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CHLORAMPHENICOL: Relation of Structure to Activity and Toxicity

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INTRODUCTION

The availability of an increasing number of effective broad spectrum antimicrobials in the past decade has diminished considerably the clinical indications for chloramphenicol (CAP). However, interest in this antibiotic has recently been revived both because of its antimicrobial activity and its toxicity. Some factors affecting its resurgence are the emergence of ampicillin-resistant *Halmophilus influenzae,* the superiority of CAP in fighting certain anaerobic infections and infections of the central nervous system, and the development of sensitive assays for the antibiotic and some of its metabolites in body fluids. Greater attention, however, has focused on hematotoxicity from CAP and its pathogenesis. The extensive use of a CAP analogue, thiamphenicol (TAP), in Europe and the Far East, without an increase in the incidence of associated aplastic anemia, has revived interest in the structure-toxicity relationship in the CAP molecule and has focused attention on the *p*-NO_2 group as the structural feature that probably underlies the development of bone marrow aplasia in association with administration of CAP. TAP, with a similar antimicrobial spectrum, has emerged as a challenging substitute to CAP. In this chapter the comparative metabolism and toxicity of CAP and TAP are reviewed with particular emphasis on the role of the *p*-NO_2 group. For a clearer perspective, the properties of CAP, its mechanism of action, and relation of structure to activity and to bacterial resistance are briefly considered.

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ANTIMICROBIAL ACTION OF CAP

Chemistry of CAP and Spectrum of Activity

CAP (D-(−)-threo-1-*p*-nitrophenyl-2-dichloroacetamido 1,3-propanediol) occurs as fine needle crystals freely soluble in ethanol but slightly soluble in water. The molecule has two centers of asymmetry: The first and second carbon atoms of the propanediol chain allow the existence of two pairs of diastereoisomers, the erythro and the threo configurations. Only the D(−) threo stereoisomer has biological activity (1, 2). CAP is active against gram-positive and gram negative organisms and against rickettsia, mycoplasma, and chlamydia. Susceptible organisms (inhibited by ≤ 10 μg/ml) include *Streptococcus pneumoniae,* group A and B hemolytic strep, *Streptococcus viridans,* enterococci, neisseria, hemophilus species, salmonella, and obligate anaerobes. CAP is bacteriostatic but is bacteriocidal for *Hamophilus influenzae, Neisseria meningitidis,* and *S. pneumoniae* (3–5). Current indications for CAP include typhoid fever and the various salmonella infections, anaerobic infections with *Bacteroides fragilis,* rickettsial infections, and bacterial meningitis. CAP has been the drug of choice for the treatment of *H. influenzae* meningitis, especially if the organism is a B-lactamase producer (6–8). A desirable property of CAP is its rapid accessibility to the central nervous system, with significant drug levels attained in cerebrospinal fluid, ventricular fluid (9, 10), and brain tissue including brain abscess (11, 12).

MECHANISM OF ACTION In sensitive bacteria, CAP inhibits peptide bond synthesis at the 50S ribosomal subunit by interfering with peptidyl transferase (13–15). The exact mechanism of CAP-ribosomal interaction remains somewhat uncertain. Both the crystal and solution structures of the drug are folded V-shaped molecules with C_1, C_2, and C_3 at the base of the V; the notrophenyl and -$COCHCl_2$ regions are located toward the end of the wings (16, 17). From this and a nuclear magnetic resonance study of CAP-ribosome interaction, a model has been proposed (17) in which the point of the V fits into the receptor region of the ribosome; immobilization of the central carbons of the molecule provides two faces of interaction. The model thus allows at least two recognition mechanisms and provides the necessary accuracy required to form a highly specific drug-receptor complex. This model is also consistent with the structure-activity relationship in the molecule (see below).

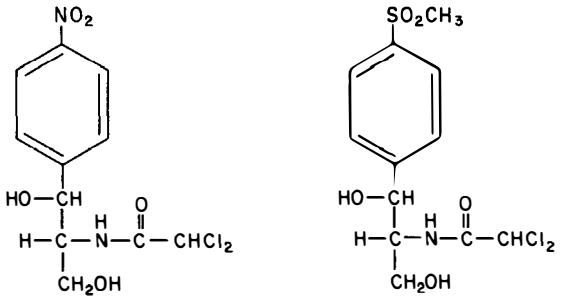
STRUCTURE-ACTIVITY RELATIONSHIP CAP has three functional groups that determine its biological activity (Figure 1): the *p*-NO_2 group, the dichloroacetyl moiety, and the primary alcoholic group at carbon 3 of the propanediol chain. The electronegativity of the *p*-NO_2 group is essential for the proper conformation. It is possible to replace the *p*-NO_2 with other

electronegative groups without drastic effects on conformation or biological activity. An example is the $H_3C\text{-}SO_2$ group in thiamphenicol (18) (Figure 1).

The methylsulfonyl group of TAP allows conformational changes in the molecule, as in CAP, and, therefore, an intact drug-ribosomal interaction. Thus TAP inhibits bacterial ribosomal protein synthesis by a similar mechanism and shares identical binding sites on the 50S ribosomal subunit (19). A number of other analogues have been synthesized in which the *p*-NO_2 has been substituted. In general, all analogues in which the substitute group is electronegative retained antimicrobial activity though at a reduced level. Except for TAP, however, none has undergone sufficient evaluation to merit clinical use (18). Metabolites of CAP in which the *p*-NO_2 is replaced with nitroso (–N = O), hydroxylamine (–NHOH), hydroxamic acid [–N(OH)–$COCH_3$], O-methylhydroxamate ester [–N(OCH_3)–$COCH_3$], and O-acetylhydroxamate [–N($OCOCH_3$)–$COCH_3$] are either an order of magnitude less active than CAP or totally inactive (20). Nitroreduction of CAP to the amino derivative results in loss of biological activity.

An intact propanediol moiety is required for full biological activity (21, 22). Consistent with Tritton's model (17), alterations in the propanediol portion of the molecules at carbons 1, 2, and 3 (CAP-ribosomes interaction site) generally lead to a loss in biological activity. However, this is not a rigid requirement since replacement of the alcoholic group at carbon 3 has been done with retention of biological activity (18). Thus, fluorinated derivatives of CAP and TAP have been prepared in which the primary alcoholic group on carbon 3 has been replaced by fluorine (F). These derivatives not only retained biological activity but were also active against CAP-resistant organisms (23) (see below).

The dichloroacetyl side chain is important for biological activity but may be replaced by other acetyl side chains. Removal of the side chain altogether results in loss of activity (18).



CHLORAMPHENICOL THIAMPHENICOL

Figure 1 The structure of chloramphenicol and thiamphenicol.

SOME DISTINCTIVE PROPERTIES OF TAP The substitution of the p-NO_2 group with a methylsulfonyl moiety in TAP confers on the molecule some distinctive pharmacokinetic properties. Whereas CAP is largely conjugated in the liver to the glucuronide derivative, TAP does not undergo glucuronidation; glucuronyl transferase is inactive with TAP as substrate (24). Thus over 60% of a given TAP dose is excreted in the urine as the unchanged active compound (25). The half-life of TAP is twice as long as that of CAP in normal rats. The half-life of CAP but not of TAP is prolonged after hepatectomy and shortened by enzymatic induction with phenobarbital (24). The half-life of TAP is prolonged in renal failure. The higher polarity of the methylsulfonyl moiety renders TAP more soluble in water but less soluble in lipids and therefore more slowly diffusible into lipid membranes than CAP (18, 26).

CAP-RESISTANCE AND MECHANISMS At least four mechanisms of bacterial resistance to CAP have been described. The most important one is the plasmid-mediated transmissible resistance conferred by the presence in resistant bacteria of CAP acetyltransferase (CAT), which catalyzes the acetyl-CoA dependent acetylation of CAP at the hydroxyl group on carbon 3 position (27–30). The enzymology and molecular biology of CAT have recently been reviewed (31). Like CAP, TAP is also inactiviated by CAT-carrying bacteria; acetylation yields 1,3-diacetoxyl, but the affinity of the enzyme appears to be greater for CAP (24). Derivatives of CAP and TAP in which the –OH group at carbon 3 is replaced by F are resistant to CAT and are therefore active against CAT-producing resistant bacteria (23).

Resistance due to altered bacterial permeability to CAP has been described in *Escherichia coli, H. influenzae,* and *Pseudomonas aeruginosa* (32–34). Both R-plasmid and chromosomally mediated resistance described in *E. coli* and *P. aeruginosa* are thought to operate via decreased uptake of CAP. Burns et al (32) described an apparently chromosomally mediated resistance determinant in *H. influenzae* conferring decreased CAP uptake and a decreased 40-kd protein in the outer membrane. More recently these authors reported the cloning of a transposon (Tn*1696*)-mediated resistance gene from *P. aeruginosa* plasmid, which encodes for the expression in *E. coli* of a permeability barrier to CAP (35). This was associated with the loss of a 50-kd protein from the outer membrane. They further found DNA homology between Tn*1696* and the CAP-resistant isolate of *H. influenzae,* suggesting a common ancestral origin for the resistance gene.

CAP resistance at the ribosomal level has also been described (36–38). Altered binding of CAP to the 50S ribosomal subunit of *Bacillus subtilis* has been reported (36).

An additional mechanism of CAP resistance is inactivation by nitroreduction, particularly in anaerobic organisms. Failure of CAP therapy for serious

B. fragilis infection in humans has been attributed to this mechanism. CAP can be rapidly inactivated in vitro by bacteroides and clostidium (39). However, some strains of *B. Fragilis* can also inactivate CAP by the CAT system (40). Since both mechanisms exist in bacteroides, the relative significance of each mechanism in clinical resistance is not known, but both may contribute to treatment failure noted in some patients with intraabdominal or brain abscesses (41). In one study (42), CAP was inactivated by 19 strains of *B. fragilis,* none of which were capable of inactivating TAP; this suggests that nitroreduction is a more important mechanism of inactivation than CAT in these organisms.

The clinical significance of CAP resistance is the subject of several recent reviews (43–45). In addition to indiscriminate clinical use of CAP in humans as a major factor in the spread of CAP resistance, animal-to-human transmission of CAP-resistant organisms, particularly salmonella, has been noted (46–49).

CHLORAMPHENICOL TOXICITY

Types of Hematotoxicity

The major toxicity of CAP involves the hematopoietic system. Two types of hematoxicity have been clearly delineated (50, 51): the common, dose-related, reversible bone marrow suppression affecting primarily the erythroid series and usually occurring at CAP blood levels of ≥ 25 μg/ml (52), and the rare but devastating complication of bone marrow aplasia characterized by pancytopenia, lack of dose-effect relationship, and often fatal outcome as a result of hemorrhage and/or infection. Signficant progress has been made in the last decade in our understanding of the pathogenesis of these two types of toxicity, and much of this progress derives from comparative studies involving CAP and TAP. The latter produces reversible bone marrow suppression but is not associated with increased incidence of aplastic anemia (53, 54).

Actions of CAP and TAP in Mammalian Cells

In order to attribute any clinical significance to a given in vitro metabolic effect of CAP, such effect must be demonstrated at concentrations that fall within therapeutic range (20–60 μg/ml or $0.5–2 \times 10^{-4}$ M). Among the extensive number of metabolic parameters tested, only mitochondrial protein synthesis is sensitive to these concentrations (55), the same concentrations that inhibit bacterial ribosomal protein synthesis. A similar degree of inhibition can be demonstrated by TAP (56–59). Inhibition of mitochondrial protein synthesis by CAP is not secondary to inhibition of respiration, since concentrations of $> 3 \times 10^{-4}$ M are needed to inhibit the latter (60, 61). Comparable levels of TAP do not inhibit mitochondrial respiration. Inhibition of mitochondrial respiration by CAP has been offered as the most likely

mechanism for the Grey syndrome (62), observed in newborns, in which high CAP levels are encountered because of defective glucuronidation (63).

Other metabolic parameters in mammalian cells (ribosomal protein synthesis, DNA synthesis, etc) are relatively resistant to CAP; for significant inhibition to occur, concentrations that are 10–20-fold therapeutic levels are required (55).

Pathogenesis of Reversible Bone Marrow Suppression by CAP

Abundant evidence indicates that reversible bone marrow suppression from CAP is a consequence of mitochondrial injury (64–67). In concentrations as low as 10 μg/ml, CAP causes profound inhibition of mitochondrial protein synthesis in bone marrow (64). When given to patients in large doses, CAP produces in mitochondria an ultrastructural lesion that, like clinical bone marrow suppression, occurs concurrently with drug therapy, is directly related to serum levels of CAP, and is likewise reversible (66). Reversibility could also be demonstrated at the mitochondrial level in vitro; restoration of protein synthesis was observed after the drug was removed by mitochondrial washing (D. R. Manyan A. A. Yunis, unpublished).

TAP is equally potent as an inhibitor of mitochondrial protein synthesis (56, 57, 59), which is consistent with its ability to produce clinical reversible bone marrow suppression as readily as CAP (53).

Because CAP and TAP inhibit specifically the synthesis of mitochondrial membranous proteins, suppressed synthesis of important membrane-associated enzymes such as cytochromes a + a_3 and b ultimately leads to suppressed mitochondrial respiration, compromised cellular synthetic machinery, and cessation of cellular proliferation. Inhibition of cellular proliferation by CAP and/or TAP can be demonstrated in a variety of in vitro culture systems including HeLa cells (58) and bone marrow (68–71).

Both CAP and TAP inhibit murine and human myeloid colony (CFU-GM) growth at concentrations within therapeutic range. The inhibition is drug concentration dependent and is completely reversible upon removal of the drug after 8 hr of cell-drug exposure (69). In addition, in both murine and human bone marrow, the degree of inhibition of CFU-GM growth by a given CAP concentration is inversely related to the level of colony stimulating factor (CSF) in the culture medium (69), e.g. inhibition is reversed by increasing the level of CSF. These observations suggest that the level of CSF in the cell milieu in vivo may determine the occurrence and/or degree of granulocytopenia from CAP.

Similarly, CAP inhibits murine and human erythroid colony (CFU-E) growth in a stereospecific, concentration-dependent manner (70). However, an important difference appears to be the greater sensitivity of human CFU-E growth to CAP, with virtually complete inhibition occurring at 10 μg/ml

($\sim 0.3 \times 10^{-4}$ M). Furthermore, the degree of inhibition of CFU-E growth is unaffected by the level of erythropoietin (EPO) in the culture medium; this is in sharp contrast to the protective effect of CSF on CFU-GM. The greater in vitro sensitivity of CFU-E to CAP and the lack of protection by EPO are consistent with the known greater vulnerability of erythroid precursors to CAP in vivo.

Biochemical Mechanisms Underlying Erythroid Sensitivity to CAP

Although the known in vivo vulnerability of erythroid precursors to CAP can also be demonstrated in vitro, the biochemical mechanism for the erythroid sensitivity remains uncertain. Suppressed synthesis of ferrochelatase, a mitrochondrial membrane-associated enzyme, and consequent block in heme synthesis has been proposed as a contributing factor (72, 73).

Since mitochondrial protein synthesis is selectively blocked by CAP, it is possibile that the difference in sensitivity between erythroid and myeloid cells resides at the mitochondrial level. However, using pure erythroid and myeloid tissues as models, no difference could be detected in the sensitivity of mitochondrial protein synthesis to CAP (74). On the other hand, when exogenous amino acids were omitted from the reaction mixture, erythroid mitochondria were more sensitive to a given CAP concentration than myeloid mitochondria (75), suggesting that mitochondrial amino acid pool may be involved in the greater sensitivity of erythroid precursors to CAP. Certain amino acids (glycine, serine, histidine) were present in higher concentations in erythroid vs myeloid mitochondria (76). Furthermore, when either serine or glycine was added to myeloid mitochondria, sensitivity to CAP was enhanced from 14 to 50%, whereas their addition to myeloid mitochondria was without effect (76). These observations suggested that erythroid cell sensitivity to CAP may be determined by the mitochondrial serine-glycine pool. Since serine and glycine are interconvertible and since glycine is a key reactant in the biosynthetic pathway of heme, the sensitivity of erythroid cells to CAP may somehow be related to heme biosynthesis, a question that deserves further study.

Pathogenesis of Aplastic Anemia from CAP

Aplastic anemia from CAP is rare, occurring in 1/10–45,000 of the exposed population (77, 78), and has no relation to dose or duration of therapy (50), which suggests an individual predisposition. The occurrence of this complication in identical twins (79) further suggests a genetically determined predisposition of CAP-induced aplastic anemia.

Studies of the pathogenetic mechanisms of CAP-induced aplastic anemia are severely limited, primarily because of the rarity and unpredictability of

this complication and the lack of a suitable experimental model. In the last 10 years, progress in this area was triggered by comparative studies with TAP and the development of state-of-the-art-technology for the isolation and identification of metabolic intermediates.

Initial comparative studies of CAP and TAP yielded some intriguing observations. Both CAP and TAP were equipotent as inhibitors of mitochondrial protein synthesis, which was consistent with their ability to suppress bone marrow reversibly. In contrast to CAP, however, which inhibits DNA synthesis when used at high concentrations ($\geq 10^{-3}$ M), TAP has no significant effect on DNA synthesis (57, 80). Examination of a number of analogues with various substitutes of the p-NO_2 group (81) suggested that the p-NO_2 group conferred on the CAP molecule the capacity to inhibit DNA synthesis. Other differences between TAP and CAP included more rapid cellular and mitochondrial uptake of CAP and greater intracellular covalent binding (82). On the basis of these early studies, Yunis et al (83) hypothesized: "The p-NO_2 group of CAP is the structural feature underlying aplastic anemia from CAP. In the predisposed subject the p-NO_2 group undergoes nitroreduction leading to the production of toxic intermediates (nitroso, hydroxylamine) resulting in stem cell damage."

Cellular Toxicity of Nitroso-CAP

In order to explore the above hypothesis, extensive studies were carried out on the metabolic effects of nitroso-CAP in vitro (83–93). The results indicated that nitroso-CAP is highly cytotoxic. In micromolar concentrations, it inhibits myeloid (CFU-GM) growth irreversibly (83) and arrests cells in the G^2M phase of cell cycle (83), which causes extensive cell death, because cells in DNA synthesis are more sensitive to the lethal effects of the drug (86). Additional effects include inhibition of proton translocation in mitochondria (88) and inhibition of mitochodrial DNA polymerase activity (90). Nitroso-CAP rapidly undergoes covalent binding to intracellular macromolecular components (85).

Inhibition of DNA Synthesis and the Induction of DNA Damage

In contrast to CAP, nitroso-CAP in small concentrations inhibits DNA synthesis with significant inhibition occurring at 5×10^{-5} M (83, 94). In the presence of NADH and copper, nitroso-CAP causes the hydrolysis of isolated *E. coli* DNA in vitro in a concentration-dependent manner: significant hydrolysis occurrs at 5 μM and is completed at 100 μM (87). At high concentrations, similar to those required for inhibition of DNA synthesis ($\geq 10^{-3}$ M), CAP also causes hydrolysis of isolated DNA under the same conditions (89) (Figure 2). Thiamphenicol, however, lacking the p-NO_2 group is incapable of damaging DNA.

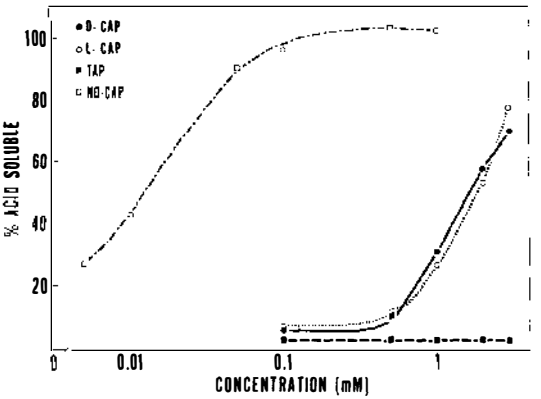


Figure 2 DNA degradation as a function of drug concentration. Double-stranded [^{3}H]-DNA (7 μg) was incubated for 60 min at 37°C in the presence of 100 μM $CuCl_2$, 5 mM NADH, 100 mM potassium phosphate, pH 8.0, and the indicated amount of CAP, L-CAP, TAP, or NO-CAP. Points represent the mean of triplicate determination. [Reprinted from Murray et al (89) by permission from the publishers.]

In order to ascribe any clinical significance to DNA damage by nitroso-CAP, one must be able to demonstrate damage in intact cells. Studies in both activated normal human lymphocytes and cultured Raji cells (92) showed that nitroso-CAP in concentrations as low as 2×10^{-5} M induces DNA single strand breaks after 3 hr of drug-cell exposure (Figure 3), as determined by the alkaline elution technique (95). Only a slight effect was observed from CAP at the high concentration of 2 mM; no effect was observed from TAP.

Mechanism of DNA Damage

In studying degradation of DNA by nitroso-CAP, Murray et al (87) observed reduced DNA degradation in the absence of O_2, suggesting that some form of oxygen plays a role in the process. Furthermore, strong inhibition of the reaction by catalase indicated a role for H_2O_2 in the DNA cleavage. In the presence of copper, which is required for the reaction, H_2O_2 can serve both as an oxidant and a source of hydroxyl radicals through a Fenton type reaction in which complexed Cu (I) is oxidized by H_2O_2 (96, 97). Protection of the DNA from damage by agents that can scavenge hydroxyl radicals suggests that hydroxyl radicals may also be involved in the strand scission. An alternate explanation for O_2 requirement is the oxidation of the –N = O to the one-electron radical anion as the direct damaging agent (98).

Both damage to isolated DNA and to DNA in intact cells from nitroso-CAP can be blocked by sulfhydryl compounds such as glutathione and N-acetylcysteine, probably by direct interaction of the thiol with the –N = O,

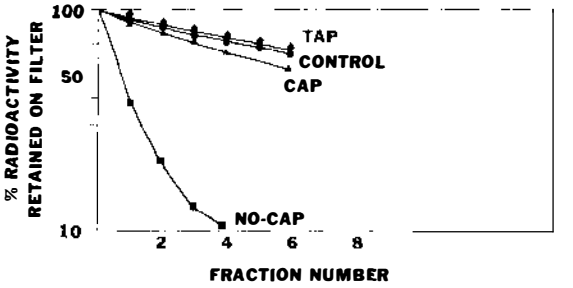


Figure 3 Comparative effects of CAP (2 mM), TAP (2 mM), and NO-CAP (0.1 mM) on the alkaline elution profile of DNA of phytohemagglutinin-stimulated normal human lymphocytes. [Reprinted from Yunis et al (92) by permission from the publishers.]

since the interaction of aromatic nitroso groups with glutathione is known to result in inactivation (99).

Evidence relating nitroreduction to DNA damage comes from several systems. Bacterial DNA damage from CAP occurs only when bacteria can reduce the *p*-NO_2 group (100). DNA damage is observed during electrochemical reduction of CAP (101). Correlation between DNA damage, cytotoxicity, and mutagenicity of nitrocompounds with their reduction potential and/or electron affinity has been described (102, 103). The mutagenic activity of metronidazol (Flagyl) has been attributed to its nitroreduction product (104, 105).

Taken together, the above studies lend further support to our hypothesis that nitroreduction is the key mechanism in the induction of aplastic anemia by CAP. However, for such a hypothesis to be tenable, evidence for nitroreduction of CAP or one of its metabolites by mammalian, and more specifically human tissues, must be provided.

Metabolism of CAP by Mammalian Tissues

Most studies on the metabolism of CAP in vitro and in vivo have been carried out using the rat. In the rat, CAP is excreted in the stools largely in the form of arylamines. The 9000 × g supernatant from rat liver homogenates will catalyze the nitroreduction of CAP under anaerobic conditions in vitro (106, 107), whereas the incubation of rat hepatocytes with CAP under aerobic conditions in vitro yields no detectable arylamines in the supernatant, the major product being the glucuronide (108). Similar observations have been made with human liver tissue, e.g. CAP reduction by the 9000 × g supernatant under anaerobic conditions (109), but there is no evidence of reduction by cultured human hepatocytes under aerobic conditions (M. Isildar and A. Yunis, unpublished results). These latter observations suggest that nitroreduction in vivo must take place in the intestinal tract catalyzed by

microbial nitroreductases. More recently, the metabolic disposition of an orally administered single dose of tritiated CAP was studied in conventional and germ-free rats using the more sensitive HPLC analysis (110). Rapid absorption, hepatic glucuronide conjugation, and biliary excretion of the conjugate were observed. CAP, CAP-oxamic acid, CAP-alcohol, and CAP-base were present in the urine in similar proportions in both the conventional and germ-free rats. Reduction products were present in much greater quantities in the urine and feces of conventional rats; this finding was consistent with a pathway of glucuronidation, biliary excretion, hydrolysis, and nitroreduction in the gut. However, in the germ-free rats, reduction products in the urine could still be detected, which suggests that some reduction was taking place independent of intestinal bacteria (the source of this reduction is undetermined).

The relevance of these results in the rat to the metabolism of CAP in man is uncertain. Data generated over 25 years ago indicated that 90 percent of a given CAP dose in man is excreted in the urine largely in the form of glucuronide conjugate. The original methodology for those studies used colorimetric and microbiological assays that are now obsolete. The application of the powerful and sensitive separation techniques of HPLC to the study of CAP metabolism has just begun, and data thus far are limited. A recent study (111) indicates that it should be possible to separate and identify CAP metabolites in urine of rat, goat, and human using simple and ion pair reverse phase HPLC combined with selective extraction of urine after the administration of ^{3}H-CAP. In this study, CAP glucuronide and CAP base were predominant in human urine, but there also were a number of unidentified peaks.

Clearly, whereas the toxicity of nitroreduction intermediates and the relationship of nitroreduction to DNA damage have been established, there is as yet no clear evidence that CAP nitroreduction takes place in vivo in animals or humans without the presence of intestinal bacteria.

For a candidate CAP intermediate to effectively mediate bone marrow damage, it must be produced in the marrow itself or must be transported in stable form to the bone marrow. Because of its extreme instability, it is unlikely that an intermediate such as nitroso-CAP can reach its target organ. Thus, in recent studies it was found that the compound disappears within seconds from the blood, and no nitroso-CAP can pass the liver (112, 113). On the other hand, it is possible that CAP can undergo nitroreduction in the marrow, particularly in the predisposed subject with in situ generation of nitroso-CAP. Attempts to demonstrate CAP nitroreduction by human bone marrow have been unsuccessful (A. A. Yunis, unpublished results).

The recent demonstration in our laboratory of a nitrobenzene reductase in rat liver and in human bone marrow mitochondria (114 and unpublished results) that is active under aerobic conditions raised an intriguing possibility:

Structural modification of CAP (such as by metabolic degradation) may render it a better substrate for nitroreduction by bone marrow. The following hypothesis was therefore formulated: One or more stable bacterial metabolites of CAP may find their way to the bone marrow where either they may be directly toxic or they may serve as a better substrate for nitroreduction with in situ production of toxic intermediate(s).

Possible Role of Bacterial Metabolites of CAP in Aplastic Anemia

In order to examine the above hypothesis, a series of CAP metabolites known to be produced by intestinal bacteria (115) were tested with respect to their cytotoxicity and capacity to induce DNA damage in intact cells (116, 117). One of four compounds examined, dehydro-CAP (DH-CAP), was found to be as toxic as nitroso-CAP. Thus in micromolar concentrations DH-CAP inhibits myeloid colony (CFU-GM) growth irreversibly and causes DNA single strand breaks in intact cells as demonstrated in activated normal human lymphocytes, cultured Raji cells, and normal human bone marrow cells. In contrast to nitroso-CAP, which becomes undetectable instantly upon mixing with human blood or liver tissues, DH-CAP is relatively stable (118). Thus after 30 min of incubation 35 and 65% of DH-CAP can be recovered from blood and liver respectively as determined by HPLC analysis (118, 119). Accordingly, any DH-CAP formed elsewhere should find its way to the bone marrow before inactivation. Perhaps the most important aspect of DH-CAP is that, in contrast to CAP, it is readily reduced by human bone marrow homogenates under aerobic conditions, and its nitroreduction is cell concentration dependent. Thus 5×10^{-6} M and $5-6 \times 10^{-5}$ M amino equivalent are generated from 5×10^{7} cells/ml and 2×10^{8} cells/ml respectively as determined by the Bratton Marshall colorimetric reaction. The production of amino-DH-CAP was confirmed by HPLC (unpublished data).

These recent observations suggest that DH-CAP-induced DNA damage in intact human bond marrow cells is mediated by nitroreduction intermediates produced from DH-CAP in situ, although a direct toxic action cannot be excluded. It is clear that both nitroso-CAP and DH-CAP are highly toxic. However, nitroso-CAP is also extremely unstable and, unless formed in the bone marrow in situ, it cannot reach its target. By contrast, DH-CAP is relatively stable. Perhaps more importantly, it can undergo nitroreduction by bone marrow, which presumably generates toxic intermediates. It would therefore appear that the cytotoxicity/genotoxicity of DH-CAP (and possibly other yet unidentified bacterial metabolites of CAP), its relative stability, and its nitroreducibility by bone marrow render it an excellent candidate mediator of CAP-induced aplastic anemia in the predisposed host.

CONCLUDING REMARKS

Chloramphenicol was once rightly considered as one of the most potent and useful antibiotics. Even today in our current antimicrobial armamentarium CAP might still enjoy an important place were it not for its potential serious toxicity. Bone marrow aplasia, though considered rare, is a devastating complication of CAP therapy. Investigations in the past decade have shed considerable light on the pathogenesis of CAP-induced aplastic anemia and have focused attention on the p-NO_2 group as the structural feature underlying the potential mutagenecity/leukemogenecity of CAP. The observation that some CAP metabolites such as DH-CAP (known to be produced by bacteria normally inhabiting the intestinal tract) are over 20-fold more cytotoxic in vitro than CAP itself, suggests that bacterial metabolites play a critical role as mediators of CAP-induced aplastic anemia. This possibility could apply to other potential myelotoxins.

The induction of DNA damage by DH-CAP in intact bone marrow cells and the observation that these cells can catalyze the nitroreduction of DH-CAP suggests that the bone marrow may be both the site of metabolic conversion as well as the target of injury from the nitroreduction intermediates produced in situ. Since CAP-induced aplastic anemia is rare, an individual predisposition must be the determining factor. The predisposed host may generate more bacterial metabolites such as DH-CAP, or his marrow may possess a greater nitroreduction capacity and thus generate more toxic intermediates. Alternatively, the host's stem cells DNA may be inherently more sensitive to the offending metabolite or may have decreased capacity for repair. Whatever the nature of predisposition, it is likely that the occurrence of aplastic anemia requires the production of stable chloramphenicol metabolite, which can serve as substrate for nitroreduction by the host's bone marrow. This could be DH-CAP itself or some other bacterial metabolite. Clearly these recent observations have provided new and exciting avenues for investigation of this important problem. The metabolism of CAP by intestinal microorganisms should be reassessed using state-of-the art technology for isolating metabolites. These metabolites should then be screened for cytotoxicity/genotoxicity, and their metabolic profiling by normal marrow and marrow from predisposed subjects should be investigated. Knowledge derived from these approaches will undoubtedly shed light on the mechanism of myelotoxicity from other agents.

Recognition of the potential role of the p-NO_2 group in CAP toxicity prompted the design of analogues with various p-NO_2 substitutes. Among these, only TAP underwent complete drug assessment and has been in extensive clinical use outside the United States. (TAP is not marketed in the USA.) That TAP is not associated with increased incidence of aplastic

anemia has been clearly indicated by clinical experience and is corroborated by recent experimental observations. Having a similar antibacterial spectrum, TAP therefore offers clear advantages over CAP. The problem of CAT-mediated bacterial resistance is common to both drugs. The synthesis of fluorinated analogues of TAP could provide a potential solution to this problem.

The picture that has emerged about CAP in the past decade leaves little doubt that the era of CAP as an antimicrobial is waning. Currently, there are no absolute indications for administration of CAP. Now that an increasing number of comparably effective broad spectrum antimicrobials are available, the risks of prescribing CAP outweigh the benefits.

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