Enzyme Systems that Metabolise Drugs and Other Xenobiotics.
Edited by Costas Ioannides

ISBNs: 0-471-894-66-4 (Hardback); 0-470-84630-5 (Electronic)

16 Nitroreductases and Azoreductases

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Introduction

Reductive drug metabolism is the least studied biotransformation pathway in comparison to enzymic oxidation, hydrolysis and conjugation. There are numerous thiol, flavin and cytochrome P-450 enzymes that await individual recognition as well as enzymes with metal centres that may serve as electron sources. Reductive drug metabolism is a means by which drug detoxification or activation, that elevates toxicity and mutagenicity, may take place.

Nitro substituents are found in compounds such as industrial solvents, insecticides, food preservatives and other xenobiotics. Azo compounds are widely used as colourants in food, cosmetics, pharmaceuticals and other industries. The azo dyes' colour is derived from conjugated double bonds of aromatic residues through an azo linkage, permitting release of energy in the visible spectrum. The last 10 years of publications associated with the metabolism of nitro compounds and azo dyes, and its implication for both activation and detoxification are discussed in this chapter.

Nitroreductases

NITROREDUCTASES AND ENZYME MULTIPLICITY

Nitro moietices ($-NO_2$) can be reduced by three sets of two electron reductions to nitroso (–NO), hydroxylamine (–NHOH) and finally primary amine ($-NH_2$) (Figure 16.1) (McLane *et al.* 1983). Nitroreductase activity is associated with a diversity of enzymes, including xanthine oxidase (De Castro *et al.* 1990; Nakao *et al.* 1991),

Figure 16.1 Reduction of nitro compounds to primary amine metabolites requiring a total of six electrons. Each reductive step requires two electrons.

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aldehyde oxidase (Belisario *et al.* 1990; Nakao *et al.* 1991), DT-diaphorase (Belisario *et al.* 1990), flavoprotein-enzymes (Bueding and Jolliffe 1946), NADPH-cytochrome P-450 reductase alone (Person *et al.* 1991) and/or in combination with cytochrome P-450s (Belisario *et al.* 1990, 1991). Consequently, multiple mechanisms are involved in nitroreduction activity. The main forms of cytochrome P-450 (CYP) which exhibit nitroreductase activity are associated with CYP1A and CYP3A subfamilies (Berson *et al.* 1993), primarily CYP1A2 (Chae *et al.* 1993; Lehman-McKeeman *et al.* 1997a), CYP3A4 (Chae *et al.* 1993; Seree *et al.* 1993) and CYP2B10 (Lehman-McKeeman *et al.* 1997a) isoforms.

The literature of the past 10 years discusses a variety of nitroreductase biotransformation pathways. Recent examples, in addition to those previously discussed in an excellent review by McLane *et al.* (1983), include studies on the anaerobic reductive metabolism of the hair dye constituent, 2-nitro-1,4-diaminobenzene (2-nitro-*p*-phenylenediamine), in rat liver microsomal and cytosolic fractions (Nakao *et al.* 1991). In the microsomal preparation, both air and carbon monoxide inhibit the nitroreduction of this compound while NADPH is more effective than NADH as an electron donor. This is consistent with the involvement of cytochrome P-450 in microsomal nitroreductase activity. Addition of FMN, together with NADPH and NADH, also elevates nitroreductase activity with 2-nitro-1,4-diaminobenzene. However, activity in the cytosolic fraction is attributed to xanthine oxidase, aldehyde oxidase and other enzymes.

De Castro *et al.* (1990) studied Nifurtimox nitroreductase activity in the microsomal, mitochondrial and cytosolic fractions of rat adrenals. Enzymic activity of all fractions was inhibited by oxygen. Carbon monoxide inhibited only 10% of the microsomal activity but did not affect the mitochondrial activity. Reduction of Nifurtimox in both the microsomal and mitochondrial fractions required NADPH. However, reduction of this drug in the cytosolic fraction required the addition of hypoxanthine and was inhibited by allopurinol, suggesting that xanthine oxidase was involved in its nitroreduction.

Belisario *et al.* (1990) studied the biotransformation of nitrofluoranthenes, mutagenic and carcinogenic environmental pollutants. These compounds undergo both oxidation and reduction by rat liver subcellular fractions. Under aerobic conditions only ring hydroxylation occurred, whereas under anaerobic conditions, reduction of the nitrofluoranthenes occurred in both cytosolic and microsomal fractions. Based on the cofactors required for the cytosolic nitroreduction, the authors attributed the activity of this fraction to DT-diaphorase, aldehyde oxidase and other unknown enzymes. Nitroreductase activity of the microsomal fraction was attributed by cytochrome P-450.

In another study (Belisario *et al.* 1991) the ring-oxidation and nitroreduction of 1-nitropyrene and 1,6-dinitropyrene were examined in human hepatoma cells, HepG2, following induction with either phenobarbital or 3-methylcholanthrene. 3-Methylcholanthrene selectively induced ring-oxidation of 1-nitropyrene, whereas phenobarbital stimulated its nitroreduction. Phenobarbital-inducible nitroreduction was consistent with cytochrome P-450, as indicated by the requirement of NADPH and the inhibition of nitroreduction activity by α-naphthoflavone and carbon monoxide.

The nitroreduction of the antiandrogen nilutamide was studied using rat liver

microsomes and NADPH (Person *et al.* 1991). Under anaerobic conditions, a nitro anion-free radical was detected by electron spin resonance (EPR) spectroscopy while in air this radical reacts with oxygen, forming a reactive oxygen species.

Since this reaction was not inhibited by SKF 525-A or carbon monoxde (cytochrome P-450 inhibitors) but was inhibited by methylene blue and 2′-adenosine monophosphate (NADPH-cytochrome P-450 reductase inhibitors), the authors concluded that nilutamide was reduced by NADPH-cytochrome P-450 reductase alone.

Oxidations versus nitroreduction biotransformation pathways were investigated to determine the possible toxicity of nitro-aromatic compounds. Nitroreduction can biologically activate nitro compounds leading to carcinogenecity or mutagenicity. In other cases, nitroreduction can serve as a detoxification pathway.

Silvers *et al.* (1994) examined the ratio of cytochrome P-450-mediated C-oxidation to nitroreduction of 1-nitropyrene in HepG2 cells. Addition of 3-methylcholanthrene to the cells increased the ratio of *C*-oxidation to nitroreduction, which was accompanied by a decrease in the formation of 1-nitropyrene adduct *via* nitroreduction. These results suggest that the cytochrome P-450-mediated *C*-oxidation is not an activation pathway in HepG2 cells, and may explain the weak carcinogenecity of 1-nitropyrene where cytochrome P-450-mediated biotransformation predominates.

Studies of lung tumours in rats induced by 3,9-dinitrofluoranthrene (3,9-DNF) and 3-nitrofluoranthrene (3-NF) were investiaged by Mitchel *et al.* (1993). The two compounds exhibited different carcinogenicity. The former was a potent pulmonary carcinogen while the latter exhibited weak carcinogenic activity. It was found that there was greater ring hydroxylation of 3-NF than of 3,9-DNF, where nitroreduction was the major pathway. Thus, a higher ratio of nitroreduction to ring hydroxylation accounted for lower carcinogenicity of 3,9-DNF.

Studies were also directed toward identification of cytochrome P-450 (CYP) isoforms involved in the biological activation of nitrocompounds. Chae *et al.* (1993) demonstrated that human liver and lung are capable of metabolising 6-nitrochrysene to known potent carcinogenic metabolites *via* ring oxidation and nitroreduction. Rates of phenacetin *O*-deethylation (CYP1A2) and nifedipine oxidation (CYP3A4) were correlated with the rates of formation of *trans*-1,2-dihydro-1,2-dihydroxy-6-nitrochrysene (oxidation) and 6-aminochrysene (nitroreduction), respectively. This suggested that both CYP1A2 and CYP3A4 were involved in the biotransformation of 6-nitrochrysene. The involvement of CYP3A4 in the nitroreduction of clonazepam was also reported by Seree *et al.* (1993). The authors synthesised an oligonucleotide specific for the CYP3A4 protein gene and used it for hybridisation on total RNA from human liver samples. The 2.2 kb transcript correlated ($r = 0.61$) with the intensity of clonazepam nitroreduction in human liver microsomes.

Li *et al.* (1995) evaluated the biotransformation pathways catalysed by CYP3A4 in human liver preparations (microsomes, cultured hepatocytes) and in yeast cells genetically engineered to express CYP3A4. These authors demonstrated that CYP3A4 pathways included both oxidation reactions (*N*-oxidation, *C*-oxidation, *N*-dealkylation and *O*-dealkylation) as well as nitroreduction.

Musk xylene, 1,3,5-trinitro-2-t-butylxylene, a synthetic nitromusk perfume ingredient that induces cytochrome P-450 enzymes, undergoes nitroreduction by intestinal flora to yield aromatic amine metabolites (Lehman-McKeeman *et al.* 1997a,b). The

authors examined the potential capability of the monoamine metabolites to induce CYP2B10 and CYP1A2 using Northern Blot analyses. mRNAs for both cytochromes were induced, but that for CYP1A2 only to a slight extent.

In vitro metabolic activation of flutamide, a nitroaromatic antiandrogen that produces hepatitis, was studied in male rat liver microsomes (Berson *et al.* 1993). The authors did not detect the presence of one-electron reduction intermediate of flutamide by electron spin resonance. However, flutamide formed reactive intermediate(s) that covalently bound to microsomal proteins. Formation of microsomal protein-flutamide adducts required NADPH and oxygen, and was suppressed by cytochrome P-450 inhibitors, e.g. SKF 525-A, piperonyl butoxide and troleandomycin (inhibitor of the CYP3A subfamily). The formation of the covalent adduct was enhanced considerably following pretreatment of the rats with dexamethasone (inducer of CYP3A subfamily) and moderately following pretreatment with β-naphthoflavone (inducer of the CYP1A subfamily). The formation of the covalent adduct(s) was enhanced with yeast microsomes expressing human CYP1A1, CYP1A2 or CYP3A4. Covalent binding was inhibited significantly by anti-CYP3A immunoglobulin G and moderately with anti-CYP1A immunoglobulin G. Thus, the authors concluded that CYP1A and CYP3A subfamilies mediated the formation of the covalent-adduct of flutamide.

NITROREDUCTASE ACTIVITY IN DIFFERENT SPECIES AND ORGANS

Nitroreductase activity was found in mammalian liver and adrenal cortex, and channel catfish (*Ictalurus punctatus*) (Washburn and Di Giulio 1988), as well as in different bacteria, such as *Bacillus licheniformis* isolated from industrial waste containing high concentrations of 5-nitro-1,2,4-triazol-3-one (Le Campion *et al.* 1999).

MECHANISM OF ENZYMATIC NITROREDUCTION

Due to the involvement of various enzymes in different tissues there is no comprehensive mechanism for enzymatic nitroreduction. Two general types of nitroreductase mechanisms are described:

(1) Oxygen-sensitive biotransformation pathway. This nitroreductase mechanism requires the formation of one-electron free-radical intermediate, which is stable enough to be detected by EPR and to react with oxygen (aerobic reduction) with the formation of the superoxide anion radical (Person *et al.* 1991; Washburn and Di Giulio 1998). This requires two separate one-electron reductions but not a single two-electron reduction.

    Studies by Mason (Orna and Mason 1989; Mason 1997) also suggested a nitroreductase mechanism involving a one-electron reduction and formation of a free-radical intermediate at the initial step. These nitro free-radicals reductive intermediates were found to be unstable in air (Mason 1997; Orna and Mason 1989). A linear correlation between $\log V_{max}/K_m$ and one-electron reduction potentials of nitro compounds found by these researchers clearly supported such a mechanism.

(2) Oxygen-insensitive biotransformation pathway. Reduction of 5-nitrofuran is rela-

tively oxygen insensitive (Holtzman *et al.* 1981). This type of mechanism probably requires either a single two-electron step reduction or a stepwise mechanism of two one-electron reductions, with the formation of a one-electron free-radical intermediate, temporarily stable in air (oxygen), that 'immediately' proceeds to an additional one-electron reduction.

UTILISATION OF NITRO COMPOUNDS AS PRODRUGS

The possible use of nitro compounds as prodrugs activated by bacterial aerobic nitroreductase is currently being investigated. This prospect was discussed in a review article by Patterson and Raleigh (1998) on reductive drug metabolism, which includes a section on nitroreductase biotransformation pathways.

Azoreductases

Azo compounds (Ar-N = N-Ar) are widely used as colourants in the pharmaceutical, food, textile and printing industries (Catino and Farris 1978). The reduction of azo dyes to primary amines is catalysed by mammalian liver microsomal cytochrome P-450 (Fugita and Peisach 1978; Hernandez *et al.* 1967), cytosolic enzymes (Huang *et al.* 1979) and colonic bacteria (Walker 1970; Scheline 1980). Chung *et al.* (1992) discuss reductive cleavage of a large number of azo dyes to primary amines by intestinal microflora (Figure 16.2).

Azoreductase activity was also detected *in vitro* (diffusion cells) following percutaneous absorption and metabolism of three azo dyes, phenylazophenol, phenylazo-2-naphthol and 5-(phenylazo)-6-hydroxynaphthalene-2-sulphonic acid, in mouse, pig and human skin (Collier *et al.* 1983). Thus, dermal risk assessments from exposures to pharmaceutical preparations should be considered.

The NAD(P)H:quinone oxidoreductase, also known as DT-diaphorase, is a flavoprotein that catalyses the two-electron reduction of quinones, quinone imines and azo-compounds (Belinsky and Jaiswal 1993). Thus, this enzyme forms no free-radical intermediates resulting from one-electron reductions and consequently, no reactive oxygen intermediates are formed. In contrast, reduction by cytochrome P-450 is associated with one electron reduction (Porter and Coon 1991).

Metabolism of azo dyes may lead to either mutagenic or toxic effects or to detoxification. Sandhu and Chipman (1991) studied the oxidation by cytochrome P-450, particularly P-448 (CYP1), which resulted in the metabolic activation of chrysoidine azo dyes. However, addition of FMN inhibited mutagenicity, suggesting that reduction of the azo dyes resulted in detoxification.

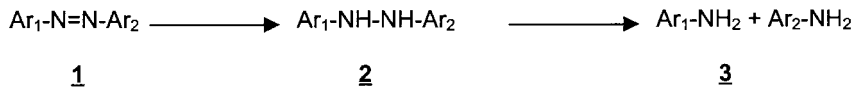
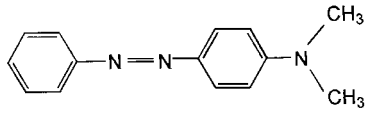


Figure 16.2 Reductive cleavage of azo dyes **(1)** to primary amine metabolites **(3)** requiring electrons. Two electrons are required for the reduction of an azo compound **(1)** to hydrazo intermediate **(2)** and an additional two electrons are required for the reduction of hydrazo compound **(2)** to primary amine metabolites **(3)**.

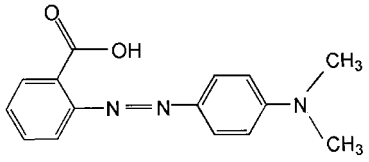
Purified rabbit liver aldehyde oxidase readily reduced water-soluble azo dyes. However, lipophilic azodyes were poor substrates or not reduced at all by this enzyme but were readily reduced by microsomal cytochrome P-450 enzymes (Stoddart and Levine 1992).

Prediction of the azoreduction biotransformation pathway in liver microsomes is possible based on electronic aspects and structure-activity relationships. Highlights of these findings, which were previously presented in a detailed review article (Zbaida 1995), are summarised below:

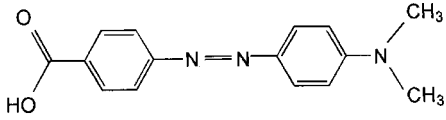
- Microsomal reduction of azo dyes requires polar electron-donating substituents (Hammett σ constant of -0.37 or lower) such as hydroxyl, amino, methylamino or dimethylamino moieties *ortho* or *para* to the azo linkages for binding to enzyme (Levine and Zbaida 1988; Zbaida *et al.* 1989). These substituents are essential for both binding and reduction of azo dyes. Type I and type II binding-spectra were observed for the reactive azo dyes but not for the poorly reactive compounds (Levine and Zbaida 1988), suggesting that the electron-donating substituents are essential for both binding and reduction of azo dyes.
- High substrate reactivity was observed for 4-dimethylamino-, 4-methylamino- and 4-amino-azobenzene and for 4-hydroxyazobenzene. In contrast, substituents that nullify the non-bonding electron donation (Hammett σ constant higher than -0.37), such as the benzoylamide derivative of 4-aminoazobenzene, unsubstituted azobenzene or non-polar electron donating substituents such as 4-isopropylazobenzene, exhibited only negligible rates of azoreduction. These results suggest that polar electron-donating substituents are obligatory for microsomal azoreduction (Levine and Zbaida 1988; Zbaida *et al.* 1988, 1989).
- Two classes of azo dyes structurally related to 4-dimethylaminoazobenzene, based on the sensitivity of their microsomal azoreduction to oxygen and carbon-monoxide, were identified: I-substrates, *insensitive* to both oxygen and carbon-monoxide, and the S-substrates, sensitive to both oxygen and carbon-monoxide (Levine and Zbaida 1991; Zbaida and Levine 1990a).
- The two classes of dyes differ in their chemical structure (Figure 16.3). The I-substrates contain *only* polar electron donating substituents, whereas the S-substrates contain *both* electron-donating and electron-withdrawing substituents [such as carboxylic, sulphonic or arsenic (AsO_3H_2) acid residues on the opposite phenyl ring] (Zbaida and Levine 1990a). The combination of both electron-donating and electron-withdrawing substituents in the S-substrates promotes electronic resonance. Substituents on azo dyes are the key in defining the mechanism of their microsomal reduction. The electron-donating substituents are mainly associated with binding to cytochrome P-450, whereas the combination of additional electron-withdrawing substituents on the opposite ring (S-substrates) alters the charge and the redox potentials, and consequently changes their rates and mechanisms of microsomal reduction. The combination of electron-donating and/or -withdrawing groups alters the relative stability of their one-electron-reduced radical intermediate towards oxygen and their tendency to interact with ferrous cytochrome P-450. This results in a different sensitivity of their microsomal azoreduction towards oxygen and carbon monoxide.



p-Dimethylaminoazobenzene



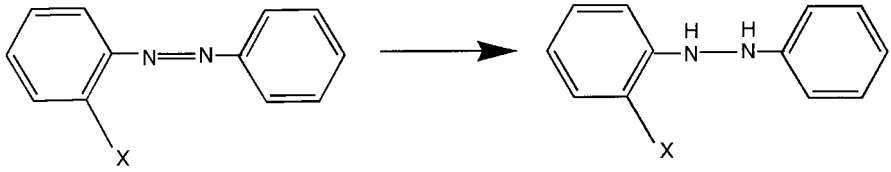
o-Methyl red



p-Methyl red

Figure 16.3 Chemical structures of a representative *I-substrate* (*p*-dimethylaminoazobenzene) containing *only* electron-donating substituent and two representative *S-substrates* (*o*- and *p*-methyl red) containing *both* electron-donating and electron-withdrawing substituents *ortho* or *para* to the azo linkage.

- Although a total of four electrons are required for the reduction of an azo linkage to primary amines, microsomal reduction of azo dyes probably involves a two-electron reduction cycle leading to a hydrazo intermediate (Zbaida *et al.* 1989). Non-substrate dyes (such as azobenzene) yield a stable hydrazo intermediate but non-reactive enzymically. Electron-donating groups *ortho* or *para* to the azo linkage are responsible for the instability of the hydrazo compounds (Figure 16.4). Non-bonding electrons from the electronegative substituent can produce a negative charge on the hydrazo nitrogen. Dissociable hydrogen from the substituent could then migrate to the negatively charged nitrogen leading to bond cleavage (Zbaida *et al.* 1989).
- The azoreduction of I-substrates is selectively induced by clofibrate, whereas the



Azo compounds

Hydrazo intermediates

X = Electron-donating groups

Figure 16.4 Azo dyes and hydrazo intermediates substituted with polar electron-donating substituents.

azoreduction of S-substrates is selectively induced by phenobarbital, β-naphthoflavone, isosafrole and pregnenolone-16α-carbonitrile as well as clofibrate (Levine *et al.* 1992; Zbaida and Levine 1990a,b).

- The S-substrates are more readily reduced both enzymically and chemically (by sodium dithionite) than I-substrates (Zbaida and Levine 1990a; 1992b).
- Inhibition of microsomal azoreduction by CN^- also distinguishes the two classes of dyes. Reduction of I-substrates is, on average, more sensitive to CN^- than is reduction of S-substrates, possibly due to the alteration of the enzyme redox potentials by CN^- (Zbaida and Levine 1992a; Zbaida *et al.* 1992).
- The electrode potential at which a chemical undergoes reduction or oxidation can be detected by cyclic voltammetry. Electrochemical techniques are suitable means of studying oxidation-reduction potentials. The electrode potential at which a chemical undergoes reduction or oxidation can be rapidly detected by cyclic voltammetry. Electrochemical reductions and oxidations generate intermediates coupled to the electrode surface reaction, which may serve as models for those biologically formed. Enzymic reduction of substrate and nonsubstrate dyes was performed anaerobically in anhydrous dimethylformamide (Zbaida and Levine 1991a). All azo dye substrates exhibit two negative and one positive redox potential, as measured anaerobically by cyclic voltammetry. The negative potentials reflect one- and two-electron reduced intermediates while the positive potential is associated with the electron transfer from the microsomal cytochrome P-450 to the dyes. Nonsubstrate azo dyes did not exhibit positive anodic potentials. The S-substrate dyes exhibited less negative oxidation-reduction potentials (Levine *et al.* 1992; Zbaida and Levine 1991a). The sensitivity of the S-substrates to oxygen is well demonstrated by the cyclic voltammetry. The one-electron reduced intermediates of S-substrates, which are stable under nitrogen, immediately quenched in air, while those of I-substrates were relatively stable in air. The I-substrates exhibit on average potentials which are approximately 0.6 Volt more negative than those for S-substrates (Levine *et al.* 1992; Zbaida and Levine 1991a). Although microsomal azoreduction of S-substrates is sensitive to both oxygen and carbon monoxide, the mechanisms of the two inhibitions are totally different. Carbon monoxide interacts with a reduced form of cytochrome P-450 (Ortiz de Montellano and Reich 1986; Stanford *et al.* 1980) whereas oxygen reacts with the one-electron-reduced intermediate of the dye forming the superoxide anion radical (Peterson *et al.* 1988).
- These two classes of dyes also differ in their Hammett sigma values. The I-substrates exhibit negative values while the S-substrates exhibit a combination of negative and positive values on opposite rings. However, an overall value of -0.37 or lower is obligatory for microsomal azoreductases (binding) (Zbaida *et al.* 1994). Chemical modification which alters the magnitude (from negative to positive) of the Hammett substituent constants of the prime substituents also alters sensitivity to both oxygen and carbon monoxide. This implies that catalytic recognition of azo dyes by hepatic microsomes is regulated by charge and redox potentials (Zbaida *et al.* 1994).
- A linear correlation between V_{max} and K_m was observed for I-substrates, suggesting inverse relationships between binding affinity and rate of reduction (Zbaida and Levine 1990a). This relationship was not obvious for the S-substrate dyes. A lower K_m was observed for all dyes (both I- and S-substrates) bearing greater number of

heteroatoms with non-bonding electrons in either electron-donating or -withdrawing groups (Levine and Zbaida 1992; Levine *et al*. 1992; Zbaida and Levine 1991b, 1992b).

- More basic dyes, which contain a higher density of non-bonding electrons, showed an inverse correlation with both K_m and V_{max} (Levine and Zbaida 1992; Levine *et al*. 1992; Zbaida and Levine 1991b; 1992b).
- NMR (nuclear magnetic resonance) studies revealed minor differences in the chemical shifts of protons attached to the phenyl ring substituted with electron-donating substituents. However, there are significant differences of the aromatic protons on the opposite prime ring, which also distinguish I- and S-substrates (Zbaida *et al*. 1992).
- This implies that the mechanism of microsomal azoreduction is critically dependent on charge and redox potentials of the dyes (Zbaida *et al*. 1994).
- Surprisingly, no significant studies of microsomal azoreduction were performed in the last 7 years. The commercial availability of cDNA expressed human cytochrome P-450s (Supersomes), chemical inhibitors and antibodies against specific forms of cytochrome P-450s and the considerable progress in molecular biology (Dachs *et al*. 1997) in recent years can provide an excellent opportunity to identify the form(s) of cytochrome P-450 involved in microsomal azo reduction. It is important to characterise the forms that metabolise I-substrate *versus* those that metabolise the S-substrate dyes. This may provide a better understanding of the enzymic mechanism of action of various forms of cytochrome P-450s.

Molecular biological approaches should also be considered for nitroreduction pathways. The nitroreductase from *E. coli* B has been studied for its use in antibody-directed enzyme prodrug therapy (ADEPT) (Dachs *et al*. 1997). Alternate prodrugs for use with *E. coli* nitroreductase in suicide gene approaches to cancer-therapy have been investigated (Bailey *et al*. 1996).

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