In conclusion, the calcium channel antagonist verapamil, which partially reverses multiple drug resistance properties exhibited by tumour cells, also reverses the resistance of Adriamycin-resistant P388 cells to positively charged 9-anilinoacridine derivatives related to amsacrine. In addition, it strongly increases the frameshift mutagenicity of these compounds in the Ames' test while decreasing the production of respiratory deficient "petite" mutants in yeast. These results suggest a similarity of microbial and mammalian cell mechanisms for the transport of positively charged DNA binding drugs. One implication of these results is that compounds which are effectively excluded by bacteria may be non-mutagenic (Ames' test) in the absence of verapamil but mutagenic in its presence. Whether this also applies to mammalian cells remains to be determined. Such a mechanism would have major implications in clinical medicine, where verapamil is in common use.

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# Production of superoxide anion radicals during the oxidative metabolism of amino-chloramphenicol

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Therapeutic use of the antibiotic chloramphenicol (CAP*) is limited due to the potential occurrence of reversible hematopoietic depression and irreversible aplastic anemia [1]. The mechanism by which CAP causes aplastic anemia has yet to be elucidated, but reactive metabolites of the *p*-nitro moiety are thought to be involved [2, 3]. Among such metabolites, nitroso-chloramphenicol (NO-CAP) has received the most attention as a possible mediator of CAP-induced aplastic anemia [3–5]. Two major pathways have been proposed for the production of the NO-CAP reactive intermediate from the parent compound. In the first pathway, NO-CAP is thought to be produced during nitro-reduction of CAP to amino-CAP ($NH_2$-CAP); this could possibly occur in the intestine, liver, and/or bone marrow. Based on current experimental evidence, it is doubtful, however, whether any NO-CAP produced in the liver could accumulate and then be transported, in the unreacted form, to the bone marrow [6–8]. Possible CAP reduction in rat bone marrow has also been examined but was not detectable [4].

An alternative pathway for the *in vivo* production of NO-CAP involves the reduction of CAP to $NH_2$-CAP and subsequent oxidative metabolism of the compound in the liver and/or bone marrow. Amino-CAP is a known metabolite of CAP and has been isolated from many species including man [9–11]. In man, reduction mainly occurs in the liver, whereas the intestine is the major site for the rat and guinea pig [9–11]. Ascherl *et al.* recently reported that $NH_2$-CAP can be oxidatively metabolized by rat liver microsomes to NHOH-CAP [8]. Evidence for the toxicity of NHOH-CAP is scant, although under aerobic conditions some covalent binding to microsomal protein does occur [8]. An alternative mechanism to covalent binding by which the metabolism of aromatic amines may give rise to reactive intermediates is free radical formation [12]. In the present investigation we have examined the production of free radicals during the metabolism of $NH_2$-CAP by rat liver microsomes.

## *Materials and methods*

Chloramphenicol, cytochrome *c* (horse heart; type VI), catalase (bovine liver; EC 1.11.1.6), xanthine oxidase (buttermilk; grade I; EC 1.2.3.2), peroxidase (horseradish; type II; EC 1.11.1.7), chloroperoxidase (EC 1.11.1.10), superoxide dismutase (bovine liver; EC 1.15.1.1), NADPH (type III), succinic anhydride, Trizma base, and xanthine (grade V) were obtained from the Sigma Chemical Co., St.

* Abbreviations: CAP, chloramphenicol; NO-CAP, nitroso-chloramphenicol; NHOH-CAP, hydroxylamino-chloramphenicol; $NH_2$-CAP, amino-chloramphenicol; PB, phenobarbital; BNF, beta-naphthoflavone; ANF, alpha-naphthoflavone; SFCC, succinoylated ferricytochrome *c*; and SOD, superoxide dismutase.

Louis, MO. Isosafrole was from ICN Pharmaceuticals Inc., Fair Lawn, NJ; beta-naphthoflavone, alpha-naphthoflavone, and sodium pentacyanoamino ferroate (ammonium disodium salt) were purchased from the Aldrich Chemical Co., Milwaukee, WI. Silica gel thin-layer plates (Si250F-PA19c) were purchased from J. T. Baker, Phillipsburg, NJ. Reverse-phase plates (RP-18 F254s, 0.25 mm) were obtained from EM Science, Gibbstown, NJ. All solvents were analytical grade and came from Burdick & Jackson Laboratories Inc., Muskegon, MI, and Fisher Scientific, Fair Lawn, NJ. Corn oil was obtained commercially (Mazola).

Synthetic NO-CAP and $NH_2$-CAP were prepared as described previously [4], and NHOH-CAP was synthesized according to Corbett and Chipko [13]. The $R_f$ values of these compounds as well as CAP itself in two thin-layer chromatography systems were as follows: (1) silica gel; $CHCl_3$–MeOH (90:10, v/v); CAP, 0.31; NO-CAP, 0.30; NHOH-CAP, 0.11; $NH_2$-CAP, 0.19, and (2) RP-18; MeOH–$H_2O$–$CH_3COOH$ (69:30:1, by vol.); CAP, 0.55; NO-CAP, 0.56; NHOH-CAP, 0.74; $NH_2$-CAP, 0.68.

Partially succinoylated ferricytochrome *c* (SFCC) was synthesized according to Kuthan *et al.* [14]. A solution consisting of 49.3 mg of cytochrome *c* in 20.0 ml of cold 0.03 M potassium phosphate buffer at pH 7.6 was first prepared. To it was added 21.0 mg of succinic anhydride. The mixture was kept at pH 7.6 by titration with 2 M KOH, stirred in an ice-water mixture for 1 hr, and then dialyzed against 1 liter of 0.1 mM EDTA at 4°. After 6 hr, the EDTA solution was changed, and the mixture was dialyzed overnight. The SFCC was reduced at only 2% the rate of native cytochrome *c* when incubated in 300 mM potassium phosphate buffer, pH 7.7, with purified NADPH-cytochrome P-450 reductase, prepared as described previously [15].

*Results and discussion*

Addition of $NH_2$-CAP to rat liver microsomes in the presence of NADPH caused an enhanced production of free radicals capable of reducing SFCC (see Table 1). Only two-thirds of the enhanced SFCC reduction was blocked by the addition of 0.70 or 1.40 μM superoxide dismutase, although these concentrations of superoxide dismutase were sufficient to completely block free radical production catalyzed by xanthine oxidase (20 μg/ml) in the presence of 0.2 mM xanthine (data not shown). These results suggest that one-third of the SFCC reduction in the microsomal system could be due to radical species other than superoxide produced from the oxidative metabolism of $NH_2$-CAP.

To determine whether the major hepatic cytochrome P-450 isozyme(s) might be involved in the oxidative metabolism of $NH_2$-CAP, three major isozymes were induced in the rat. Isosafrole mainly induces the cytochrome P-450d isozyme along with some P-450c; BNF preferentially induces the P-450c isozyme relative to the P-450d; and phenobarbital mainly induces the P-450b isozyme [16, 17]. Both isozymes cytochrome P-450c and P-450d have been implicated in the metabolic activation of a number of aromatic amines [18]. As seen in Table 1, control, isosafrole- and BNF-induced microsomes catalyzed significantly faster SFCC reduction in the presence than in the absence of $NH_2$-CAP. There was no significant enhancement of SFCC reduction by $NH_2$-CAP with phenobarbital microsomes (data not shown). This could be due to a phenobarbital-mediated decrease in the levels of the enzyme that metabolizes $NH_2$-CAP, or to the high levels of endogenous superoxide radicals in these microsomes, such that the relatively small amount of superoxides produced from the metabolism of $NH_2$-CAP is masked. Since the control microsomes metabolized $NH_2$-CAP as efficiently as the BNF and isosafrole microsomes, it is unlikely that the cytochrome P-450 isozymes induced by these compounds are responsible for the metabolism observed. The lack of involvement of P-450c was confirmed by the finding that addition of the selective inhibitor, alpha-naphthoflavone (ANF, 0.014 mM), caused no decrease in the rate of SFCC reduction catalyzed by BNF-microsomes in the presence of $NH_2$-CAP, although this concentration of ANF decreased the ethoxycoumarin deethylase activity of similar microsomes by 70%.

Autoxidation studies were done on $NH_2$-CAP, NHOH-CAP, and NO-CAP to determine if superoxide radicals were spontaneously generated. Only the NHOH-CAP reference compound was found to spontaneously reduce SFCC in the absence of NADPH and microsomes. Ascherl *et al.* [8] recently reported that NO-CAP can be reduced

Table 1. Reduction of succinoylated ferricytochrome *c* (SFCC) by rat liver microsomes in the presence and absence of amino-chloramphenicol

| Microsomes | Cytochrome P-450 content (nmoles/mg protein) | (nmoles SFCC reduced/min/mg protein) | | |
|---|---|---|---|---|
| | | +$NH_2$-CAP | −$NH_2$-CAP | Δ |
| Control | 0.7 ± 0.1 | 2.0 ± 0.2* | 1.0 ± 0.2 | 1.0 ± 0.1 |
| Isosafrole | 0.9 ± 0.1 | 3.0 ± 0.3* | 1.9 ± 0.3 | 1.1 ± 0.4 |
| BNF | 1.3 ± 0.1 | 2.6 ± 0.5* | 0.9 ± 0.1 | 1.7 ± 0.6 |

Male Sprague–Dawley rats (170–190 g) were obtained from Charles River Breeding Laboratory, Wilmington, MA. BNF (40 mg/kg) or isosafrole (150 mg/kg) was administered intraperitoneally in 0.45 ml corn oil once daily for 3 days. Controls received corn oil only. Individual liver microsomes were prepared from four animals in each group as described previously [19]. Protein was determined by the method of Lowry *et al.* [20] and cytochrome P-450 according to Omura and Sato [21]. Reactions were performed at 25° under an air atmosphere. Each incubation mixture contained 1.4 mM $NH_2$-CAP, 0.2 mg/ml microsomal protein, 50 μM SFCC, 1200 units of catalase/ml, 0.1 mM EDTA, and 70 mM Tris–HCl, pH 7.7. The reduction was initiated by the addition of 0.2 mM NADPH and was monitored at 550 nm using an extinction coefficient of 21 $mM^{-1}$ $cm^{-1}$. No SFCC reduction was observed in the absence of microsomes or of NADPH. Statistical analysis of SFCC reduction was conducted using a 3 × 2 factorial analysis of variance (ANOVA); subsequent comparisons between individual means were conducted using the Newman–Keuls' multiple-range tests. Values are means ± SEM of four microsomal samples.

* Statistically significant ($P < 0.05$) compared with incubations lacking $NH_2$-CAP.

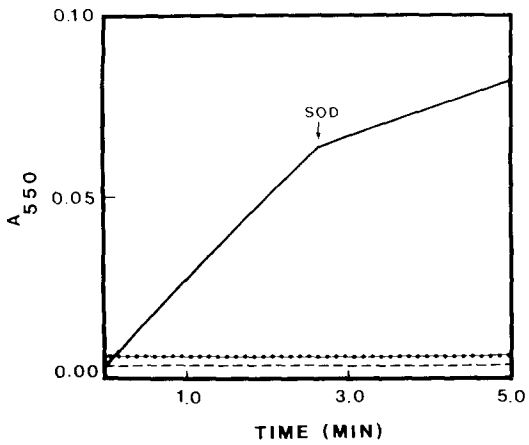


Fig. 1. Reduction of succinoylated ferricytochrome *c* (SFCC) by NO-CAP in the presence of NADPH. Incubations of 1 mM NADPH (––) or 0.05 mM NO-CAP (●●) and of both together (—) were done at 25° in 0.2 M sodium potassium phosphate, pH 8.2. Superoxide dismutase (SOD), 0.70 μM, was added as indicated.

by NADPH to NHOH-CAP. However, they did not determine if superoxide radicals were also produced. We confirmed by spectral measurements and by thin-layer chromotography that NHOH-CAP (237 nm) is produced from NO-CAP reduction by NADPH. Furthermore, Fig. 1 shows that, while NADPH and NO-CAP each had no effect on the SFCC, both compounds together caused a rapid reduction of the cytochrome *c* derivative. Addition of 0.70 μM SOD to the NO-CAP–NADPH incubation mixture blocked 65% of the SFCC reduction, again indicating the production of another species besides superoxide capable of reducing the SFCC. This amount of inhibition by SOD was the same as that in the earlier microsomal incubation studies, suggesting that the production of free radicals from NHOH-CAP in both the microsomal incubation and NO-CAP–NADPH studies involved similar mechanisms. The presence of Cu(II) had no effect on SFCC reduction (data not shown), although this metal ion is required for DNA degradation by the NO-CAP–NADPH system [5].

In summary, oxidative metabolism of $NH_2$-CAP by rat liver microsomes was found to produce superoxide anion radicals. Such superoxide formation was not enhanced by pretreatment of the animals with phenobarbital, β-naphthoflavone, or isosafrole, suggesting no major role for cytochrome P-450 isozymes P-450b, P-450c, or P-450d. Superoxide was also produced during the spontaneous autoxidation of the NHOH-CAP metabolite as well as during the chemical reduction of NO-CAP by NADPH. The oxidative metabolism of $NH_2$-CAP to NHOH-CAP and NO-CAP and the reduction of these metabolites back to the arylamine constitute a redox cycle which produces superoxide radicals. Subsequent formation of hydroxyl radicals could account for the known ability of nitro-reduction products of chloramphenicol to damage DNA [5].

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