

## Characterization of the myelotoxicity of chloramphenicol succinate in the B6C3F1 mouse

John A. Turton\*, Rajni Fagg<sup>†</sup>, William R. Sones\*<sup>1</sup>, Thomas C. Williams<sup>†</sup> and C. Michael Andrews<sup>†2</sup>

\*Centre for Toxicology, Department of Pharmacology, The School of Pharmacy, University of London, London, UK, and

<sup>†</sup>GlaxoSmithKline Research and Development, Ware, Herts, UK

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### Summary

Chloramphenicol (CAP) is haemotoxic in man, inducing two types of toxicity. First, a dose-related, reversible anaemia with reticulocytopenia, sometimes seen in conjunction with leucopenia and thrombocytopenia; this form of toxicity develops during drug treatment. The second haemotoxicity is aplastic anaemia (AA) which is evident in the blood as severe pancytopenia. AA development is not dose-related and occurs weeks or months after treatment. We wish, in the longer term, to investigate CAP-induced AA in the busulphan-pretreated mouse. However, as a prelude to that study, we wanted to characterize in detail the reversible haemotoxicity of CAP succinate (CAPS), administered at high dose levels in the mouse, and follow the recovery of the bone marrow in the post-dosing period. Female B6C3F1 mice were gavaged with CAPS at 0, 2500 and 3500 mg/kg, daily, for 5 days and sampled ( $n = 5$ ) at 1, 7, 14 and 21 days post-dosing. Blood, bone marrow and spleen samples were analysed and clonogenic assays carried out. At day 1 post-dosing, at both CAPS dose levels, decreases were seen in erythrocytes and erythrocyte precursors; marrow erythroid cells were reduced. Reductions were also evident in splenic nucleated cell counts, blood high fluorescence ratio (HFR) reticulocyte counts and total reticulocyte counts; burst-forming units-erythroid and colony-forming units-erythroid showed decreases. At day 7 post-dosing (2500 mg/kg CAPS), there was regeneration of erythrocyte production, with marked splenic erythropoietic activity, and raised blood HFR reticulocytes. At day 7, at 3500 mg/kg CAPS, erythrocyte and reticulocyte parameters remained depressed. At 14 days post-dosing (2500 mg/kg CAPS), many erythrocyte parameters had returned to normal; at 3500 mg/kg CAPS, there was erythroid regeneration. By 21 days post-dosing, at both CAPS dose levels, most erythrocytic parameters were equivalent to control values. For leucocyte parameters, there was some depression at day 1 post-dosing (at both CAPS dose levels) and signs of recovery at day 7. At days 14 and 21 post-dosing, most leucocyte

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#### Correspondence:

J.A. Turton  
Centre for Toxicology  
Department of Pharmacology  
The School of Pharmacy  
University of London  
29-39 Brunswick Square  
London WC1N 1AX, UK.  
Tel.: +44 20 7 753 5832;  
Fax: +44 20 7 753 5811;  
E-mail: john.turton@ulsop.ac.uk

<sup>1</sup>Present address: W. R. Sones, Department of Biosciences, University of Hertfordshire, C.P. Snow Building, College Lane, Hatfield, Hertfordshire AL10 9AB, UK.

<sup>2</sup>Deceased.

parameters were close to control values. Marrow smears at day 1 post-dosing (at both CAPS dose levels) showed vacuolation of early normoblasts, of myeloid and of monocytic precursors. We conclude that the administration of CAPS at 2500 and 3500 mg/kg for 5 days induced significant myelotoxicity in female B6C3F1 mice, with cessation of erythropoiesis at day 1 post-dosing; recovery was seen over the following 7/14 days. The blood HFR reticulocyte count was a precise indicator of CAPS-induced depressive effects and subsequent recovery. It is concluded that the administration of five daily doses of CAPS at 2500 and 3500 mg/kg to the female B6C3F1 mouse induces an anaemia with reticulocytopenia, in conjunction with leucopenia, in the immediate post-dosing period; no evidence was seen at 21 days post-dosing of peripheral blood pancytopenia or a hypocellular/acellular bone marrow, which are both characteristic features of AA in man.

### Keywords

chloramphenicol, haemotoxicity, mouse, myelotoxicity, reticulocyte, spleen

Aplastic anaemia (AA) in man is a disease characterized by pancytopenia of the peripheral blood and bone marrow hypocellularity (Young & Alter 1994; Jandl 1996). The condition is associated with a variety of aetiological agents including viruses, irradiation and a wide range of drugs and chemicals (Heimpel 2000; Young & Maciejewski 2000). However, the basic pathophysiology of AA is not clearly understood, and a factor that has contributed to this lack of understanding is that there are currently no animal models of the drug-induced disease that are convenient to use (Benestad 1979; Haak 1980; Vincent 1984; Young & Maciejewski 1997). Nevertheless, in a series of papers, Morley and his colleagues (Morley & Blake 1974a, b; Morley *et al.* 1975, 1976) developed and assessed a model of busulphan (BU)-induced chronic hypoplastic marrow failure (CHMF, i.e. AA) in the female Swiss and BALB/c mouse. In this model, BU was administered on four fortnightly occasions (i.e. over a 6-week period) at 20, 20, 20 and 10 mg/kg, respectively, and the animals were investigated over the following 300–400 days. An essential feature of this mouse model was that BU produced a form of 'residual' or 'late stage' marrow injury, which was characterized by mild or moderate marrow failure. Morley *et al.* (1976) proposed that such a residual marrow injury might be a factor in predisposing individuals to develop AA, following exposure to drugs such as the antibiotic chloramphenicol (CAP), a compound widely known to induce AA in man (Young & Alter 1994; Young & Maciejewski 2000). To investigate this proposal, Morley *et al.* (1976) evaluated the capacity of CAP succinate (CAPS) to induce CHMF in the female BALB/c mouse pretreated with BU. With CAPS administered in the drinking water at 5 mg/ml, the antibiotic was reported to cause a progressive fall in the number of pluripotent stem

cells and granulocytic progenitor cells in BU-pretreated mice, but there was no effect of CAPS on the marrow cells of normal (i.e. not BU-pretreated) animals (Morley *et al.* 1976).

CAP is a highly active, broad-spectrum antibiotic. The drug became widely used in the treatment of serious infections including typhoid fever and other forms of salmonellosis, in severe infections due to *Haemophilus influenzae* (particularly meningitis), and in other life-threatening infections of the central nervous system and respiratory tract (Holt *et al.* 1993; Parfitt 1999). The therapeutic dose of CAP is generally 50 mg/kg daily, in divided doses; the time periods of treatment are often 10–15 days (Chaplin 1986). CAP is widely used today in the UK and USA in the topical treatment of ear and eye infections (Fraunfelder *et al.* 1993; Dollery 1999; Parfitt 1999) and is also used extensively in the developing world (Trevett *et al.* 1992; Kumar & Verma 1993; Kushwaha *et al.* 1994).

However, CAP is haemotoxic in man, inducing, in particular, two major effects on the blood (FAO/WHO 1988; IARC 1990; Young & Alter 1994). The first haemotoxicity is an often-occurring (i.e. predictable), mild anaemia with reticulocytopenia, sometimes seen in conjunction with leucopenia and thrombocytopenia (Yunis & Bloomberg 1964). This predictable myelosuppression, primary affecting erythropoiesis, is dose-related, develops during drug therapy (particularly with relatively high-dose levels given over a short time period), and is quickly reversible following drug withdrawal (Krakoff *et al.* 1955; Scott *et al.* 1965; Best 1967). The bone marrow generally shows a normal cellularity, but reduced numbers of late erythroid cells may be seen (Rubin *et al.* 1958; Chaplin 1986). The second haemotoxicity of CAP in man is AA (Rich *et al.* 1950; Welch *et al.* 1954; Hugley *et al.* 1961; Wallerstein *et al.* 1969). This toxicity is seen

relatively infrequently (i.e. it is unpredictable) but is evident in the blood as a severe pancytopenia and a hypocellular or acellular marrow. CAP-induced AA does not generally appear to be related to the dose level of drug administered, develops weeks or months after treatment and is often irreversible and fatal (Yunis & Bloomberg 1964; Yunis 1978). The development of AA is unrelated to the reversible form of haemotoxicity (Yunis & Bloomberg 1964; Wallerstein *et al.* 1969).

To examine the capacity of CAPS to induce AA in the BU-pretreated mouse, we carried out a series of experiments following the protocols of Morley and his co-workers (Morley & Blake 1974a; Morley *et al.* 1976) but using the female B6C3F1 mouse rather than the inbred BALB/c strain used by Morley & Blake (1974a). However, we were not able to demonstrate that CAPS caused AA (Andrews *et al.* 1993, 1997, 1998; Andrews 2000). In our studies, CAPS was administered in the drinking water at 4 mg/ml which was the maximum tolerated dose in the B6C3F1 strain of mouse (at dose levels of 5 and 6 mg/ml CAPS in the drinking water, there were significant reductions in body weight, a decrease in the reticulocyte count and an increase in RBC count, which were possible indications of a haemoconcentration effect). At 4 mg/kg, water consumption data demonstrated that over the course of a 13-month study, the animals consumed about 176–370 mg/kg/day of CAPS. It has not been possible to determine why our results contrasted with the earlier positive findings of Morley *et al.* (1976). However, it was subsequently shown that CAPS, administered at dose levels of 176–370 mg/kg/day in the drinking water, was not haemotoxic (Holt *et al.* 1998; Andrews 2000), and in, for example, the CD-1 mouse, dose levels of 1400–1700 mg/kg CAPS, administered by gavage, daily, were required to induce significant haemotoxicity (Turton *et al.* 1999). In a similar way, it was shown that in several inbred mouse strains, including the BALB/c, dose levels of 500–2500 mg/kg CAPS administered daily for 7 days by gavage are necessary to cause haemotoxicity (Festing *et al.* 2001).

Yunis & Bloomberg (1964), in a review of CAP-induced AA in man, observed that in 37 out of 46 cases of fatal marrow aplasia, the therapeutic administration of CAP to the patient had been on an intermittent basis, rather than as a continuous daily exposure. However, in 36 cases of the reversible form of anaemia and bone marrow depression, in only eight cases had CAP been taken intermittently (Yunis & Bloomberg 1964). It was therefore considered that our earlier failure to induce AA in the B6C3F1 mouse (Andrews *et al.* 1998; Andrews 2000) may have been related to the dosing of CAPS using a continuous low-level administration regimen in the drinking water. For this reason, it was thought possible that an intermittent

dosing regimen with CAPS given in repeated cycles of dosing and recovery (i.e. dosing and non-dosing), at high (i.e. haemotoxic) dose levels, by gavage, might be successful in the induction of AA in the BU-pretreated B6C3F1 mouse.

As a prelude to carrying out such a study in the BU-pretreated mouse using the high-dose, intermittent, gavage administration of CAPS, we wished to investigate in detail the haemotoxicity of the drug given orally at 2500 and 3500 mg/kg daily for 5 days, followed by an observation of the haematological recovery in the following 21 day post-dosing period. It was considered that recovery would be complete by day 21 post-dosing, as Turton *et al.* (1999) had shown that in CD-1 mice given 1400 mg/kg CAPS daily for 10 days, there was a return to control values after 15 days. Furthermore, in man, erythropoietic recovery in the reversible form of CAPS haemotoxicity occurs at 1 week after drug administration (Saidi *et al.* 1961).

## Materials and methods

### Animals

Female B6C3F1 mice (Charles River UK, Margate, Kent), 12–14 weeks old, were housed in groups of five, bedded on wood shavings, and given diet (Rat and Mouse No.1, SDS, Witham, Essex) and drinking water *ad libitum*. A temperature of  $22 \text{ }^{\circ}\text{C} \pm 1 \text{ }^{\circ}\text{C}$  was maintained, with a relative humidity of 45–70% and a 12 : 12 h light : dark cycle (lights on at 06:00 hours). Animals were acclimatized for 5 days before the start of the experiment and were observed daily for signs of ill health.

### Administration of CAPS

Solutions of CAPS (Sigma Chemical, Poole, Dorset) were prepared in water for irrigation (BP) and administered by gavage in dose volumes of approximately 10 ml/kg; control animals were given water at the same dose volume.

### Tissue sampling

Mice were killed by CO<sub>2</sub> overdose and 0.5 ml blood samples for cell counting were taken from the posterior vena cava and anticoagulated with 1.5 mg dipotassium EDTA/ml of blood (Teklab, Sacriston, Durham, UK). Bone marrow cellularity was assessed by flushing the contents of one femur into 10 ml of phosphate-buffered saline (PBS). A bone marrow smear was prepared from the contents of one tibia. The spleen was removed, weighed and sectioned to prepare spleen imprints (touch preparations). Here, the spleen is sectioned transversely

with a razor blade and with each half held with forceps, the cut surface is lightly touched repeatedly on a glass microscope slide. Spleen cell suspensions were prepared by scissor-mincing after removal of the capsular material, and dispersion in PBS is achieved by cavitation in a 50 ml syringe. For bone marrow progenitor cell assays, the contents of one femur were flushed into 1.0 ml of Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies, Paisley, UK) under sterile conditions.

### Sample analysis

*Peripheral blood: full blood count, differential leucocyte count and reticulocyte count.* Full blood counts and differential leucocyte counts were obtained with a Bayer H\*1 haematology analyser (Bayer Diagnostics, Newbury, Berkshire, UK). Leucocytes, excluding basophils, were differentiated by cluster analysis of peroxidase content and volume, measured by absorption of right-angle scatter, and forward scatter, of tungsten light, respectively, against an archetype for mouse leucocytes (Davies & Fisher 1991). Basophils were counted in a separate channel as phthalic acid-resistant intact cells; other cells in this channel were detected as stripped nuclei. The H\*1, with mouse-specific software (Versions 1.0–3.0; Bayer, Swords, Dublin, Ireland), was validated in-house for use with mouse blood. Reticulocytes were counted using a Sysmex R-1000 reticulocyte analyser (Sysmex, Milton Keynes, Buckinghamshire, UK) (Tichelli *et al.* 1990), with photomultiplier voltage gain adjusted optimally for mouse blood (Fuchs & Eder 1991). Reticulocytes were differentiated from mature erythrocytes by fluorescence after staining with auramine O (Tichelli *et al.* 1990; Fuchs & Eder 1991). Reticulocyte maturity was assessed according to fluorescence intensity which is divided into three fractions: the high-, mid- and low-fluorescence ratio (HFR, MFR and LFR). In-house validation for the R-1000 has been performed for mouse blood. A small proportion of leucocytes fall into an area on the H\*1 cytogram indicating a greater cell size than most lymphocytes, but having a lower peroxidase activity than monocytes. Such cells are classified as large unstained cells (LUC) (Andrews *et al.* 1997; Knoll 2000). In human blood, it has been shown that assigning half the LUC count to the lymphocytes and half to monocytes produces a differential leucocyte count very close to manual (i.e. visual) examination of the Romanowsky-stained smear (Kinsey & Watts 1988).

*Bone marrow cellularity.* The femoral bone marrow suspension in PBS was used to obtain a nucleated cell count from the basophil channel of the H\*1. Where the presence of fat particles was evident in the analyser cytogram, correction of the count was performed according to Bentley *et al.* (1995).

*Bone marrow smears, spleen touch preparations and spleen cell suspensions.* Bone marrow smears were stained with May-Grünwald-Giemsa (MGG) stain (Dacie & Lewis 1975; Hall & Malia 1984; Smith *et al.* 1994; Freeman 2000) and examined microscopically for morphological assessment and the enumeration of myeloid, erythroid, lymphoid and 'other' cells in 200 cell-differential counts (other cells include monocytes and monocyte precursors, mast cells, megakaryocytes, plasma cells and unidentifiable cells). Spleen touch preparations (imprints) were stained with MGG stain and assessed histologically; erythropoietic activity was graded as: 0 = absent; 1 = very slight; 2 = slight; 3 = moderate; 4 = marked. The nucleated cell count of spleen cell suspensions in PBS was obtained from the basophil channel of the H\*1.

*Bone marrow progenitor cell analysis.* 0.3 ml of femoral bone marrow cell suspension in IMDM was, within 1 h of sampling, resuspended in 3.0 ml of Methocult GF M3434 culture medium (Metachem Diagnostic, Piddington, Northampton, UK) containing 0.9% methylcellulose in IMDM, 15% fetal calf serum (PAA Laboratories GmbH, Linz, Austria),  $10^{-4}$  M2-mercaptoethanol, 2 nM L-glutamine, 1% bovine serum albumin, 10 µg/ml bovine pancreatic insulin, 200 µg/ml human transferrin, 10 ng/ml recombinant murine IL-3, 10 ng/ml recombinant human IL-6, 50 ng/ml recombinant murine stem cell factor and 3 U/ml recombinant murine erythropoietin (all last ingredients: Sigma). The tubes were vortexed and allowed to stand (5 min; 37 °C), dispensed into cell culture multiwell plates and incubated at 37 °C. Burst-forming units-erythroid (BFU-E) were counted after 10 days incubation, and colony-forming units-erythroid (CFU-E) and colony-forming units-granulocyte-macrophage (CFU-GM) after 12 days, on the same plates. Each sample was assayed in triplicate.

### Statistical analysis

Student's *t*-test was used to compare treated and control groups at the same time point using RS/1 software (BBN, Cambridge, MA, USA) and Microsoft Excel.

### Experimental design

Sixty mice were divided into three equal groups and dosed orally with CAPS by gavage at 0, 2500 and 3500 mg/kg, daily for 5 days. On days 1, 7, 14 and 21 after the final dose, five mice per group were killed and samples of blood, femoral bone marrow and spleen were taken for a full blood count, nucleated cell count (marrow; spleen), femoral progenitor cell assays (BFU-E, CFU-E and CFU-GM) and microscopy of

marrow smears and spleen touch imprints. Body weights of animals were determined twice weekly.

## Results

### *Clinical observations*

Control and CAPS-treated mice appeared well at all times and there was no evidence of drug-induced toxicity. One animal in the group receiving CAPS at 3500 mg/kg died on the first day of treatment following a dosing accident (oesophageal perforation).

### *Haematology*

*Erythroid series.* At 1 day post-dosing, marked reductions were seen in erythrocytes and bone marrow erythroid precursors at both 2500 and 3500 mg/kg CAPS (Table 1). HCT and Hb were also significantly decreased. MCV, MCH and MCHC were not affected. The femoral bone marrow erythroid cell count was reduced at 2500 mg/kg CAPS to 20.8% of the control mean value, and at 3500 mg/kg to 9.5% of the control mean. In the bone marrow smears, at both CAPS dose levels, the only representatives of the erythroid series were pronormoblasts and early normoblasts; the majority of early normoblasts contained a single large cytoplasmic vacuole (Figures 1 and 2).

On day 1 post-dosing, the splenic nucleated cell count was reduced to 57.7% and 43.0% of the mean control value at 2500 and 3500 mg/kg CAPS, respectively (Table 1); examination of the spleen touch preparations (erythropoietic grading) showed an absence of erythroid activity at both CAPS dose levels (Table 1). Similarly, the total reticulocyte count and the HFR reticulocyte count (i.e. the most immature reticulocytes) were both markedly decreased at each dose level. RDW and HDW were also significantly reduced at this time in both CAPS treatment groups, reflecting the depletion of immature erythrocytes in the peripheral circulation. Femoral bone marrow BFU-E and CFU-E were reduced at day 1 post-dosing at both CAPS dose levels. Platelet counts were not affected at either CAPS dose level at this time point.

At day 7 after CAPS administration at 2500 mg/kg, the regeneration of erythrocyte production was evident. Assessment of the spleen touch preparations for erythropoietic grading at this dose level showed marked erythropoietic activity (Table 1), and there were significant increases in the splenic nucleated cell count; HFR reticulocytes and RDW were raised in value (Table 1). The relationship between the grade of splenic erythropoietic activity and the peripheral blood HFR reticulocyte count is illustrated in Figure 3. The total reticulocyte count and the femoral bone marrow erythroid count were both also

elevated at this time in animals treated at 2500 mg/kg, but the increases were not statistically significant.

At 3500 mg/kg CAPS, at day 7 post-dosing, significant depressions of the total reticulocyte count and the HFR reticulocyte count were still evident. Similarly, many other erythrocyte parameters including RBC, HCT and Hb were also still significantly depressed at this time in the 3500 mg/kg CAPS group. However, the nucleated cell count of the spleen compared with the control figure at day 7 post-dosing, but the splenic erythropoietic grading (in touch preparations) showed only slight or moderate activity (Table 1). However, platelet counts were significantly elevated at both 2500 and 3500 mg/kg CAPS on day 7 post-dosing, and at 3500 mg/kg the increase was to 181.9% of the control value.

On day 14 after CAPS administration, at 2500 mg/kg, many erythrocyte parameters had returned to normal (Table 1). At 3500 mg/kg CAPS, there was a similar trend with evidence of regeneration; the splenic nucleated cell count, the total reticulocyte count and the HFR reticulocyte count all showed elevations (to 118.6, 141.6 and 155.4% of the control values, respectively), but these increases were not statistically significant. However, at both 2500 and 3500 mg/kg CAPS, at day 14 post-dosing, RDW and HDW were significantly increased.

In overall terms, therefore, by day 21 post-dosing, at both CAPS levels, parameters had returned to normal; however, RDW and HDW still remained significantly elevated at this time.

*Leucocytes.* At the 2500 mg/kg dose level of CAPS, on day 1 post-dosing, the total WBC count was statistically significantly depressed, as was the count for CFU-GM (Table 2). However, the femoral bone marrow myeloid count at day 1 post-dosing was normal. The numbers of 'other' femoral marrow cells (this included especially monocytes and monocyte precursors), and peripheral blood LUC counts, were both elevated at day 1 post-dosing in mice treated at 2500 mg/kg, but these increases did not reach statistical significance. Peripheral blood neutrophils, monocytes, eosinophils and basophils all showed reductions at day 1 post-dosing at 2500 mg/kg, but these decreases did not reach statistical significance. At day 7 post-dosing in the 2500 mg/kg CAPS group, there were significant elevations in LUC and basophil counts; monocytes and 'other' femoral bone marrow cells were also increased, but not statistically significantly. At 14 and 21 days post-dosing, in the 2500 mg/kg CAPS mice, the majority of leucocyte parameters were close to control values.

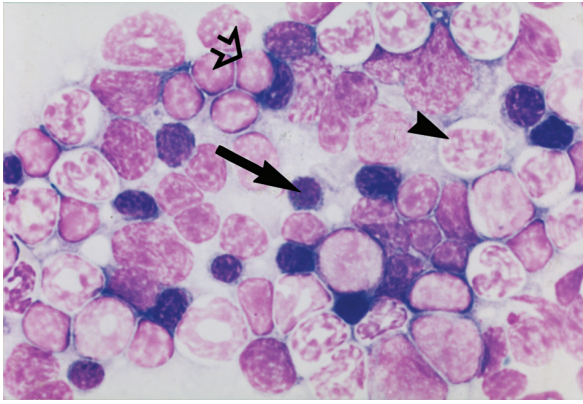
In mice treated at 3500 mg/kg CAPS, on day 1 post-dosing, significant reductions were evident in total leucocyte, neutrophil, lymphocyte, eosinophil and CFU-GM counts (Table 2).

**Table 1** Mean erythroid parameters in female B6C3F1 mice dosed with chloramphenicol succinate (CAPS) at 2500 and 3500 mg/kg on five consecutive days and sampled at 1, 7, 14 and 21 days after the final dose

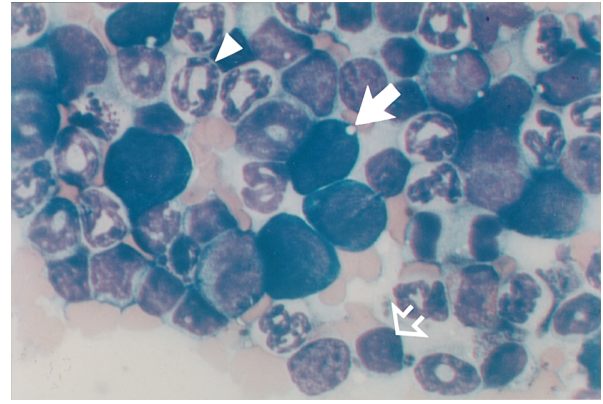
Day post-dosing	CAPS dose (mg/kg)											
	Control				2500				3500			
	1	7	14	21	1	7	14	21	1	7	14	21
RBC	9.848	9.770	9.688	9.900	8.855**	8.058**	9.682	10.043	8.968**	7.642**	9.955	9.658
HCT	0.542	0.538	0.533	0.531	0.480**	0.448**	0.535	0.546	0.487**	0.411**	0.545	0.512
Hb	15.45	15.38	15.13	15.36	13.93**	12.42**	15.24	15.58	14.16**	11.70**	15.53	14.86
MCV	55.05	55.06	55.00	53.60	54.23	55.58	55.30	54.35	54.32	53.78**	54.75	52.98
MCH	15.70	15.74	15.60	15.52	15.70	15.42	15.74	15.50	15.78	15.32*	15.60	15.38
MCHC	28.53	28.60	28.35	28.94	28.98	27.74	28.42	28.58	29.02	28.50	28.48	29.04
RDW	11.60	11.84	11.85	11.66	11.10**	12.70**	12.86**	12.71**	10.92**	11.32	13.80**	13.26**
HDW	1.500	1.514	1.558	1.492	1.430*	1.550**	1.622**	1.660**	1.392*	1.410**	1.665**	1.708**
Retic	318.4	274.1	280.9	249.4	6.0**	414.1	275.4	249.2	6.1***	19.5***	397.8	212.6
HFR	77.5	54.1	49.6	39.4	0.1**	170.8**	50.8	39.2	0.2***	1.5***	77.1	28.3
BM cells	1.180	1.258	1.053	1.106	0.960	1.588	1.073	1.398	1.050	1.196	0.983	1.378
BM eryth cells	0.423	0.380	0.253	0.300	0.088**	0.558	0.345	0.400	0.040**	0.260	0.305	0.326
BFU-E	42.67	nd	45.33	30.00	33.67**	nd	46.00	25.00*	26.33***	nd	46.33	28.33
CFU-E	47.00	nd	48.67	60.00	35.00*	nd	49.67	60.00	33.33*	nd	39.00	58.67
Splenic cells	8.68	8.82	9.82	9.91	5.01***	17.44***	11.94	11.86	3.73***	8.31	11.65	10.59
Splenic eryth	3.2	1.8	1.8	2.0	0.0	4.0	1.6	2.0	0.0	2.6	3.0	2.2
Plt	847.3	970.4	1072.5	987.0	897.8	1261.8*	1254.0	1084.0	770.4	1765.0*	1066.0	973.0

Abbreviations and units: RBC, red blood cells ( $\times 10^{12}/l$ ); HCT, haematocrit ( $l/l$ ); Hb, haemoglobin (g/dl); MCV, mean cell volume (fl); MCH, mean cell haemoglobin (pg); MCHC, mean cell haemoglobin concentration (g/dl); RDW, red cell (volume) distribution width femoral (%); HDW, haemoglobin (cellular concentration) distribution width (g/dl); Retic, absolute reticulocyte count ( $\times 10^9/l$ ); HFR, high fluorescence ratio reticulocytes ( $\times 10^9/l$ ); BM cells, bone marrow nucleated cell count ( $\times 10^7$ ); BM eryth cells, femoral bone marrow erythroid cells ( $\times 10^7$ ); BFU-E, burst-forming units-erythroid, absolute number of colonies per plate; CFU-E, colony-forming units-erythroid, absolute number of colonies per plate; Splenic cells, spleen suspension nucleated cell count ( $\times 10^7$ ); Splenic eryth, splenic erythropoietic grading, histological grading: 0, absent; 1, very slight; 2, slight; 3, moderate; 4, marked; Plt, platelets, ( $\times 10^9/l$ ); nd, not determined.  $n = 5$  for control and CAPS groups at all time points, except  $n = 4$  for 3500 mg/kg CAPS group at day 14 and for BFU-E and CFU-E where  $n = 3$ .

Significantly different from control animals, \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ . Data for splenic erythropoietic grading was not analysed statistically.



**Figure 1** Romanowsky-stained bone marrow smear from a control (vehicle-treated) mouse at day 1 post-dosing to illustrate the normal distribution of erythroid (arrow), myeloid (arrowhead) and lymphoid (open arrow) cells; original magnification  $\times 1000$ .



**Figure 2** Vacuolation in a Romanowsky-stained bone marrow smear from a mouse treated with chloramphenicol succinate at 3500 mg/kg for 5 days and sampled at day 1 post-dosing; early normoblasts are present and often contain a single cytoplasmic vacuole (arrow). There is a relative depletion of late normoblasts. Later myeloid forms appear normal (arrowhead). The lymphoid series is also represented (open arrow). No megakaryocytes are present. Original magnification  $\times 1000$ .

Basophils and LUC were also decreased, but not significantly. Femoral marrow myeloid cells, however, were slightly increased (NS), but other femoral marrow cells were increased to nearly 300% of the control value ( $P < 0.05$ ). At 7 days post-dosing with 3500 mg/kg CAPS, all peripheral blood WBC counts except the basophil count were elevated; however, only the elevation of the LUC count was statistically significant ( $P < 0.05$ ). The femoral marrow count of other cells was elevated to about 260% of the control value at 7 days post-dosing (NS).

At 14 days and 21 days post-dosing in the 3500 mg/kg CAPS group, most leucocyte parameters were close to the corresponding control values.

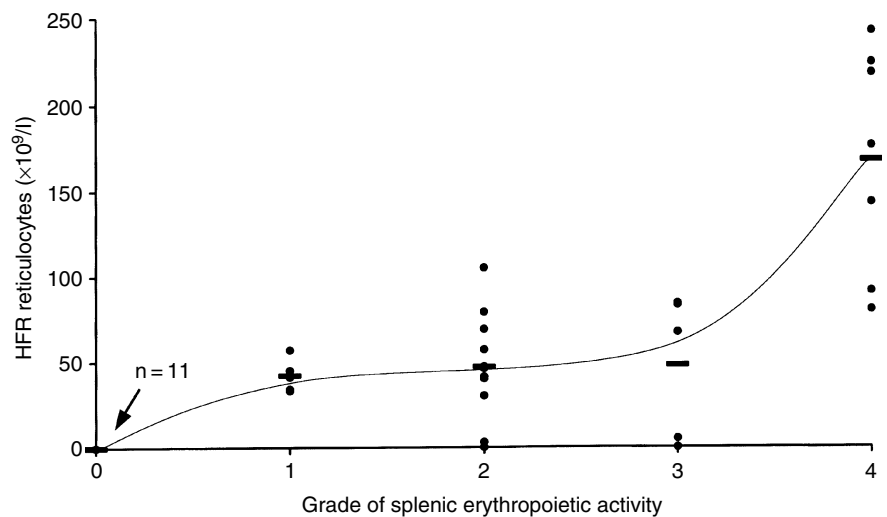
An examination of the marrow smears from mice at day 1 post-dosing (2500 and 3500 mg/kg CAPS) showed that many myeloblasts, promyelocytes, myelocytes and

monocyte precursors contained a small number of cytoplasmic vacuoles. Furthermore, bone marrow taken from the mouse killed accidentally after a single dose of CAPS at 3500 mg/kg illustrated marked vacuolation of the myeloid precursors (Figure 4).

### Discussion

In the present study with female B6C3F1 mice, significant myelotoxicity was induced with CAPS using a dosing regimen of drug administration by gavage at 2500 and 3500 mg/kg for five consecutive days. At both CAPS dose levels, cessation of

**Figure 3** Comparison of the grade of splenic erythropoietic activity in spleen touch preparations and the peripheral blood high fluorescence ratio (HFR) reticulocyte count, in individual mice treated with chloramphenicol succinate at 2500 and 3500 mg/kg for 5 days, and sampled at day 1, 7, 14 and 21 after the final dose. Splenic erythropoietic activity was graded in spleen touch preparations as: 0, absent; 1, very slight; 2, slight; 3, moderate; 4, marked. Horizontal bars indicate the mean HFR reticulocyte value at each grade of splenic erythropoietic activity.

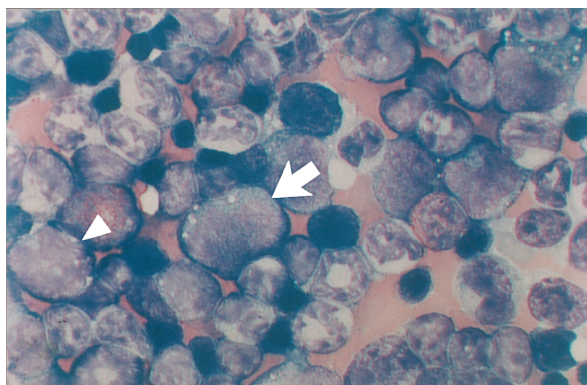


**Table 2** Mean leucocyte parameters in female B6C3F1 mice dosed with chloramphenicol succinate (CAPS) at 2500 and 3500 mg/kg on five consecutive days and sampled at 1, 7, 14 and 21 days after the final dose

Day post-dosing	CAPS dose (mg/kg)											
	Control				2500				3500			
	1	7	14	21	1	7	14	21	1	7	14	21
WBC	4.43	4.54	4.78	5.80	360**	6.44	5.60	5.80	2.60**	5.66	4.35	6.06
Neut	0.510	0.530	0.563	0.652	0.265	0.643	0.754	0.660	0.116**	0.708	0.640	0.690
Lymph	3.635	3.704	3.862	4.762	3.158	4.884	4.388	4.843	2.374*	4.000	3.416	5.012
Mono	0.108	0.125	0.075	0.120	0.048	0.295	0.107*	0.097	0.078	0.244	0.088	0.107
Eo	0.100	0.080	0.100	0.140	0.065	0.084	0.070	0.100	0.040*	0.109	0.120	0.140
Baso	0.010	0.020	0.010	0.020	0.000	0.090*	0.020	0.020	0.000	0.020	0.010	0.020
LUC	0.028	0.024	0.078	0.064	0.065	0.070*	0.070	0.060	0.010	0.036*	0.053	0.048
BM myel cells	0.440	0.478	0.433	0.404	0.426	0.506	0.380	0.460	0.598	0.608	0.363	0.604
BM lymph cells	0.403	0.378	0.323	0.360	0.280	0.472	0.323	0.498	0.315	0.274	0.288	0.422
BM other cells	0.035	0.022	0.045	0.044	0.084	0.052	0.028	0.046	0.100*	0.058	0.028	0.026
CFU-GM	28.7	nd	30.3	30.7	19.0*	nd	27.3	29.0	14.3*	nd	26.7	35.3

Abbreviations and units: WBC, white blood cells ( $\times 10^9/l$ ); Neut, neutrophils ( $\times 10^9/l$ ); Lymph, lymphocytes ( $\times 10^9/l$ ); Mono, monocytes ( $\times 10^9/l$ ); Eo, eosinophils ( $\times 10^9/l$ ); Baso, basophils ( $\times 10^9/l$ ); LUC, large unstained cells ( $\times 10^9/l$ ); BM myel cells, femoral bone marrow myeloid cells ( $\times 10^7$ ); BM lymph cells, femoral bone marrow lymphoid cells ( $\times 10^7$ ); BM other cells, femoral bone marrow other cells ( $\times 10^7$ ); CFU-GM, colony-forming units-granulocyte-macrophage, absolute number of colonies per plate; nd, not determined. All other information as Table 1, except  $n = 3$  for CFU-GM. Cells categorized as 'other' include monocytes and monocyte precursors, mast cells, megakaryocytes, plasma cells and unidentifiable cells.

erythropoiesis was evident at day 1 post-dosing. A recovery was seen at day 7 post-dosing at the 2500 mg/kg CAPS dose level and at between 7 and 14 days at the 3500 mg/kg dose level (Table 1). Myelotoxicity was most pronounced in the erythroid series at each dose level. However, toxic effects were not restricted to the erythroid lineage, as some reductions in CFU-GM counts and peripheral blood leucocyte counts were also evident at both CAPS dose levels (Table 2). In addition,



**Figure 4** Bone marrow smear (Romanowsky-stained) from a mouse given a single dose of chloramphenicol succinate at 3500 mg/kg and which died on the day of treatment (following a dosing accident). Cytoplasmic vacuolation is evident in the myeloid (arrow) and monocytic (arrowhead) precursors. Original magnification,  $\times 1000$ .

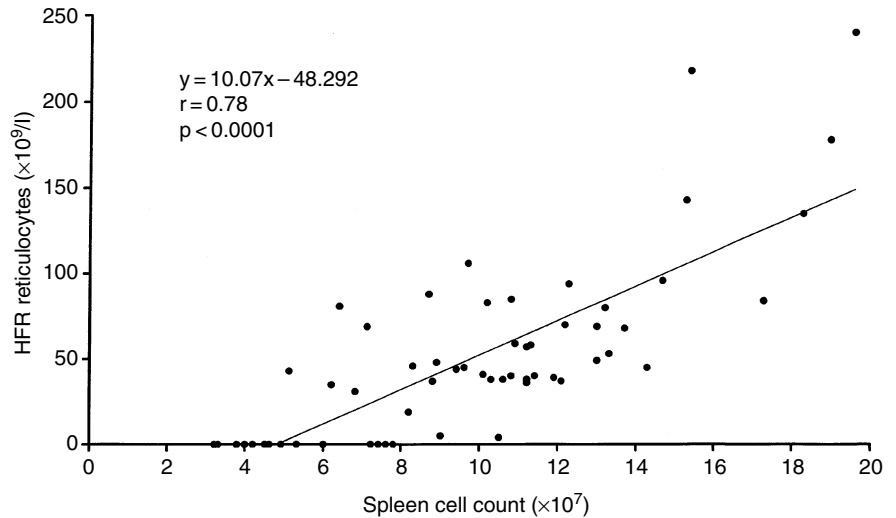
although cytoplasmic vacuolation was demonstrated in cells of the erythroid series (Figure 2), precursors of the myeloid lineage were also affected (Figure 4). Although femoral marrow BFU-E and CFU-E were depressed by CAPS administration at day 1 post-dosing, the myelosuppressive effect of the drug was more clearly identified in the morphologically recognizable committed erythroid precursor cells in the bone marrow and in the cellularity of the spleen.

However, of the many parameters investigated in the present study, the CAPS-induced depressive change was most marked in the response of the HFR reticulocyte fraction, that is, in the most immature reticulocytes. The HFR reticulocyte count was also the most sensitive indicator of erythroid recovery and showed a maximal amplitude of response as erythropoiesis returned to normal in the post-dosing period.

The pattern of CAPS-induced erythroid suppression and recovery in the present experiment compares closely with the reversible toxicity to cells of the erythroid series reported in man (Volini *et al.* 1950; Saidi *et al.* 1961; Jiji *et al.* 1963; Scott *et al.* 1965). The maturation arrest (and vacuolation) at the level of the pronormoblast and early normoblast in the present study is also consistent with findings in man (Rosenbach *et al.* 1960; Scott *et al.* 1965; Yunis *et al.* 1970; Skinnider & Ghadially 1976), and also in the rat (Schrober *et al.* 1972), and in the mouse (Miura *et al.* 1980).

In the present experiment with CAPS, and in previous investigations with other myelotoxic drugs such as





**Figure 5** Relationship between splenic nucleated cell count and the peripheral blood high fluorescence ratio reticulocyte count (with line of best fit) for individual control mice and animals treated at 2500 and 3500 mg/kg chloramphenicol succinate for 5 days and sampled at 1, 7, 14 and 21 days after the final dose.

methotrexate (Andrews 1995), a clear relationship has been shown to exist between the peripheral blood HFR reticulocyte count and the histological assessment of splenic erythropoiesis in touch preparations (Figure 3). These results also find a parallel in the relationship between the HFR reticulocyte count and the splenic nucleated cell count; this is illustrated in Figure 5. However, it was demonstrated previously (Andrews 2000) that changes in the response of the peripheral blood HFR reticulocyte levels tend to lag behind the response of the spleen and marrow by about 1 day. A delay in response of the blood HFR reticulocyte fraction was also seen in the present study, for mice which had received 3500 mg/kg CAPS, at day 7 post-dosing, showed slight/moderate erythropoietic activity in spleen touch preparations, whereas the HFR reticulocyte counts in the peripheral blood at this time remained exceptionally low (Table 1).

In the post-dosing recovery period, erythroid parameters exhibited a rebound effect, with some values increasing to levels above those in the control animals. For example, in mice treated with 2500 mg/kg CAPS at 7 days post-dosing, the HFR and total reticulocyte counts, and the spleen cellularity, all showed some evidence of a rebound effect to values above control levels (Table 1); this pattern of response was also seen in the bone marrow erythroid cells. Similarly, at the 3500 mg/kg CAPS dose level, at 14 days post-dosing, some evidence of a rebound effect was seen in the HFR and total reticulocyte counts. Rebound reticulocytosis in the BALB/c and CD-1 mouse strains in the recovery period after CAPS administration was also reported by Havard *et al.* (1999a) and Turton *et al.* (1999, 2000); a similar response was also evident after CAPS dosing in the guinea pig (Havard *et al.* 1999b; Turton *et al.* 2003) and after CAP administration in the duck (Rigdon *et al.* 1954). In the report of Rigdon *et al.* (1954), an

analysis of reticulocyte morphology suggested that the response involved the most immature reticulocyte forms, and this result therefore confirms the changes seen in the HFR reticulocyte fraction in the present study.

The findings in the present investigation on CAPS myelotoxicity support the observation that in states of drug- and chemical-induced myelosuppression in the mouse, compensatory erythropoiesis occurs in the spleen. In mice which developed marrow aplasia following the administration of  $^{89}\text{Sr}$  (Klassen *et al.* 1972), normal levels of reticulocyte production and  $^{59}\text{Fe}$  incorporation were maintained by splenic erythropoiesis, with splenectomized animals dying with pancytopenia. Likewise, Anagnostou *et al.* (1976) reported that in mice treated with estradiol benzoate, reduction in the uptake of  $^{59}\text{Fe}$  into the bone marrow occurred, but significantly increased uptake took place in the spleen, which maintained a normal haematocrit and red cell mass in the peripheral blood. Similarly, in mice treated with the antibiotic thiamphenicol (TAP) (Goris *et al.* 1990) or human granulocyte colony-stimulating factor (de Hann *et al.* 1992), suppression of femoral marrow BFU-E and CFU-E was accompanied by the stimulation of splenic erythroid progenitors; this response was interpreted as a net migration of erythroid precursor cells from the marrow to the spleen.

At day 7 post-dosing in mice treated with CAPS at both 2500 and 3500 mg/kg, there was a significant increase in platelet count (Table 1). A similar effect on platelet numbers has previously been reported in the CAPS- and TAP-treated mouse (Festing *et al.* 2001; Turton *et al.* 2002). This thrombocytosis, in the period after a drug-mediated inhibition of erythrocyte maturation, is consistent with the suggestion that this change is an erythropoietin (EPO)-stimulated effect and appears to relate to the frequently observed clinical finding of

an inverse correlation between HCT and platelet count (Jackson *et al.* 1974). An explanation for the response may be that in an anaemia, EPO production by the kidney increases, and EPO as well as stimulating RBC output from the marrow also has an enhancing effect on megakaryopoiesis (Sakaguchi *et al.* 1987; Hunt 1995).

Peripheral blood total leucocyte counts were significantly depressed at day 1 post-dosing with CAPS given at 2500 and 3500 mg/kg in the present study, returning to normal values at day 7. However, this was in contrast to erythroid counts which only returned to control values at day 14 post-dosing. This suggests a lower specificity of CAPS toxicity for cells of the myeloid series, possibly in association with the shorter maturation time of mouse leucocytes (Filmanowicz & Gurney 1960; Lee *et al.* 1979). However, although counts of CFU-GM were also significantly reduced at day 1 post-dosing at both CAPS dose levels (Table 2), the numbers of morphologically recognizable myeloid precursors in the bone marrow were not decreased. Indeed, monocytes and monocyte precursors (recorded as 'other' bone marrow cells, Table 2), were present at increased levels in marrow smears at both 2500 (NS) and 3500 mg/kg CAPS ( $P < 0.05$ ) at day 1 post-dosing, and this was followed at day 7 by increased peripheral blood monocyte (NS) and LUC ( $P < 0.05$ ) counts. Adams (1983) described a neutrophilia following CAP administration in a patient with chronic neutropenia, and a stimulation of human CFU-GM levels was demonstrated *in vitro* with sub-therapeutic concentrations of CAP by Bostrom *et al.* (1986). Turton *et al.* (2000) also described elevated neutrophil counts in the immediate post-dosing period in BALB/c mice treated with CAPS at 2000 mg/kg for 17 days.

In the present study, the formation of cytoplasmic vacuoles in early normoblasts, and in myeloblasts, promyelocytes and myelocytes, and in monocyte precursors, is of interest. CAP-induced cytoplasmic and nuclear vacuolation has been described in patients treated with the antibiotic (Krakoff *et al.* 1955; Rosenbach *et al.* 1960; Saidi *et al.* 1961; Jiji *et al.* 1963; Scott *et al.* 1965; Ferrari & Pajola 1981), with the change generally involving the erythroblast, but granulocytes, megakaryocytes and plasma cells may also be affected. Schrober *et al.* (1972), reported the induction of vacuoles in the pronormoblasts of rats dosed with 150 mg/kg CAP twice daily for 5 weeks, and Turton *et al.* (2000, 2002) described the formation of TAP-induced vacuoles in the bone marrow precursors of the mouse and in the CAPS-treated guinea pig (Turton *et al.* 2003).

The present experiment was carried out to characterize in detail the myelotoxicity of CAPS administered at high-dose levels, with a view to using repeated cycles of a 5-day dosing period followed by a 16- or 23-day non-dosing recovery

period (i.e. an intermittent dosing regimen), in a later study to investigate the possible development of CAPS-induced AA in the BU-pretreated B6C3F1 mouse. In general terms, all the blood and marrow parameters in the present study returned to normal by 14 days post-dosing at both the 2500 and 3500 mg/kg CAPS dose level. However, the particularly sensitive indices of erythrocyte volume (RDW) and intracellular haemoglobin distribution (HDW) still illustrated some residual effects at day 21 post-dosing, reflecting the earlier perturbation of erythropoiesis. It was therefore considered that in a future experiment with BU-pretreated mice, an intermediate dose level of 3000 mg/kg CAPS would be well tolerated, with repeated 21 day cycles consisting of a 5-day dosing period followed by a 16-day recovery period (Andrews 2000).

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