	18.6	16.5	17.4	16.8	18.5	19.5	17.3	1.13	0.85	0.61	0.51	0.53	0.18	0.07	0.12
Hematocrit	66.1	67.8	59.3	64.3	59.9	68.5	66.5	62.5	4.14	0.35	0.99	0.81	0.26	0.29	0.21	0.13
Platelet	314.3	340.2	732.9	304.7	533.5	196.1	178.8	354.6	161.3	0.23	0.22	0.48	0.58	0.86	0.10	0.02

[1]Mice were treated with or without C and P 7 days prior to the collection of a single blood sample for the measurements of hematology values. [2]F = female, M = male. [3]Largest standard error of the mean (SEM) among treatments.

received C compared with those that did not (**Table 5**). A 2-way interaction of C × P treatment combination showed an increase in RBCs (P = 0.05) and a decrease in hemoglobin (P = 0.05) in all mice, both male and female. In addition, the C × P treatment showed a trend of decrease in number of platelets (P = 0.06) compared to the control group (**Table 5**). All three treatment groups had a slightly increased neutrophilia in comparison to the control group but were still within the normal reference range (data not shown).

The hematological values at the 7-day time point (subacute phase) showed that the WBC counts were slightly increased in all treatment groups compared to the groups that received no C or P, but they were still in the normal reference range (**Table 6**). P caused an elevation in the WBC counts (P = 0.002) compared to the group receiving no C or P (**Table 6**). This increase showed an equal elevation in neutrophils and lymphocytes contributing to the overall WBC elevation. There was also a 3-way interaction involving sex × C × P on the platelets (P = 0.02) in which males had an elevation and females had a decrease in platelet numbers. There were no 2- or 3-way interactions involving C × P treatment. Only trends involving elevations of RBCs (P = 0.08) and hemoglobin (P = 0.07) were observed.

Histological findings

There were no significant histopathological changes of the 9 organs examined microscopically in all treatment groups tested at either the 24-hour or 7-day time point in comparison with control groups. There were sporadic findings of mild inflammation in the gallbladder of mice from the C and P combination-treated groups, suggesting a minimal injury of the drugs in the process of detoxification and elimination through the bile. Moderate or marked atrophy of the retina with loss of photoreceptors and inner molecular layer affecting one or both eyes was observed with similar frequency in the control groups and treated groups, indicating that this condition was related to the strain/genotype of these mice. The bone marrow of 4 out of the 5 females treated with C and P combination had minimal to moderate

decrease in the number of erythroid precursor cells 24 hours post treatment, suggesting acute mobilization of immature RBCs from bone marrow into systemic blood circulation. In addition, the mice in this treatment group also had decreased hematopoietic cells and increased number of hemosiderin-laden macrophages in the spleen, all of which suggested mild hemolytic injury of the RBCs. However, since the RBCs count were within the normal range values without clinical hemolytic anemia, this mild reduction of red cells precursors from bone marrow and spleen is not considered a significant adverse effect.

Discussion

A previous study evaluated the IC_{50} of the C, P, and sildenafil combination *in vitro*, but did not evaluate the *in vivo* toxicity or clinical effects of these drugs in combination [10]. However, there is a paucity of research that describes the effects of combined administration of C and P on male and female laboratory mice. To our knowledge, we are the first to describe such effects not only on blood chemistry and hematology values but also on histopathology, and body and organ weight. The findings of this study can benefit clinical trials using this combination of drugs in the treatment of cancer, and researchers and veterinary clinicians who are using this treatment in other animal models for cancer research. The combined treatment with C and P had only minimal adverse effects on serum chemistry values, but no significant adverse effects on hematology and overall health of the mice.

In reference to the blood chemistry, ALT, an enzyme associated with the liver, was elevated in the 24-hour chemistry time point for C × P but was not high enough to be considered abnormal in mice. Likewise, globulin was also affected by C × P but was only mildly decreased compared to the control mice and still not out of the range of normal. It appeared that the C and P combination affected the blood to a lesser degree than did each drug alone. Treatment of C by itself had more effects on the blood chemistry than did the combination with P. This was unexpected since the drugs were given in the same dose together as they were separately. It would seem that the same results would be attained or higher values due to the combination. The C and P combination also caused an increase in RBCs and decrease in hemoglobin at the 24-hour and 7-day time points. In addition, sex appeared to have an effect on platelets where males had an increase while females had a decrease in number. Typically, lower numbers of RBCs, platelets, and WBCs is expected in the P treatment group, but we had an overall increase in RBCs, and increase in platelets in males.

Our results also showed that there were no adverse effects of the combination treatment on clinical signs, body and organ weight, and gross changes of examined organs. Body weight of both male and female mice in this study remained consistent compared to the control groups (control group), suggesting that the C and P combination treatment did not cause any harmful changes in the mice body weight.

The blood samples had mild elevations of liver serum enzymes, which could attributed to mild hemolysis of some of the blood samples. Unfortunately, this tends to happen with mouse blood quickly if not placed in blood tube expeditiously and/or placed on a blood tube rocker. There were a total of 10 samples from various study groups that had to be removed as outliers due to abnormally high elevation of enzymes representative of the liver (AST and ALT). This was attributed to various degrees of hemolysis of the blood sample since the histological examination of liver revealed no significant hepatic lesions or injury.

With regard to the hematological results, it is worthwhile to point out that the C and P combination caused neutrophilia 24 hours post treatment in both male and female groups in comparison with the control groups. P treatment is commonly associated with neutropenia in humans. However, it is unclear why the mice experienced this even though the levels were still within the normal reference range for this mouse strain [13]. There were also increases in RBC counts of mice from the combination treatment in both male and female 24 hours post treatment. This is unexpected as decreased RBCs or anemia is usually associated with P [13]. Beyond these findings, there were no significant findings indicating any significant toxicity from the drugs given.

We hypothesized that the C and P combination would be more toxic than each drug alone.

Interestingly, however, the combination treatment had no significant toxicity in both male and female mice groups. There were no significant adverse findings in this combination treatment group indicating either acute or subacute toxicity in the mice. Although this study provided evidence of a relatively safe oral drug combination of C and P, more analyses are to acquire safety information such as what the maximum tolerated dose (MTD) of the drugs combined is or its effectiveness toward destroying breast cancer cells. The current study only focused on identifying any possible severe acute and subacute toxic effects of a single oral dose on the body, specifically of the blood, liver, kidneys and eyes, since these organs have side effects in humans when treated with either C or P. Further investigation into these areas as well as a longer timeframe of study involving daily HED dosing may provide even a more accurate description of how viable this drug combination would be if used in humans.

Both of the drugs, C and P, have been tested separately in mice for toxicity by Pfizer pharmaceuticals. We evaluated these drugs at relatively lower doses compared with the higher doses at which were shown to cause impairment. C was tested in mice at 200 mg/kg for 28 days, and there were no observed adverse effect level according to the Pfizer Pharmaceuticals Inc. materials safety data sheet (MSDS) as well as a previous cancer study at 200 mg/kg in which the mice survived well [14]. P has also been given at much higher doses for longer periods of time in cancer studies, e.g., 150 mg/kg for 14 days, and the mice survived without significant evidence of toxicity [15, 16]. Based on the findings of those studies, higher doses of both drugs should be tested *in vivo* together in order to identify the adverse reactions or an LD_{50} determined in subsequent studies, which would allow better evaluation of adverse toxic effects of the C and P combination on the body, including the brain, heart and reproductive organs.

The findings from the current in vivo toxicity study in mice, which encompassed two time points at 24 hours and 7 days, may help build upon the base knowledge of what is already known in reference to C and P and provide helpful information for future studies of these drugs. We have shown that the combination of C and P given orally together at HED does not induce acute or subacute toxicity in mice. However, more research will be required to substantiate this combinational drug therapy as a viable option for treatment of cancer in human patients.

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Disclosure of conflict of interest

None.

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