# Preclinical toxicological examination of a putative prostate cancer-specific 4-methyl-1-nitroacridine derivative in rodents

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**Nitroacridines are potent DNA-binding and cytotoxic agents in cancer cells, but could not be developed clinically due to high systemic toxicities. We are developing a 1-nitroacridine derivative, 9-(2′-hydroxyethylamino) -4-methyl-1-nitroacridine (C-1748), as an effective chemotherapeutic agent for prostate cancer. C-1748 demonstrates high antitumor efficacy against human prostate cancer xenografts with markedly low mutagenicity and toxicity in dogs compared with its parent 9-(2′-hydroxyethylamino)-1-nitroacridine (C-857). A surprising feature of C-1748 is the 40-fold difference in 50% inhibitory concentration between DU145 prostate cancer and HL-60 leukemia cells. In this study, we report the preclinical toxicity study of a single acute dose of C-1748 in Copenhagen rats and BALB/c mice, intraperitoneally and intravenously for 24 h and 7 days. The effect of C-1748 on hematology, cardiac and liver enzymes, and renal electrolytes was assessed by blood and serum analysis. The $LD_{50}$ (lethal dose, 50%) for C-1748 was 9 and 13.42 mg/kg compared with 2.2 and 3 mg/kg for C-857 intraperitoneally and intravenously, respectively, in mice. In Copenhagen rats, $LD_{50}$ was 15 and 14.4 mg/kg intraperitoneally and intravenously, respectively, compared to 4 and 1.3 mg/kg for C-857. No changes in blood cell counts were observed, which were in the normal range for rodents. No changes were observed in clinical chemistries of enzymes such as aspartate aminotransferase, alkaline phosphatase and creatine phosphokinase, which were within the normal range of values. No genome alterations were seen in prostate cancer cell lines by comparative genomic hybridization together with a lack of systemic toxicity, making it a unique cancer cell-type-specific drug that needs further clinical evaluation for toxicity and synergy in combination chemotherapy regimens.**
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## Introduction

Acridines have been used as medicinal agents since the early part of the 20th century and have been investigated as anticancer agents, as they are potent DNA intercalating and binding agents [1]. Derivatives of acridines having substituted nitro groups in carbon position '1' ($C_1$) have been shown to have antitumor activity against mammalian tumors, excluding leukemia, [2–11]. Clinical trials in humans with ledakrin (nitracrine) demonstrated antitumor activity against solid tumors [12]. It, however, produced side-effects such as intense nausea and vomiting, and further clinical development of these potent cytotoxic drugs was not undertaken.

Subsequently, analogs of ledakrin were synthesized that would retain the anticancer activity with lower toxicity. One such derivative of 1-nitroacridine with an amino alkyl substitution in the $C_9$ position was synthesized and designated as 9-(2′-hydroxyethylamino)-1-nitroacridine (C-857). This derivative too has potent anticancer activity; however, the systemic toxicity was retained. This led to synthesis of further derivatives, one of which was the introduction of a methyl group substitution at position $C_4$ leading to the generation of 4-methyl-1-nitroacridines. One such derivative is designated 9-(2′-hydroxyathylemino)-4-methyl-1-nitroacridine (C-1748) [13]. The nitro group at carbon '1' is critical for the biological activity of 1-nitroacridines. The nitro group is, however, conducive to reduction and leads to generation of highly reactive metabolites that contribute to toxicity of 1-nitroacridines [6]. The methyl group at position $C_4$ is electron donating that lowers the ability of the derivative to undergo reduction and, consequently, lowers the toxicity compared with the parent compounds [13,14]. Our studies in prostate cancer (CaP) xenografts indicated that C-1748 has a high degree of efficacy for CaP [14,15]. The therapeutic index, defined by the ratio of systemic toxicity [lethal dose, 50% ($LD_{50}$)] to anticancer activity,

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is 4–11-fold greater for C-1748 than for C-857 [14–16]. Our earlier studies also showed the lack of toxicity of C-1748 in male Beagle dogs [17], which allowed us to identify the maximal tolerable dose. Another study documented the lowered mutagenic potential of C-1748 compared with other 1-nitroacridine derivatives such as C-857 [18].

We present here the preclinical toxicology profile of C-1748 administered by a single intravenous (i.v.) and intraperitoneal (i.p.) dose in Copenhagen rats and Balb/c mice. The possible clinical and/or morphological adverse events associated with drug administration were investigated at 24 h and at 7 days. The major findings were that C-1748 exhibited very minimal toxicity with no acute renal, cardiac or liver toxicity associated with the administration of C-1748.

## Materials and methods

Balb/c mice (6–8 weeks old) were purchased from the National Cancer Institute and Copenhagen rats (4–6 weeks old) were purchased from Harlan (Indianapolis, Indiana, USA). They were allowed to acclimatize for 1 week, and were fed standard rodent chow and water *ad libitum*. The preclinical toxicity studies were carried out in the Department of Comparative Medicine, at New York Medical College (Valhalla, New York, USA), and was conducted with the prior Institutional Animal Care and Use Committee approval following standard practices.

### Drugs

C-1748 and C-857 were synthesized in the laboratory of Dr J. Konopa at (Gdansk University of Technology, Gdansk, Poland) following the method as described previously [13].

### Determining $LD_{50}$ for C-857 and C-1748

C-857 was administered at doses of 1, 3 and 5 mg/kg, and C-1748 at doses of 1, 3, 5 and 10 mg/kg i.p. in mice. In rats, C-857 was administered i.p. at 1, 3, 5, 9, 12 and 15 mg/kg body weight, and C-1748 at 1, 3, 5 and 10 mg/kg. The survival of the animals was recorded and Kaplan–Meier survival plots were generated.

### Administration of C-1748 for toxicity studies

The test formulations were aseptically prepared in double-distilled water [14,15] and the dose volume was 100 µl/animal. The number of animals used was between four and six per group, and the experiments were repeated at least twice. The Copenhagen rats were injected i.v. and i.p. at 3, 5, 12 and 15 mg/kg body weight of C-1748. Balb/c mice were administered i.v. (1, 3 and 5 mg/kg) and i.p. (1, 3 and 5, 12 mg/kg) with C-1748. Blood and sera were collected at 24 h and 7 days after dosing. All animals were monitored twice daily for adverse effects and monitored for weight loss. Hematological parameters and serum chemistries investigated are given in Table 1 [17,19].

**Table 1 Hematological and serum chemistries investigated**

| Hematodynamics | Liver function | Kidney function |
|---|---|---|
| WBC | AST (SGOT) | BUN |
| RBC | ALT (SGPT) | |
| Hemoglobin | ALP | |
| Hematocrit | protein (total) | |
| MCV | | |
| MCH | | |
| MCHC | | |
| Platelet | | |
| Lymphocytes | | |
| Segmented neutrophils | | |

WBC, white blood cells; AST, aspartate aminotransferase; BUN, blood urea nitrogen; RBC, red blood cells; ALT, alanine aminotransferase; MCV, mean corpuscular volume; MCH, mean corpuscular HGB; MCHC, mean corpuscular HGB concentration; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.

### Hematology and clinical chemistry

Blood was collected from mice and rats for serum and complete blood counts with differential and platelet counts and coagulation parameters. Samples were collected predose, and at day 1 and day 7. Serum was analyzed for enzymes and electrolytes including creatinine phosphokinase (CPK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and blood urea nitrogen (BUN). All animals were killed on day 7. The data were statistically analyzed by two-tailed Students *t*-test and $P \leq 0.05$ was considered statistically significant. In addition, the analysis included comparing the test parameters with the normal reference range and historical values provided for rats and mice. Statistically significant values (either above or below the normal range) were considered as drug induced adverse events.

### Comparative genomic hybridization analysis

DU145 and HL-60 cells were either untreated or treated with 10 and 500 nmol/l C-1748, respectively, for 24 h followed by isolation of genomic DNA. Test and reference genomic DNA were labeled with Fluorescein-12-dUTP and Texas Red-5-dUTP, respectively (NEN Life Science Products, Boston, Massachusetts, USA), using standard nick-translation procedures. Comparative genomic hybridization (CGH) was performed as described previously [20,21] with slight modifications. Briefly, equal amounts of the labeled test DNA and reference DNA were mixed and hybridized in the presence of 20 µg of human Cot-1 DNA (Invitrogen, Carlsbad, California, USA) for 48 h to normal human metaphase chromosomes. After hybridization, the slides were washed and the chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (VYSIS, Downers Grove, Illinois, USA). Images were captured with a cold charge-coupled device camera (Photometrics, Tucson, Arizona, USA) connected to a Zeiss Axioskop fluorescent microscope (Carl Zeiss

MicroImaging, Thornwood, New York, USA). These images were analyzed with Quips GGH software (VYSIS). In regions where there are no amplifications or deletions in the target cell genome, binding of both samples was equal and the equal emission of light from both fluorochromes resulted in a perceived yellow fluorescence. Gain and loss of DNA was determined by calculating green : red average ratio profiles from at least six metaphases. If the average ratio is $> 1.2$ or $< 0.8$, the region of alteration was considered as a gain or a loss, respectively. High-level amplification was defined if the ratio exceeded 1.5.

## Results

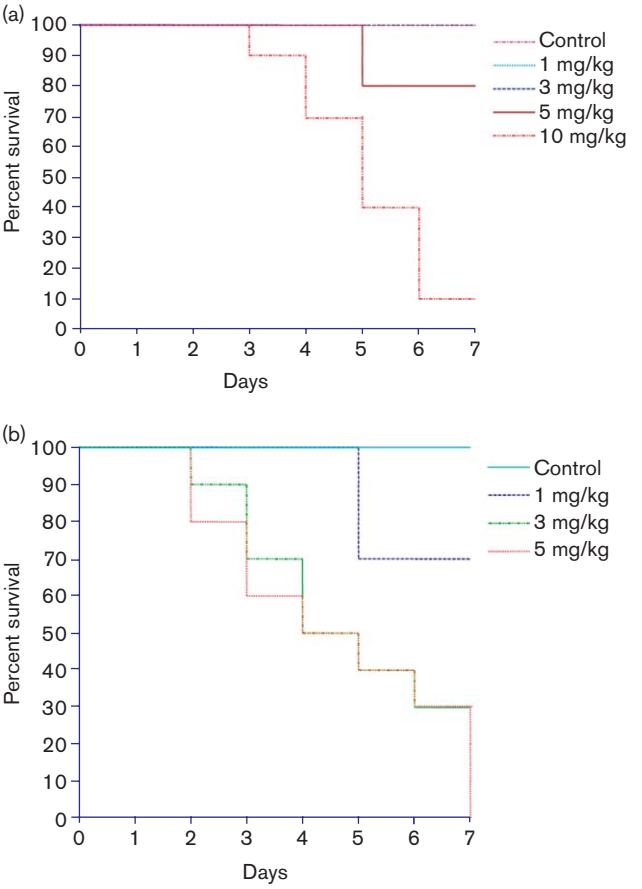
### Determining $LD_{50}$ of C-857 and C-1748

None of the Balb/c mice that received 1 and 3 mg/kg of C-1748 i.p. died during the course of the study. In all these experiments, Kaplan–Meier analysis was performed to assess survival. Two mice died on day 5 after dosing when administered 5 mg/kg, and in the group that received highest dose of 10 mg/kg, one mouse died on day 3, two on day 4, three on day 7 and 90% animals died on day 6 (Fig. 1a). In sharp contrast, the mice receiving 1 mg/kg C-857 showed 30% mortality by day 5; in 3 and 5 mg/kg groups, survival started to decline by 10 and 20% by day 2 (Fig. 1b). A 50% decrease in survival was observed in these two groups by day 4 and 100% on day 7. The results clearly indicate that C-857 is more toxic than C-1748. Any surviving mice were killed at the end of the experiment by $CO_2$ asphyxiation. They also had no changes in their body weights during the study.

In the case of Copenhagen rats that received 1, 3 and 5 mg/kg of C-1748 i.p., none of the rats died during the course of the study (Fig. 2a). Only two rats died on day 6 after dosing when administered 10 mg/kg. In contrast, rats that received C-857 showed more deaths with 3, 5, 9, 12 and 15 mg/kg (Fig. 2b). Ninety percent of deaths were observed in rats that received 15 mg/kg C-857 on day 4, whereas 80% of rats died on day 6 with 9 mg/kg; 70% on day 6 with 5 and 12 mg/kg C-857. The results again clearly demonstrate that C-857 is a more toxic agent than C-1748.

The $LD_{50}$ values for C-1748 in Balb/c mice were determined to be 13.42 and 9.0 mg/kg i.v. and i.p. compared with 3.0 and 2.2 mg/kg, respectively, for C-857. Similarly, the $LD_{50}$ values for C-1748 in Copenhagen rats are 14.4 and 15.0 mg/kg, and for C-857 are 1.3 and 4.0 mg/kg i.v. and i.p., respectively. These results indicate a higher toxicity associated with C-857 than with C-1748. If one considers the therapeutic dose that produced antitumor effects (0.8 mg/kg) and compare the therapeutic index (ratio of $LD_{50}$ to the therapeutic dose), C-1748 is 4–10-fold more preferable than C-857. The index is 11.3 and 16.8 i.p. and i.v., respectively, for C-1748 in BALB/c mice compared with 2.8 and 3.8 with C-857. In rats, the therapeutic index was 18.8 (i.p.) and 18 (i.v.) compared with 5 (i.p.) and 1.6 (i.v.) with C-857.

**Fig. 1**




Kaplan–Meier survival curves of Balb/c mice administered varying doses of C-1748 (a) and C-857 (b) intraperitoneally for 7 days and determination of $LD_{50}$. Each group comprised 10 mice.

### Clinical pathology

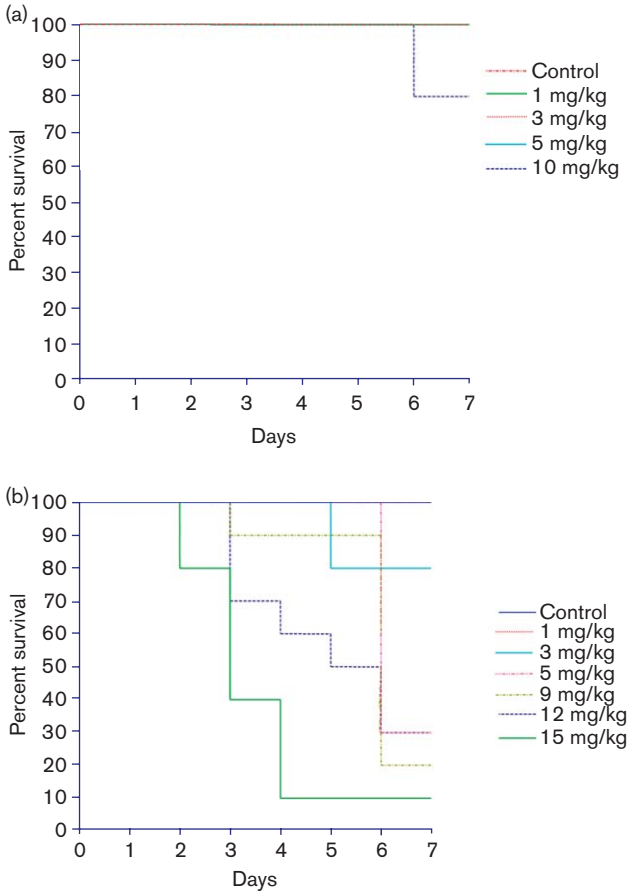
The hematological parameters were studied for Balb/c mice and Copenhagen rats that were administered with varying doses of C-1748, both i.v. and i.p. for 24 h (short term) and 7 days (long term). Significant changes over the normal values induced by C-1748 were observed at a dose of 12 mg/kg, and the data are shown in Tables 2 and 3.

#### *Effect of intravenous administration of C-1748*

*Short term*

(i) Hematology. No significant changes in hematological parameters were seen with i.v. administration of C-1748 in Balb/c mice at all three doses 24 h after dosing. Statistically significant increases in the hematocrit, lymphocytes and white blood cells (WBCs) were observed in C-1748-infused mice over the control group, but these values were in the normal reference range.

**Fig. 2**




Kaplan–Meier survival curves of Copenhagen rats administered varying doses of C-1748 (a) and C-857 (b) intraperitoneally for 7 days and determination of $LD_{50}$. Each group comprised 10 mice.

In Copenhagen rats, a slight decrease in hematocrit, mean corpuscular volume (MCV) was seen with 3 and 5 mg/kg C-1748. A decrease in WBCs, red blood cells (RBCs), hematocrit, MCV and lymphocytes, whereas small increases in mean corpuscular hemoglobin (HGB) (MCH), mean corpuscular HGB concentration (MCHC), platelet counts and neutrophils were observed at a dose of 12 mg/kg. All these changes were, however, in the normal range of values for rats.

(ii) Serum chemistries. In mice, ALP and ALT were lowered, which were, however, in the normal reference range. In rats, CPK was highly elevated almost 4-fold (2044.2 ± 391 IU/l) over control (584 ± 29.7 IU/l) with 3 mg/kg and 3.1-fold with 12 mg/kg (Table 2). A 1.6-fold increase (264.9 ± 41.3 IU/l) over control (169.8 ± 13.3 IU/l) in ALP with 3 mg/kg C-1748 was seen, which is higher than the reference values (Table 2). No changes were seen with ALT, AST, BUN and total protein. At a dose of 5 mg/kg, a 1.5-fold increase (73.3 ± 13.8 IU/l) in ALT levels was observed over control (50.3 ± 8.2) and above the normal reference values.

### *Long term*

(i) Hematology. In mice, a decrease in platelets (thrombocytopenia) was seen at a dose of 1 and 3 mg/kg, decreases in lymphocytes at 3 and 5 mg/kg, and decreases in WBCs (leucopenia) at 3 mg/kg doses (data not shown, but similar to 12 mg/kg dose). These decreases were again compared with the controls, but within normal range for the species.

In Copenhagen rats, increases in WBCs, MCV, MCH and lymphocytes, and a decrease in RBC counts were seen

**Table 2 Effect of i.v. administration of C-1748 to Copenhagen rats 24 h and 7 days after dosing**

| Parameters | C-1748 administered i.v. for 24 h | | | C-1748 administered i.v. for 7 days | | | Reference range |
|---|---|---|---|---|---|---|---|
| | Control | C-1748 (12 mg/kg) | *P* value | Control | C-1748 (12 mg/kg) | *P* value | |
| WBC count | 14.2 ± 2.04 | 10.1 ± 2.7 | 0.012[a] | 10.7 ± 2.28 | 14.3 ± 1.8 | 0.018[a] | 9.4–14.9 |
| RBC count | 7.02 ± 0.16 | 6.92 ± 0.29 | 0.484 | 7.13 ± 0.22 | 6.67 ± 0.47 | 0.079 | 6.2–9.0 |
| Hemoglobin | 14.17 ± 0.33 | 14.61 ± 0.39 | 0.05[a] | 14.04 ± 0.55 | 14.1 ± 1.08 | 0.91 | 13.4–16.4 |
| Hematocrit | 41.92 ± 0.84 | 38.01 ± 1.63 | 0.0003[a] | 42.3 ± 1.55 | 41.05 ± 2.67 | 0.398 | 40.0–49.0 |
| MCV | 59.7 ± 0.38 | 54.9 ± 0.37 | < 0.0001[a] | 59.28 ± 0.48 | 54.98 ± 0.56 | | 52.0–66.0 |
| MCH | 20.17 ± 0.187 | 21.1 ± 0.51 | 0.0015[a] | 19.68 ± 0.28 | 21.1 ± 0.55 | 0.0005[a] | 17.7–19.1 |
| MCHC | 33.8 ± 0.32 | 38.5 ± 1.04 | < 0.0001[a] | 33.2 ± 0.19 | 34.4 ± 0.96 | 0.0304 | 32.0–33.5 |
| Platelet | 1086.2 ± 54.4 | 1194.9 ± 55.09 | 0.0044[a] | 1214.2 ± 119.5 | 1334.8 ± 247.1 | 0.35 | 780–1400 |
| Segmented neutrophils | 1.69 ± 0.94 | 2.03 ± 1.04 | 0.559 | 0.74 ± 0.105 | 3.56 ± 2.9 | 0.059 | 0.58–6.30 |
| Lymphocytes | 12.39 ± 1.67 | 8.07 ± 3.3 | 0.015[a] | 9.9 ± 2.21 | 10.7 ± 2.7 | 0.61 | 3.78–14.9 |
| AST (SGOT) | 203.8 ± 12.94 | 174.6 ± 43.8 | 0.15 | 139.2 ± 22.7 | 136.3 ± 40.2 | 0.89 | 68–192 |
| ALT (SGPT) | 50.3 ± 8.2 | 63.7 ± 21 | 0.17 | 44.4 ± 6.8 | 83.3 ± 20.8 | 0.0032[a] | 20–56 |
| ALP | 169.8 ± 13.3 | 174.1 ± 23.5 | 0.7 | 174.8 ± 26.3 | 185.2 ± 40.3 | 0.64 | 17–245 |
| Protein (total) | 5.42 ± 0.38 | 5.5 ± 0.3 | 0.67 | 5.84 ± 0.59 | 5.58 ± 0.34 | 0.389 | 4.8–9.2 |
| Creatine phosphokinase | 584 ± 29.7 | 1826 ± 643.2 | 0.0006 | 1603 ± 407.9 | 1033.2 ± 451.4 | 0.058 | 0–1600 |
| BUN | 21 ± 2.4 | 23.4 ± 2.8 | 0.12 | 20.2 ± 3.5 | 5.58 ± 0.34 | 0.39 | 12–23 |

i.v., intravenous; ALP, alkaline phosphatase; WBC, white blood cells; AST, aspartate aminotransferase; BUN, blood urea nitrogen; RBC, red blood cells; ALT, alanine aminotransferase; MCV, mean corpuscular volume; MCH, mean corpuscular HGB; MCHC, mean corpuscular HGB concentration; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.
[a]Statistically significant.

**Table 3 Effect of i.p. administration of C-1748 to Balb/c mice 24 h and 7 days after dosing**

| Parameters | C-1748 administered i.p. for 24 h | | | C-1748 administered i.p. for 7 days | | | Reference range |
|---|---|---|---|---|---|---|---|
| | Control | C-1748 (12 mg/kg) | *P* value | Control | C-1748 (12 mg/kg) | *P*value | |
| WBC_count | 7.62 ± 1.02 | 4.12 ± 0.97 | <0.0001 | 3.98 ± 0.79 | 8.75 ± 0.85 | 0.0001[a] | 2.6–10.69 |
| RBC_count | 8.126 ± 0.50 | 8.57 ± 0.85 | 0.305 | 6.92 ± 0.18 | 7.59 ± 0.19 | 0.0001[a] | 6.4–9.4 |
| Hemoglobin | 15.24 ± 1.14 | 15.24 ± 0.602 | 0.99 | 12.98 ± 0.33 | 15 ± 0.52 | 0.0002[a] | 11.5–16.1 |
| Hematocrit | 41.06 ± 2.44 | 44.5 ± 4.1 | 0.11 | 35.3 ± 0.57 | 38.4 ± 1.21 | 0.0014[a] | 36.1–49.5 |
| MCV | 50.52 ± 1.03 | 51.98 ± 0.95 | 0.021[a] | 51 ± 1.07 | 50.58 ± 0.75 | 0.53 | 45.4–60.3 |
| MCH | 18.74 ± 0.37 | 17.87 ± 0.97 | 0.08 | 18.8 ± 0.81 | 19.75 ± 0.44 | 0.07 | 14.1–19.3 |
| MCHC | 37.1 ± 0.92 | 34.38 ± 1.83 | 0.0098[a] | 36.76 ± 1.0 | 39.1 ± 0.86 | 0.007[a] | 25.4–34.1 |
| Platelet | 1314.6 ± 204.4 | 1025.2 ± 77.8 | 0.0022[a] | 1548.2 ± 248.3 | 1205.25 ± 144.4 | 0.045[a] | 592–2972 |
| Segmented neutrophils | 1.97 ± 0.86 | 1.42 ± 1.15 | 0.36 | 0.488 ± 0.35 | 1.625 ± 1.51 | 0.14 | 1.43–9.94 |
| Lymphocytes | 5.65 ± 0.99 | 2.7 ± 1.08 | 0.0003[a] | 3.47 ± 0.71 | 7.1 ± 1.9 | 0.0051[a] | 1.43–9.94 |
| AST (SGOT) | 103 ± 18.85 | 138.56 ± 53.3 | 0.187 | 76.6 ± 10.2 | 53.3 ± 12.3 | 0.017[a] | 0–1600 |
| ALT (SGPT) | 60.6 ± 26.5 | 109.3 ± 64.2 | 0.14 | 39.8 ± 16.9 | 57.3 ± 39.9 | 0.4 | 24–140 |
| ALP | 41.4 ± 26.4 | 133.56 ± 36.03 | 0.0003[a] | 167.2 ± 30.3 | 110 ± 34.9 | 0.034[a] | 45–222 |
| Protein (total) | 5.18 ± 0.46 | 4.59 ± 0.36 | 0.02[a] | 5.24 ± 0.17 | 4.43 ± 0.3 | 0.0012[a] | 4–6.2 |
| Creatine phosphokinase | 333.6 ± 154.5 | 364.4 ± 159.2 | 0.73 | 372 ± 48.04 | 263 ± 215.6 | 0.302 | 0–800 |
| BUN | 21 ± 3.1 | 27.9 ± 4.5 | 0.0104[a] | 39.2 ± 4.7 | 21.3 ± 2.5 | 0.0002 | 9–28 |

i.p. intraperitoneal; ALP, alkaline phosphatase; WBC, white blood cells; AST, aspartate aminotransferase; BUN, blood urea nitrogen; RBC, red blood cells; ALT, alanine aminotransferase; MCV, mean corpuscular volume; MCH, mean corpuscular HGB; MCHC, mean corpuscular HGB concentration; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.
[a]Statistically significant.

within normal values with 3 and 5 mg/kg. At 12 mg/kg, WBCs and MCH were significantly elevated and increases in WBCs, MCV, MCH, platelets (Table 2), and decreases in RBCs and hematocrit were noted with 15 mg/kg.

(ii) Serum chemistries. AST levels were lowered at 1 mg/kg dose in mice compared with control, whereas ALP was lowered at 5 mg/kg. CPK was lowered by around 50% at 1 mg/kg. BUN was increased over control at 1, 3 and 5 mg/kg, and total protein decreased at a dose of 5 mg/kg. As observed earlier, these alterations were within normal historical values.

In rats, no changes in enzymes were seen with 3 and 5 mg/kg, whereas ALT increased by around 1.9-fold with 12 mg/kg dose (Table 2) and 1.6-fold (70.2 ± 23.6) with 15 mg/kg over control (44.4 ± 6.8 IU/l). These increases were above the normal reference range for rats. A 1.4-fold elevation (27 ± 4.9 mg/dl) in BUN was observed with 12 mg/kg over control (20.2 ± 3.5 mg/dl), which was also above the reference range (Table 3).

### *Effect of intraperitoneal administration of C-1748*

#### *Short term*

(i) Hematology. In mice, a decrease in WBCs, mean corpuscular HGB concentration, platelets and lymphocytes was observed with 1 and 3 mg/kg C-1748. Hemoglobin and mean corpuscular HGB were lowered with 1 mg/kg. WBCs, MCV, MCHC, platelets, neutrophils and lymphocytes were lowered with 5 mg/kg C-1748 (data not shown, but similar to 12 mg/kg dose) and at 12 mg/kg except for neutrophils (Table 3). These changes again were in the normal reference range for mice.

In rats, a decrease in RBC counts was observed, whereas increases in MCV, MCH, MCHC and platelet counts were seen with 3 mg/kg, all within the normal range. Similar increases were seen in MCV, MCH, MCHC and platelets with 5 and 12 mg/kg. Lymphocytes were found to be decreased and neutrophils were increased with 12 mg/kg C-1748. An increase in MCV, MCH and platelets was seen with 15 mg/kg in the normal values for Copenhagen rats.

(ii) Serum chemistries. In mice with C-1748 administration, a 2-fold increase in ALT, 3-fold increase in ALP and an increase in BUN, but a decrease in protein were observed. These changes were statistically significant over control, but were within the normal reference range for mice for these parameters (Table 3). CPK and AST were increased but not significantly with 3, 5, 12 and 15 mg/kg C-1748. ALT was increased 2-fold over controls at a dose of 12 mg/kg in rats.

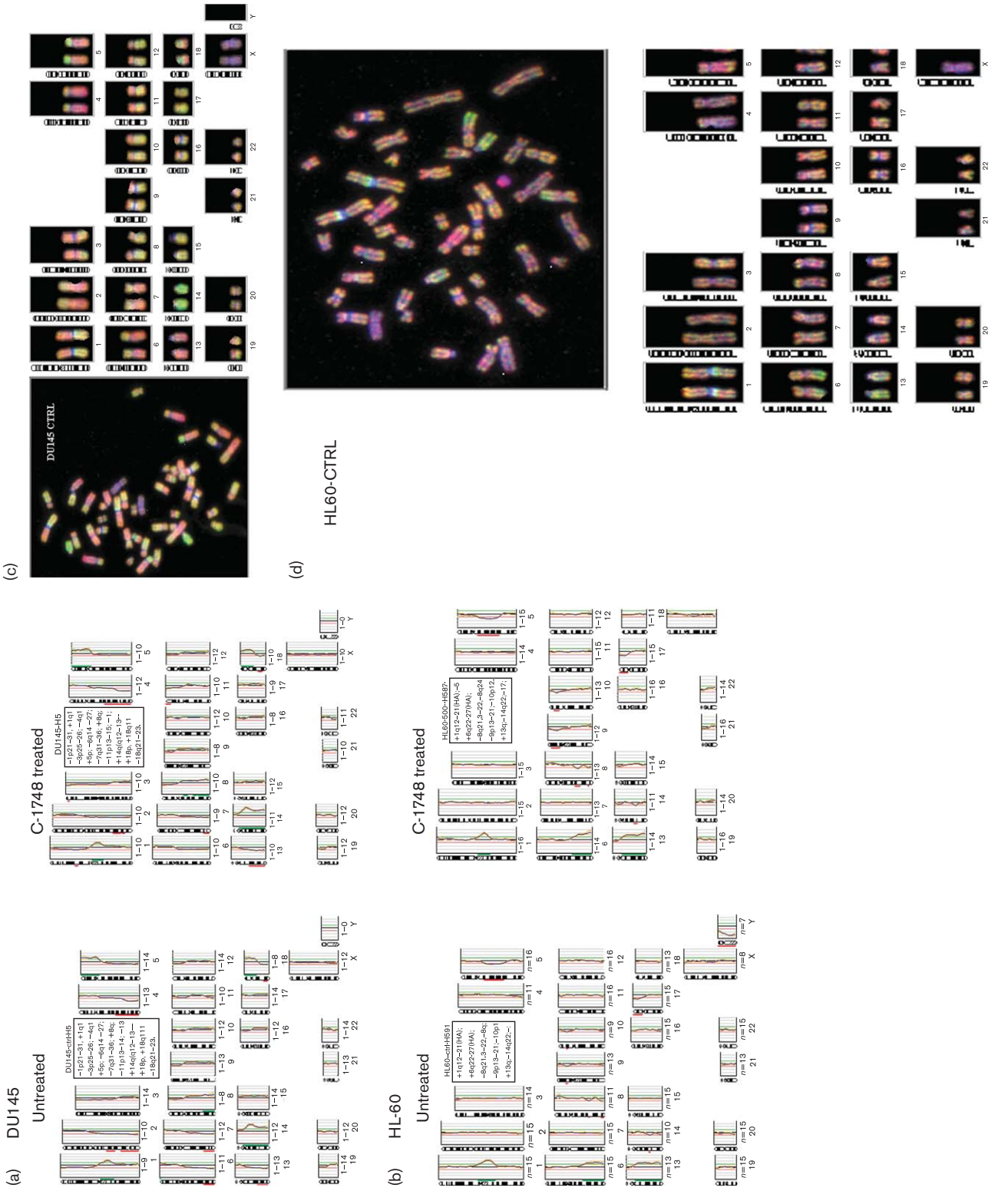
#### *Long term*

(i) Hematology. WBCs, RBCc, hemoglobin, hematocrit, MCH, MCHC, neutrophils and lymphocytes were found to be elevated over control with 1 and 12 mg/kg C-1748 (Table 3). The increases could be compensatory following drug administration. Neutrophils were increased with 3 mg/kg and lymphocytes with 5 mg/kg of C-1748. Significant thrombocytopenia was observed with 12 mg/kg dose, which, however, was in the normal range (Table 3).

In rats, increases in hemoglobin, MCV, MCH and neutrophils were seen with 3 mg/kg. Hematocrit, MCV, MCH MCHC, neutrophils and lymphocytes were increased, whereas RBC counts were lowered with 5 and 15 mg/kg. At a dose of 12 mg/kg, an increase in WBCs was seen above normal values, along with MCV, MCH, MCHC, platelets and neutrophils, which are in the normal range.

(ii) Serum chemistries. AST, BUN and total protein were lowered with 1 mg/kg as well as ALP (3 mg/kg) and CPK

**Fig. 3**




Changes in DNA sequence copy number in untreated and 10 nmol/l C-1748-treated DU-145 (a) and HL-60 cells (b) detected by comparative genomic hybridization. Gains are shown on the right side of the chromosome ideograms and losses on the left. (c) Cytogenetic profile of human prostate cancer DU145 cells and (d) human leukemia HL-60 cells.

(5 mg/kg). AST, ALP, BUN and total protein were lowered over control with 12 mg/kg C-1748 in the normal value range (Table 3). BUN was decreased with 3 and 5 mg/kg C-1748 in Copenhagen rats. CPK was found to be significantly higher in 5 (2241 ± 321 IU/l), 12 (2257.2 ± 419 IU/l) and 15 (2713.3 ± 312.8 IU/l) mg/kg group over the control (1647.6 ± 402.6 IU/l). AST was increased above the normal range with 12 (200.6 ± 24.1 IU/l) and 15 (238.8 ± 38.5 IU/l) mg/kg over controls (142.3 ±22.1 IU/l).

### Comparative genomic hybridization analysis of C-1748-treated prostate cancer and leukemia

C-1748 exhibited a preferential activity for CaP in the in-vitro cancer cell line screen and showed anti-CaP activity in animal xenograft models of human CaP. An in-vitro cell line screen demonstrated a 20-fold difference in the 50% inhibitory concentration ($IC_{50}$) values of C-1748 in CaP (DU145) and leukemia (HL-60) cells. These studies were undertaken to assess the genotoxicity of C-1748 and for comparative purpose we chose two cancer cell lines, DU145 and HL60. Both these cell lines have high genetic instability and as C-1748 preferentially showed enhanced activity towards CaP, a comparison in the CGH profile of DU145 and HL60 was studied at $IC_{50}$ values for the respective cell lines. Our studies on the genomic profile of DU145 using CGH revealed significant cytogenetic changes in untreated DU145 cells compared with normal epithelial cells (Fig. 3 and Table 4). The results obtained revealed deletions in chromosomes 1, 3, 4, 6, 7, 11, 13 and 18 (Fig. 3), and gains in chromosomes 1, 5, 8, 14 and 18 (Fig. 3). The long arm of chromosome 14 in region q12–13 was highly amplified. Of particular interest was the gain in chromosome 8q, which is a characteristic of highly aggressive hormone-independent CaP (Fig. 3). DU145 treated with C-1748 had an unchanged cytogenetic profile (Fig. 3). This study confirms an earlier report on the lowered mutagenic potential of C-1748 compared with its parent compound C-857 [18]. The results demonstrate that C-1748 is not associated with chromosomal alterations or genomic instability owing to its DNA crosslinking ability.

The effects of C-1748 gross chromosomal changes were investigated on the myeloblastic cell line, HL-60. Untreated HL-60 revealed significant cytogenetic changes in comparison with normal cells (Fig. 3), with gains in chromosomes 1, 6 and 13, and losses in chromosomes 5, 8, 9, 10, 14 and 17 (Fig. 3 and Table 4). Treatment with 500 nmol/l C-1748, however, showed no change in the cytogenetic profile of HL-60 (Fig. 3).

**Table 4 Comparative genomic hybridization (CGH) analysis of genomic alterations induced by C-1748 in DU145 and HL-60**

| Cell line | CGH analysis |
|---|---|
| DU145 control | −1p21–31, +1q12–22; −3p25–26; −4q13–35; +5p; −6q14–27; −7q31–36; +8q; −11p13–15; −13q14–34; +14q(q12–13–HA); +18p, +18q11.2–12, −18q21–23 |
| DU145 + 10 nmol/l C-1748 | −1p21–31, +1q12–22; −3p25–26; −4q13–35; +5p; −6q14–27; −7q31–36; +8q; −11p13–14; −13q14–34; +14q(q12–13–HA); +18p, +18q11.2–12, −18q21–23 |
| HL-60 control | +1q12–21(HA); −5q12–22; +6q22–27(HA); −8q21.3–22, −8q24.1–24.3; −9p13–21; −10p12–13; +13q; −14q22; −17p |
| HL-60 + 500 nmol/l C-1748 | +1q12–21(HA); −5q12–22; +6q22–27(HA); −8q21.3–22, −8q24.1–24.3; −9p13–21; −10p12–13; +13q; −14q22; −17p |

## Discussion

A frequent limitation for chemotherapy is the toxicity that ranges from emesis, gastrointestinal hemorrhage, infection, anemia and altered liver and renal function to more complicated ones like cystitis, myocardial ischemia and pulmonary fibrosis. A common toxicity associated with anticancer agent regimens is myelosuppression [22], which can result in febrile neutropenia, thrombocytopenia or anemia that can cause death. Therefore, preclinical toxicity testing is necessary for two major reasons. The first is to establish a safe dose for human trials and secondly to predict toxicity owing to multiple doses in humans [17]. Toxicology studies are generally conducted in two animals; the most preferred being rodents and dog. These studies form the basis for an investigational new drug application for conducting clinical trials in humans and lead to a therapeutic-to-dose ratio applicable to humans [23].

In this study, we report the preclinical toxicity study of C-1748 in rodents. The $LD_{50}$ values for C-1748 for mice and rats are comparable and the i.p. or i.v. route of administration did not significantly alter $LD_{50}$. This is significant as the clinical administration of the drug may be performed using either route. Hematology investigations showed no significant changes in the counts of blood cells. All the experimental values, except the WBC counts and platelets, fall within the normal historical data for each of the parameters provided by the clinical laboratory for the mice and rats, indicating that the drug does not affect clinical and pathological parameters.

Liver function tests by measurements of serum aminotransferases, AST and ALT were normal, suggesting the lack of hepatotoxicity of C-1748. The ALP level was transiently increased, but was still in the normal range and is also indicative of no hepatotoxicity. BUN was in the normal range and indicates lack of renal damage by C-1748. CPK is expressed in the brain, heart and skeletal muscle, and any changes observed indicate adverse changes to the areas. In our study, we found CPK levels to be normal and it seems that C-1748 does not indicate any muscle stress at the doses injected. In an earlier study of toxicology of C-1748 in dogs, we did not observe any changes in the cardiac isozyme CK MB levels after C-1748 administration [17]. The toxicity profile of C-1748 is in contrast to doxorubicin, a drug that is used for CaP therapy, which has high bone marrow toxicity and

cardiotoxicity [24–26]. The older age group of men with CaP would be particularly susceptible to hepatic/renal compromise. Hence, C-1748 is likely to be well tolerated and combined with its efficacy, represents a feasible alternative and/or adjuvant to surgery/radiotherapy.

An important feature of cancer chemotherapy is an increase in the risk of development of 'secondary cancers' in the patients as many drugs are DNA-damaging agents. We used CGH to measure DNA sequence copy number gains and losses and map these aberrations to the appropriate location on the chromosome [20,21]. Gains in a certain region could indicate overexpression of oncogenes, whereas losses could imply loss of tumor suppressor genes, both of which can increase the tendency of the cell towards deregulated proliferation [20,21]. Several recent studies have implicated gene deletions and amplifications as primary events in the initiation and progression of CaP. One such change that has been shown to be particularly important in the progression of CaP to androgen-resistant subsets is the gain of chromosome 8q and is considered a marker of aggressiveness in CaP. The differences in the genome of DU145 and normal epithelium could provide clues to malignant transformation in CaP. It is, however, of significance that the treatment with C-1748 did not alter the cytogenetic profile of the target cells, although nitroacridines are potent DNA intercalators [27].

The structure–activity relationship indicates that the 1-nitro group of C-1748 is crucial for biological activity. It is, however, conducive to reduction, leading to the formation of highly reactive and unstable products contributing to the high toxicity of 1-nitroacridines [6]. The introduction of a methyl-electron-donating group, at position $C_4$, led to derivatives that exhibit lower toxicity than parent compounds by lowering their ability to undergo reduction [13], while demonstrating specificity for CaP and more importantly with lowered systemic toxicity [15] (K. Tadi, Y. Chen, B.T. Ashok, D. Banerjee, B. Wysocka-Skrzela, J. Konopa, *et al.*, 2006, personal communication). Our preclinical studies in dogs indicate that C-1748 is at low risk to cause myelosuppression [17]. Our results show that the $IC_{50}$ of C-1748 for human CaP is around 40-fold lower than human leukemia and is therefore extremely significant in the clinical context. The toxicity profile of C-1748 in this study, our earlier report in dogs [17], the lack of mutagenicity [18], in conjunction with the CGH data suggest that at efficacious anticancer doses, this drug has minimal systemic toxicity and needs to be developed further clinically in a phase I human study, plans for which are underway.

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