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COPPER(II) SALICYLALDEHYDE-N,N-DIBENZYLSEMICARBAZONE – A POTENTIAL ANTITUMOUR COMPOUND

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ABSTRACT

From QSAR studies, a series of salicylaldehyde semicarbazones, $HOC_6H_4CH{=}N\text{-}NHCONR_2$ (H_2R_2) and their Cu(II) complexes were synthesized, characterized and investigated for their biological activities previously. Results obtained showed that one of the most active compounds in this series is [Cu($HBnz_2$)Cl]. Thus, in this study, research was then carried out to investigate the anti-tumour property of this compound. The LD_{50} of [Cu($HBnz_2$)Cl] was determined to be at 75 mg/kg. Anti-tumour property was observed when this compound was introduced to tumours of human origin (MOLT-4 on SCID mice) and murine origin (3LL on normal mice).

KEYWORDS

Salicylaldehye semicarbazone, copper(II) complexes, antitumour, *in vivo*, LD_{50}.

INTRODUCTION

Since the advent of Cisplatin, the search for new transition metal complexes with potential biological activity has been the focus of extensive investigation. It has also been shown that complexation with copper enhances the biological activity of a wide variety of organic ligands[1-4]. The discovery of the antiinflammatory and analgesic properties of acetylsalicylic acid (aspirin) in the 19th century has lead to a large body of research into the chemistry and properties of salicylates[5,6]. More recently, it was found that the copper complexes of non-steroidal antiinflammatory agents were more effective than the parent drugs[7,8]. This suggested that copper complexes were the active metabolites of these drugs. In order to verify this, Sorenson et al. made a comparison of the properties of salicylic acid, anthranilic acid and 3,5-diisopropylsalicylic acid (Hdips) against their respective copper(II) complexes[9]. The complex $[Cu(dips)_2]$ was discovered to be only as effective as Hdips whereas the copper complexes of the other two compounds were more effective than their organic counterparts. However, the compound $[Cu(dips)_2]$ is one of the most promising of a number of lipid soluble copper complexes of salicylates in which the observed activity is attributed to the complexed form of copper[10].

In 1982 Johnson et al. studied, *in vivo*, a range of tridentate ONO hydrazones as iron-chelating drugs[11]. This promising class of compounds, with demonstrated bioactivity, are hydrazones of pyridoxal and the structurally related salicylaldehyde. An example is the ligand salicylaldehyde benzoylhydrazone (H_2sb, Figure 1).

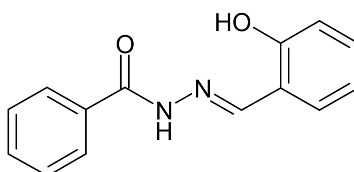

Figure 1
Salicylaldehyde benzoylhydrazone (H_2sb).

The copper(II) complex of H_2sb, $[Cu(Hsb)Cl]\cdot H_2O$, was shown to be a potent inhibitor of DNA synthesis and cell growth and is more effective than the metal-free chelator in a number of human and rodent cell lines[12-14]. The cytotoxicity of this complex was also discovered to exceed many other compounds which were previously known to possess such properties, including those used clinically[12]. This compound showed some discrimination towards different cell types and is non-toxic to mice. It was suggested that the Cu(II) complex is the biologically active species. Therefore, a series of H_2sb analogues, their copper complexes, and a range of transition metal complexes of the unsubstituted ligand have been synthesized, and a QSAR investigation of cytotoxicity against a human adenocarcinoma cell line carried out[15]. The copper(II) complex of salicylaldehyde acetyl hydrazone (H_2as), which has a structure resembling H_2sb except that the phenyl ring of H_2sb is replaced by a methyl group, also showed biological activity[16-18]. The mechanism(s) of biological

activity for the hydrazones is still unclear. As the copper complexes of these ligands tend to be more active than the free ligands, it has been suggested that the active form of the drug is the metal complex.

Previously, we reported the syntheses of a series of salicylaldehyde semicarbazones (H_2R_2) and their Cu(II) complexes[19,20] (Table 1). The structure of H_2R_2 resembles H_2sb except that the phenyl ring of H_2sb is replaced by an amide group (Figure 2).

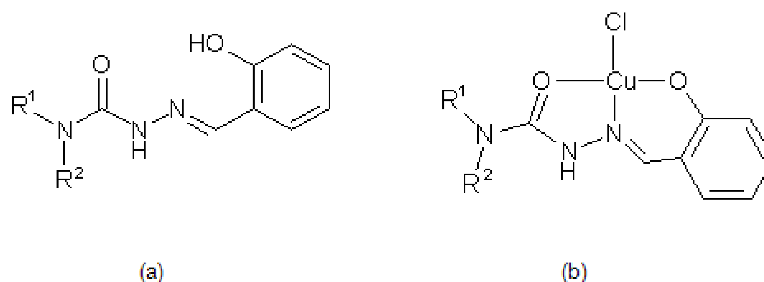

Figure 2
General structure of (a) salicylaldehyde semicarbazone (H_2R_2) and (b) its copper(II) complexes.

An amide group was chosen because it is more electron donating (due to the presence of N) than the phenyl ring in H_2sb and the methyl group in H_2as. Moreover, this electron donating effect could be further tuned by varying the electronic properties of R^1 and R^2. There is also scope for varying the lipophilicity and steric properties of R^1 and R^2.

Table 1
Summary of compounds synthesized.

Abbreviation		R^1	R^2
Ligand	Complex		
H_2Me_2	$[\{Cu(Me_2)\}_2]\cdot H_2O$	Methyl	Methyl
H_2Et_2	$[\{Cu(HEt_2)Cl\}_2]$	Ethyl	Ethyl
H_2iPr_2	$[\{Cu(HiPr_2)Cl\}_2]\cdot 4H_2O$	i-Propyl	i-Propyl
H_2Hex_2	$[\{Cu(HHex_2)Cl\}_2]\cdot 2H_2O$	Hexyl	Hexyl
H_2Bnz_2	$[Cu(HBnz_2)Cl]\cdot H_2O$	Benzyl	Benzyl
H_2Bu_2	$[Cu(HBu_2)Cl]\cdot 0.5H_2O$	Butyl	Butyl
H_2Pip	$[\{Cu(HPip)Cl\}_2]\cdot 0.5H_2O$	Piperidyl	
H_2MeP	$[\{Cu(HMePh)Cl\}]\cdot 0.5H_2$	Methyl	Phenyl
H_2Ph_2	$[Cu(Ph_2)\cdot H_2O]$	Phenyl	Phenyl
H_2PhH	$[\{Cu(HPhH)Cl\}_2]$	Phenyl	H

The cytotoxic activities of the ligands and complexes were tested on a panel of human cancer cell lines whereby the 50 % inhibitory concentration (IC_{50}) was obtained by performing the MTT assay. Results obtained showed that the two most active compounds in this series are [Cu($HBnz_2$)Cl] (IC_{50} = 2 μM) and [Cu(HBu_2)Cl] (IC_{50} = 2.5 μM), complexes of the ligand H_2R_2 such that R^1 and R^2 are substituted by benzyl and butyl groups respectively[20]. Hence, a series of biochemical assays were conducted, *in vitro*, using these two complexes to better understand their cytotoxicities on MOLT-4 cells, a human leukemia cell line. From these studies, it was demonstrated that the mode of cell death induced by [Cu($HBnz_2$)Cl] and [Cu(HBu_2)Cl] was attributed to apoptosis[20], also known as programmed cell death. Proteomic investigations revealed that these complexes down-regulate proteins that are associated with transcription, translation, protein folding, glucose metabolism, signal transduction and mitosis[20].

Henceforth, the next important and interesting step was to look into the *in vivo* studies of these complexes. [Cu($HBnz_2$)Cl] was thus chosen to investigate the toxicity and anti-tumour property of these complexes using animal models simply because it had a slightly better IC_{50} value than [Cu(HBu_2)Cl].

MATERIALS AND METHODS

It has to be emphasized that all the animal work were carried out with permission from, and according to strict rules laid down by, the National University of Singapore's Animal Holding Unit's animal ethics committee.

1. ***Acute Toxicity (LD_{50}) studies***

   For the initial round, 25 male ARC Swiss mice weighing between 18-20 g were used. Widely differing concentrations of the compound [Cu($HBnz_2$)Cl] were given to the animals. The concentrations tested were 200 mg/kg, 100 mg/kg and 50 mg/kg. The animals were treated with the compound after at least 5 days of adaptation. They were divided into five equal groups. The first two groups were control groups, one of which was treated with only the solvent (1 % DMSO in saline) and the other group was just treated with saline. The other three groups were treated with the compound at the three concentrations mentioned above (one concentration per group). The compounds were administered intraperitoneally.

   Results obtained from the first experiment (Table 2 and Figure 3) enabled us to narrow down the range of the concentrations used in order to produce a more accurate LD_{50} value. Hence, the experiment was repeated using concentrations of 40 mg/kg, 60 mg/kg, 80 mg/kg, 90 mg/kg and 180 mg/kg of [Cu($HBnz_2$)Cl].

2. ***C57 BL/6J mice bearing murine 3LL tumours.***

   13 C57 BL/6J female mice, 5-6 weeks old were used after 5 days of adaptation. The 3LL (Lewis Lung Carcinoma) murine cell line was grown in suspension culture in RPMI-1640 medium supplemented with 5 % heat-inactivated FCS. Cells to be injected were taken from culture during the log phase of growth. The cells were injected subcutaneously into the flanks of mice for a total dosage of one million cells per mouse.

   The 13 mice were divided randomly into 3 groups. The first two groups consisted of 5 mice each and they were used for treatment with the compound [Cu($HBnz_2$)Cl] at two different concentrations, 40 mg/kg and 80 mg/kg (one concentration per group). The last group was a control group consisting of 3

mice treated with only the solvent (1 % DMSO in saline).

The mice were observed to note the beginning of visible tumour growth. Administration of [Cu(HBnz$_2$)Cl] started as soon as the tumours were visible and could be measured fairly accurately (= Day 1). [Cu(HBnz$_2$)Cl] was introduced intra-tumourly according to a treatment schedule consisting of five consecutive days of [Cu(HBnz$_2$)Cl] administration (either at 40 mg/kg or 80 mg/kg) followed by two consecutive days without treatment. Tumour sizes were measured daily until Day 28. Tumour volume was calculated according to the formula: Tumour volume = length × width^2 x ½. % $\Delta T/\Delta C$ values were calculated where $\Delta T = (T - \Delta_0)$ and $\Delta C = (C - \Delta_0)$. Δ_0 is defined as the average tumour volume at Day 1 and both T and C are tumour volumes at a specified day for treated and control tumours, respectively[21]. According to the Division of Cancer Treatment, NCI, NIH[22], a % $\Delta T/\Delta C$ value of ≤ 42 % is an indication of a significant antitumour activity. The animals were sacrificed when their tumours reached an approximate volume of 1,500 mm^3.

3. ***Immunodeficient SCID mice bearing human MOLT 4 tumours.***
Experiment 1
15 male immunodeficient SCID mice, 6-7 weeks old were obtained from The Jackson Laboratory, Maine, USA. They were kept under sterile conditions in cages with filter bonnets and were fed with sterilized pellets and sterilized distilled water. The animals were used after 5 days of adaptation.

The human T-cell leukemia line MOLT-4, was grown in suspension culture in RPMI-1640 medium supplemented with 5 % heat-inactivated FCS. Cells to be injected were taken from culture during the log phase of growth. In order to promote tumour growth, the MOLT-4 cells were mixed with another cell line, the HT-1080 cells, a human fibrosarcoma cell line[23]. This cell line was grown in monolayer culture in RPMI-1640 medium containing 5 % FCS. Initially, the HT-1080 cells were stored in liquid nitrogen and were thawed and X-irradiated with a lethal dose of 6000 cGy radiation from a ^{137}Cs source and resuspended in serum–free media at a concentration of 50 x 10^6 cells/ml. MOLT-4 cells were checked for viability by trypan blue dye exclusion, spun and resuspended at a concentration of 50 x 10^6 cells/ml. The MOLT-4 cells were then mixed 2:1 with HT-1080 and injected subcutaneously into the flanks of mice for a total dose of 10 x 10^6 MOLT-4 cells and 5 x 10^6 HT-1080 cells in 0.3 ml of media.

The 15 mice were divided randomly into four groups. The first group was a control consisting of two mice that were injected only with the X-irradiated HT-1080 cells. The second group consisted of three mice that were treated with the solvent (1 % DMSO in saline) only. The last two groups were each made up of five mice that were subjected to treatment of the compound [Cu(HBnz$_2$)Cl] in two different concentrations – one group received 40 mg/kg of [Cu(HBnz$_2$)Cl] while the other group received 80 mg/kg of [Cu(HBnz$_2$)Cl].

The mice were observed to note the beginning of visible tumour growth. Administration of [Cu(HBnz$_2$)Cl] started as soon as the tumours were visible and could be measured fairly accurately (Day 1). [Cu(HBnz$_2$)Cl] was introduced intra-tumourly according to a treatment schedule consisting of five consecutive days of [Cu(HBnz$_2$)Cl] administration (either at 40 mg/kg or 80 mg/kg) followed by two consecutive days without treatment.

Tumour sizes were measured daily until Day 28. Tumour volume was calculated according to the formula: Tumour volume = length × width^2 x ½. The values of % ∆T/∆C were also calculated[21]. According to the Division of Cancer Treatment, NCI, NIH[22], a % ∆T/∆C value of ≤ 42 % is an indication of a significant antitumour activity. The animals were sacrificed when their tumours reached an approximate volume of 1,500 mm^3.

Experiment 2
The experiment was repeated under similar conditions described in Experiment 1 but only a single dosage of 80 mg/kg of [Cu(HBnz$_2$)Cl] was tested instead.

4. Statistical Analysis.
Tumour volumes were statistically evaluated by the Student's *t*-test using the PASW Statistics 18 (18.0.0, IBM Corporation, USA) software.

RESULTS AND DISCUSSION

1. Acute Toxicity (LD$_{50}$)
From Table 2 and Figure 3, we could derive the LD$_{50}$ value of [Cu(HBnz$_2$)Cl] to be ca. 70 mg/kg. Similarly, from Table 3 and Figure 4, after narrowing down the range of dosages used, the LD$_{50}$ was more accurately approximated to be ca. 75 mg/kg. Incidentally, this LD$_{50}$ value was also obtained for the ligand, H$_2$sb[11].

In the first experiment, for the 200 mg/kg group, a total of 4 mice died, of which, one died one hour after the injection of [Cu(HBnz$_2$)Cl], two were found dead at the third hour and the last one the next day. As for the 100 mg/kg group, one died after the first hour, another died during the sixth hour and the other two were found dead the next day. Finally, only one mouse was found dead from the 50 mg/kg group (the next day).

Table 2
Results obtained from the acute toxicity experiment.

Treatment	50mg/kg	100mg/kg	200mg/kg	Saline	Solvent
No. before treatment	5	5	5	5	5
No. after treatment	4	1	1	5	5
No. dead	1	4	4	0	0
% Mortality	20	80	80	0	0

Table 3
Results obtained from the second acute toxicity experiment.

Treatment	40mg/kg	60mg/kg	80mg/kg	90mg/kg	180mg/kg
No. before treatment	5	5	5	5	5
No. after treatment	5	4	2	2	0
No. dead	0	1	3	3	5
% Mortality	0	20	60	60	100

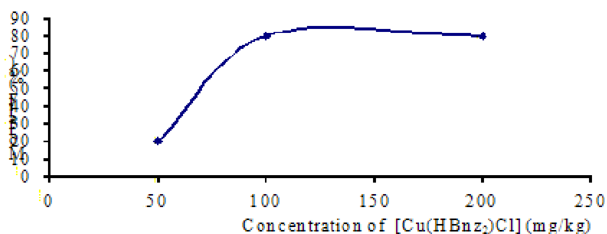

Figure 3

A mortality vs concentration curve to obtain the LD_{50} value using $[Cu(HBnz_2)Cl]$ on male ARC Swiss mice.

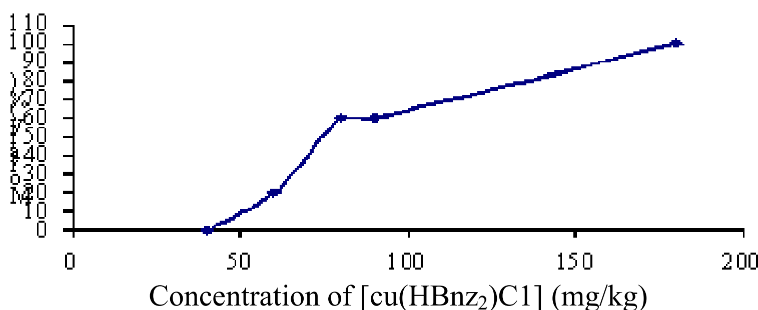

Figure 4

A second mortality vs concentration curve to obtain a more accurate LD_{50} value using $[Cu(HBnz_2)Cl]$ on male ARC Swiss mice.

Based on these experiments, we were able to arrive at the LD_{50} value of $[Cu(HBnz_2)Cl]$ (75 mg/kg). Toxicity of this copper complex was on par with the ligand, H_2sb.

2. C57 BL/6J mice

The results obtained from this experiment are summarized in Table 4 and presented graphically in Figures 5, 6 and 7.

Table 4

Comparison of means across groups in experiment with C57 BL/6J mice.

Day	40 mg/kg (n=5)	80 mg/kg (n=5)	Solvent (n=3)	Difference between groups[a] (based on t-test comparisons)
1	158.60	79.20	75.33	40mg/kg = Solvent 80mg/kg = Solvent 40mg/kg > 80mg/kg*
2	95.00	0	93.00	40mg/kg = Solvent 80mg/kg < Solvent** 40mg/kg > 80mg/kg**
3	62.00	0	111.67	40mg/kg = Solvent 80mg/kg < Solvent** 40mg/kg > 80mg/kg*

4	47.60	0	122.33	40mg/kg < Solvent* 80mg/kg < Solvent** 40mg/kg > 80mg/kg*
5	24.20	0	272.67	40mg/kg < Solvent** 80mg/kg < Solvent* 40mg/kg = 80mg/kg
6	11.60	0	385.33	40mg/kg < Solvent*** 80mg/kg < Solvent** 40mg/kg = 80mg/kg
7	33.60	0	536.33	40mg/kg < Solvent* 80mg/kg < Solvent* 40mg/kg > 80mg/kg**
8	103.20	0	655.33	40mg/kg < Solvent* 80mg/kg < Solvent* 40mg/kg > 80mg/kg***
9	71.80	0	901.33	40mg/kg < Solvent* 80mg/kg < Solvent* 40mg/kg > 80mg/kg*
10	58.80	0	1359.33	40mg/kg < Solvent* 80mg/kg < Solvent* 40mg/kg > 80mg/kg**
11	22.00	0	-[b]	40mg/kg = 80mg/kg
12	13.20	0	-	40mg/kg = 80mg/kg
13	8.20	0	-	40mg/kg = 80mg/kg
14	29.00	0	-	40mg/kg > 80mg/kg***
15	107.60	0	-	40mg/kg > 80mg/kg**
16	91.00	0	-	40mg/kg > 80mg/kg**
17	70.00	0	-	40mg/kg > 80mg/kg***
18	47.60	0	-	40mg/kg > 80mg/kg*
19	29.60	0	-	40mg/kg = 80mg/kg
20	17.00	0	-	40mg/kg = 80mg/kg
21	40.60	0	-	40mg/kg > 80mg/kg*
22	96.00	0	-	40mg/kg > 80mg/kg**
23	54.60	0	-	40mg/kg > 80mg/kg*
24	34.00	0	-	40mg/kg = 80mg/kg
25	21.40	0	-	40mg/kg = 80mg/kg
26	14.20	0	-	40mg/kg = 80mg/kg
27	9.20	0	-	40mg/kg = 80mg/kg
28	27.20	0	-	40mg/kg > 80mg/kg**

[a] * $p<.05$, ** $p<.01$, *** $p<.001$ [b] Data unavailable as animals were sacrificed

As seen in Table 4 and Figures 5 and 6 the tumours on the C57 BL/6J mice in the 80 mg/kg group were not different from the solvent group on Day 1, they were completely regressed from Day 2 to Day 10 of treatment. The animals in the control group were sacrificed on Day 11 (tumour volumes > 1500 mm^3) hence comparison of tumour volumes between this 80 mg/kg [Cu(HBnz$_2$)Cl] treated group with that of the control group stopped on this day.

In the case of the 40 mg/kg group, although there appears to be a downward trend in tumour sizes between Day 1 to Day 3 (see Figure 5), they were not statistically different from the tumour sizes of the solvent group. However, this reduction in size was evident from Day 4 where the smaller tumour sizes in the 40 mg/kg group were statistically significant. Again, as the animals in the control group were sacrificed on Day 11 (tumour volumes > 1500 mm^3) hence comparison of tumour volumes between this 40 mg/kg $[Cu(HBnz_2)Cl]$ treated group with that of the control group stopped on this day.

Next, we compared the tumour volumes between the 40 mg/kg group and the 80 mg/kg group. The tumour volumes were reduced to zero for all five mice in the 80 mg/kg group, from Day 5. The volumes of the tumours were statistically larger in the 40 mg/kg group compared to the 80 mg/kg group ($p<0.05$) for Days 1 to 4, 7 to 10, 14 to 17, 21 to 23 and 28 but not Days 5 to 6, 11 to 13, 18 to 20, and 24 to 27. This might, firstly, be due to the relatively higher variances between tumour sizes in the 40 mg/kg group and secondly, the effects of the treatment cycle where reduction in tumour sizes typically took place the day after introducing $[Cu(HBnz_2)Cl]$ into the tumours and a "re-growth" of the tumour volumes in this 40 mg/kg group about two days following the termination of treatment.

Figure 7 shows the % $\Delta T/\Delta C$ of tumour growth in the C57 BL/6J mice undergoing treatment based on 40 mg/kg or 80 mg/kg of $[Cu(HBnz_2)Cl]$. According to the Division of Cancer Treatment, NCI, NIH[22], a % $\Delta T/\Delta C$ value of ≤ 42 % is an indication of a significant antitumour activity. From Day 1 to Day 10, the % $\Delta T/\Delta C$ values ranged from about −8 % to –360 % for the 40 mg/kg group and −6 % to −450 % for the 80 mg/kg group. These results confirmed the significant antitumour activity of $[Cu(HBnz_2)Cl]$ on the tumours formed by murine 3LL cells, *in vivo*.

In Figure 5, we can also see that the solvent treated group showed uncontrolled tumour growth. Their volumes increased significantly throughout the whole experiment. Thus we can deduce that without the effect of $[Cu(HBnz_2)Cl]$, the tumours would continue to grow unchecked and this would eventually lead to the death of the animals.

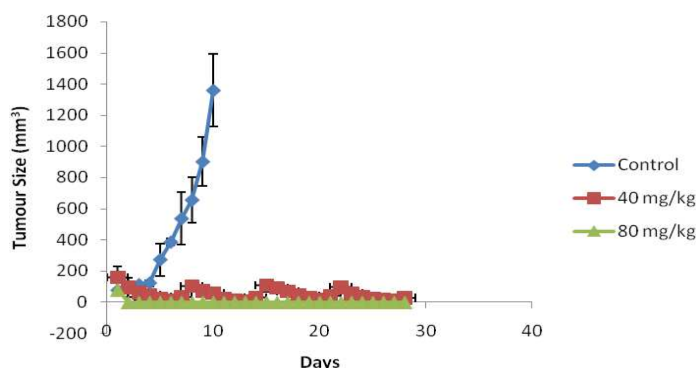

Figure 5
Tumour volumes of the three groups of C57 BL/6J mice tested: control group (1 % DMSO in Saline), 40 mg/kg group and the 80 mg/kg group. Vertical bars denote the respective standard deviations.

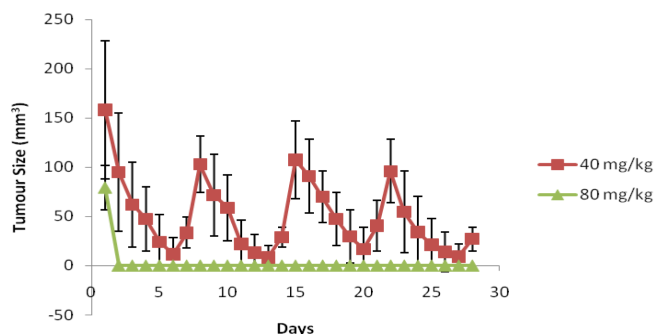

Figure 6

An expanded view of the tumour volumes of the two groups of C57 BL/6J mice tested (without the control group): 40 mg/kg group and the 80 mg/kg group. Vertical bars denote the respective standard deviations.

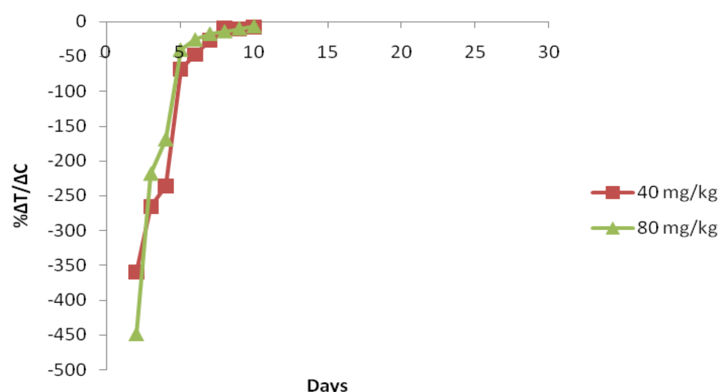

Figure 7

% ∆T/∆C of tumour growth in the C57 BL/6J mice undergoing treatment based on 40 mg/kg or 80 mg/kg of [Cu(HBnz$_2$)Cl].

3. SCID mice

Experiment 1

Since our results for treating mice that carried tumours of murine origin with 40 mg/kg and 80 mg/kg of [Cu(HBnz$_2$)Cl] were both exciting and promising, we decided to extend our study into looking at the effects of [Cu(HBnz$_2$)Cl] on mice that carried tumours of human origin. Hence a similar experiment was set up using thirteen mice that were each inoculated with human MOLT-4 cells instead. The results obtained from this experiment are summarized in Table 5 and illustrated in Figures 8, 9 and 10.

Table 5
Comparison of means across groups in experiment with SCID mice.

Day	40 mg/kg (n=5)	80 mg/kg (n=5)	Solvent (n=3)	Difference between groups[a] (based on t-test comparisons)
1	78.80	71.20	87.00	40mg/kg = Solvent 80mg/kg = Solvent 40mg/kg = 80mg/kg
2	64.20	28.60	127.00	40mg/kg < Solvent* 80mg/kg < Solvent* 40mg/kg > 80mg/kg**
3	41.20	12.20	173.00	40mg/kg < Solvent*** 80mg/kg < Solvent** 40mg/kg > 80mg/kg*
4	36.20	2.40	225.33	40mg/kg < Solvent** 80mg/kg < Solvent** 40mg/kg > 80mg/kg***
5	26.60	0	261.67	40mg/kg < Solvent** 80mg/kg < Solvent** 40mg/kg > 80mg/kg**
6	16.40	0	319.67	40mg/kg < Solvent** 80mg/kg < Solvent** 40mg/kg > 80mg/kg***
7	67.20	0	386.00	40mg/kg < Solvent** 80mg/kg < Solvent** 40mg/kg > 80mg/kg***
8	86.60	2.80	420.00	40mg/kg < Solvent** 80mg/kg < Solvent** 40mg/kg > 80mg/kg***
9	71.20	0	579.00	40mg/kg < Solvent** 80mg/kg < Solvent** 40mg/kg > 80mg/kg***
10	50.20	0	738.00	40mg/kg < Solvent** 80mg/kg < Solvent** 40mg/kg > 80mg/kg**
11	38.80	0	853.00	40mg/kg < Solvent** 80mg/kg < Solvent** 40mg/kg > 80mg/kg*
12	28.20	0	1057.67	40mg/kg < Solvent** 80mg/kg < Solvent** 40mg/kg > 80mg/kg*
13	20.60	0	1255.00	40mg/kg < Solvent** 80mg/kg < Solvent** 40mg/kg > 80mg/kg**
14	42.60	0	1375.67	40mg/kg < Solvent**

				80mg/kg < Solvent** 40mg/kg > 80mg/kg***
15	70.40	0	1689.67	40mg/kg < Solvent** 80mg/kg < Solvent** 40mg/kg > 80mg/kg**
16	62.80	0	2002.67	40mg/kg < Solvent** 80mg/kg < Solvent** 40mg/kg > 80mg/kg**
17	39.80	0	-[b]	40mg/kg > 80mg/kg**
18	34.80	0	-	40mg/kg > 80mg/kg**
19	24.60	0	-	40mg/kg > 80mg/kg**
20	18.00	0	-	40mg/kg > 80mg/kg***
21	62.20	0	-	40mg/kg > 80mg/kg***
22	79.80	0	-	40mg/kg > 80mg/kg***
23	60.20	0	-	40mg/kg > 80mg/kg**
24	47.00	0	-	40mg/kg > 80mg/kg**
25	37.60	0	-	40mg/kg > 80mg/kg**
26	23.80	0	-	40mg/kg > 80mg/kg**
27	15.80	0	-	40mg/kg > 80mg/kg**
28	54.80	0	-	40mg/kg > 80mg/kg***

[a] * *p*<.05, ** *p*<.01, *** *p*<.001 [b] Data unavailable as animals were sacrificed

Within the 80 mg/kg condition, there was a statistically significant reduction in tumour volumes as of Day 2 (see Table 5), some mice had tumours that were completely regressed starting from Day 3 (Figures 8 & 9), and all the five mice had tumours that were completely regressed by Day 5.

In the 40 mg/kg group, there was a notable reduction in the volumes of the tumours especially after every introduction of [Cu(HBnz$_2$)Cl] into the tumours and a statistically significant reduction in tumour volumes as of Day 2 (see Table 5).

The control group (1 % DMSO in Saline), on the other hand, reflected an increasing trend in the volumes of the tumours measured representing unchecked growth of the tumour cells. The animals in this group were sacrificed on Day 17 (tumour volumes > 1500 mm^3).

There were no statistical difference in the tumour volumes between the two [Cu(HBnz$_2$)Cl] treated groups (i.e., the 40 mg/kg and 80 mg/kg groups) on Day 1. However, the impact of the 80 mg/kg dosage was evident from Day 2 and 28 where the tumours obtained after treatment with 40 mg/kg were statistically larger when compared to the tumours treated with 80 mg/kg.

Figure 10 shows the % $\Delta T/\Delta C$ of tumour growth in the SCID mice undergoing treatment based on 40 mg/kg or 80 mg/kg of [Cu(HBnz$_2$)Cl]. From Day 1 to Day 16, the % $\Delta T/\Delta C$ values ranged from about +2 % to – 44 % for the 40 mg/kg group and −4 % to −110 % for the 80 mg/kg group. These results confirmed the significant antitumour activity of [Cu(HBnz$_2$)Cl] on the tumours formed by human MOLT-4 cells, *in vivo*.

It is also noteworthy that we had also included another control group consisting of two mice that were injected only with the X-irradiated HT-1080 cells (data not shown). No tumour growths were observed on both mice at all. Hence, we can confirm that the tumours growing on the SCID mice consisted of only MOLT-4 cells and not a mixture of MOLT-4 and HT-1080.

Experiment 2

In this study, Experiment 1 above was repeated using only the 80 mg/kg concentration together with the solvent control. Treatment with [Cu($HBnz_2$)Cl] resulted in a statistically significant regression of the tumours on some mice starting from Day 2 (see Table 6 and Figure 11). On Day 4, all the five mice had tumours that were completely regressed. In contrast, the group that was treated with only the solvent again showed an expected increasing trend in the volumes of the tumours as the experiment proceeds thus displaying uncontrolled growth of the tumour cells.

Figure 12 shows the % $\Delta T/\Delta C$ of tumour growth in these SCID mice in response to treatment with [Cu($HBnz_2$)Cl]. From Day 1 to Day 14, the % $\Delta T/\Delta C$ values ranged from about −5 % to –161 % Hence, these results again confirmed the significant antitumour activity of [Cu($HBnz_2$)Cl] on the tumours formed by human MOLT-4 cells, *in vivo*.

Table 6

Comparison of means across groups in experiment with SCID mice.

Day	80 mg/kg (n=5)	Solvent (n=5)	Difference between groups[a] (based on t-test comparisons)
1	67.40	84.50	80mg/kg = Solvent
2	22.60	146.00	80mg/kg < Solvent**
3	8.00	121.50	80mg/kg < Solvent*
4	0	169.00	80mg/kg < Solvent**
5	0	215.50	80mg/kg < Solvent**
6	0	271.00	80mg/kg < Solvent***
7	0	324.00	80mg/kg < Solvent***
8	0	406.50	80mg/kg < Solvent***
9	0	574.00	80mg/kg < Solvent***
10	0	840.00	80mg/kg < Solvent***
11	0	1093.00	80mg/kg < Solvent***
12	0	1184.50	80mg/kg < Solvent***
13	0	1335.50	80mg/kg < Solvent***
14	0	1549.00	80mg/kg < Solvent***

[a] *** p<.05, ** p<.01, *** p<.001***

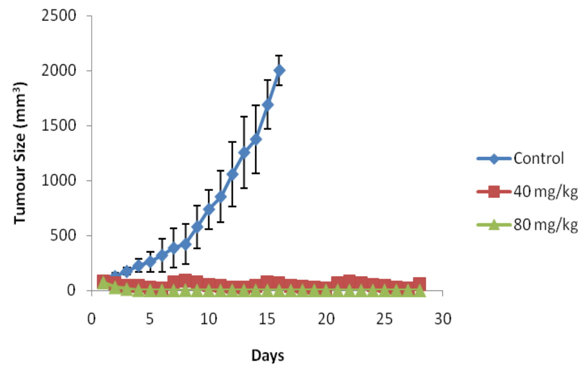

Figure 8
Tumour volumes of the three groups of SCID mice tested: control group (1 % DMSO in Saline), 40 mg/kg group and the 80 mg/kg group. Vertical bars denote the respective standard deviations.

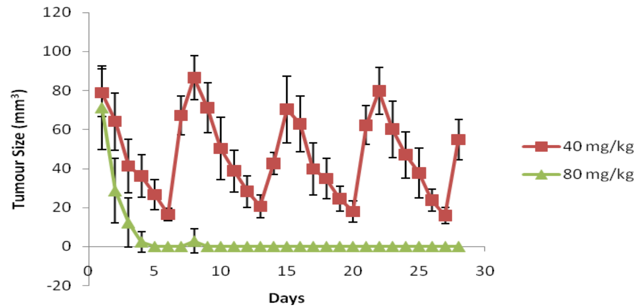

Figure 9
An expanded view of the tumour volumes of the two groups of SCID mice tested (without the control group): 40 mg/kg group and the 80 mg/kg group. Vertical bars denote the respective standard deviations.

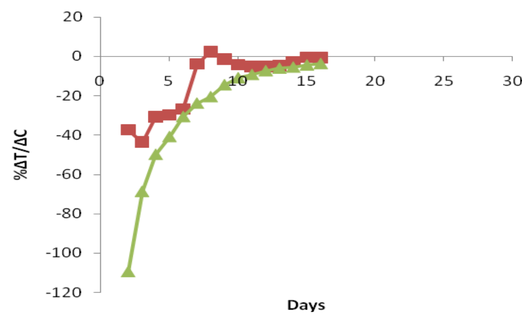

Figure 10
% ΔT/ΔC of tumour growth in the SCID mice undergoing treatment based on 40 mg/kg or 80 mg/kg of [Cu(HBnz$_2$)Cl].

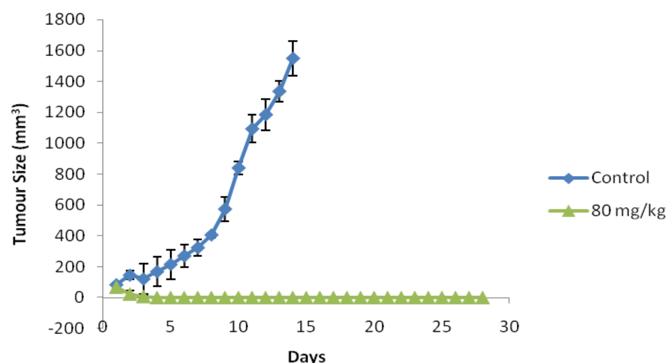

Figure 11

Tumour volumes of the two groups of SCID mice tested: control group (1 % DMSO in Saline) and the 80 mg/kg group. Vertical bars denote the respective standard deviations.

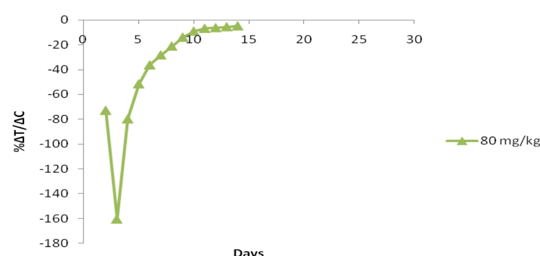

Figure 12

% ΔT/ΔC of tumour growth in the SCID mice undergoing treatment based on 80 mg/kg of [Cu(HBnz$_2$)Cl].

4. [Cu(HBnz$_2$)Cl] induces apoptosis on the cells in the tumours

Based on the studies above, it is obvious that [Cu(HBnz$_2$)Cl] indeed possesses antitumour activity. From our earlier *in vitro* studies[20], we confirmed that the mode of cell death induced by [Cu(HBnz$_2$)Cl] on MOLT-4 cells was attributed to apoptosis. Hence, by triggering apoptosis in the tumour cells of the mice, *in vivo*, this complex might have great potential to be developed into a chemotherapeutic drug.

CONCLUSION

The antitumour property of [Cu(HBnz$_2$)Cl] was investigated. The LD_{50} of this copper complex was determined to be 75 mg/kg. When [Cu(HBnz$_2$)Cl] was introduced to tumours of mouse origin (3LL on normal mice) and human origin (MOLT-4 on SCID mice), it was found that it indeed exhibited antitumour property. There was an obvious reduction of the tumour volumes in the 40 mg/kg treated group of mice, an effect that was not seen in the control group of mice. As for the 80 mg/kg treated group of mice, it was possible to reduce tumour volumes to zero hence demonstrating the effectiveness of [Cu(HBnz$_2$)Cl] as an antitumour agent. From here on, conditions should be further optimized in order to bring about a more significant therapeutic effect of this compound based on this antitumour property, e.g., alternate routes of administration of the

compounds, optimizing the best dosage and frequency of treatment, as well as improving the specificity of the compounds toward malignant cells.

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