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COMMENTARY

MUTAGENICITY, CARCINOGENICITY AND TOXICITY OF β-NAPHTHOFLAVONE, A POTENT INDUCER OF P448

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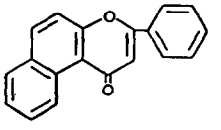
The cytochrome P450-dependent mixed-function oxidase enzyme system is responsible for the metabolism of a wide range of foreign compounds including various chemicals, pesticides, carcinogens, environmental pollutants and many drugs [1]. This enzyme complex also has an important role in the biosynthesis and degradation of endogenous substrates such as steroids, fatty acids, prostaglandins, leukotrienes, vitamins, thyroxine and biogenic amines [2, 3]. The broad specificity of the mixed-function oxidase system is due to the existence of multiple forms of P450 which exhibit different but overlapping substrate specificities [4]. It is now recognised that there are at least ten mammalian P450 gene families; these may consist of more than one subfamily each containing several P450 genes [5, 6]. At present, there is evidence for the existence of over seventy distinct genes coding for specific mammalian P450 enzymes.

The spectral characteristic which gave rise to the descriptive name, P450, was first reported independently by Klingenberg [7] and Garfinkel [8]. Using hepatic microsomes from the rat and pig, these workers observed a broad absorbance band with a maximum at 450 nm when carbon monoxide was added to dithionite- or NADPH-reduced microsomal suspensions. However, it was not until 1962 that Omura and Sato [9] recognised the haemoprotein nature of this carbon monoxide-binding pigment with the characteristic absorbance maximum at 450 nm; as a result, they proposed that this protein should be termed cytochrome P450. The heterogeneity of this haemoprotein was initially observed not only by its catalytic activity but also by its spectral characteristics. Induction of the microsomal enzymes by certain polycyclic aromatic hydrocarbons produced a protein where the wavelength maximum of the carbon monoxide difference absorption spectrum had shifted to 448 nm [10]. Various workers referred to this induced protein either as cytochrome P448 or as cytochrome $P_1$450 [11]. However, as new P450 isozymes were discovered and reported, this type of notation was of little use and became quite confusing; the nomenclature of P450 isozymes has been revised recently by a collaboration of many of the leading exponents in this field [5, 6]. For the purposes of this review, the original descriptive term, P448, will be used to refer to the haemoproteins of the P450I gene family, which is known to consist of at least two P448 isozymes.

Induced in animals by the administration of polycyclic aromatic hydrocarbons, P448 is one of the most widely studied families of P450 [1, 11]. This isozyme is also induced by polyhalogenated aromatic compounds [12, 13], as well as by a number of other chemicals; many of these xenobiotics are mutagenic, carcinogenic, teratogenic or produce some form of toxicity in laboratory animals [14, 15]. In contrast to the induction of other forms of P450, there appears to be a marked structure–activity relationship associated with the induction of P448 [16, 17]. Both the substrate and inhibitor specificities of the isozyme show a similar structural selectivity, requiring the molecule to be flat, planar and highly lipophilic. Induction of P448 has been studied extensively since this isozyme has been shown to catalyse the activation of polycyclic aromatic hydrocarbons to the ultimate carcinogenic metabolite [18]; indeed induction of P448 has also been associated with both the carcinogenicity and potential toxicity of the inducing agent [14, 19, 20].

Many studies into various aspects of P448 activity and induction have included β-naphthoflavone (BNF) as a specific and potent inducer of this isozyme.



β-Naphthoflavone

The inducing properties of BNF, which was the most potent of a number of natural and synthetic flavonoid compounds, were first described by Wattenberg *et al.* [21]. Since BNF shows a similar potency and specificity of induction to the carcinogenic polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and benzo[*a*]pyrene [22, 23], it is used routinely within ICI Pharmaceuticals to provide a positive control for comparing the potency and profile of enzyme induction produced by test compounds. BNF is also used in many laboratories as the P448 inducer of choice, probably because BNF, unlike most other P448 inducers, is widely regarded as being non-carcinogenic. Although many

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authors have referred to BNF as having no carcinogenic potential, this view has not been supported by any data or references in these papers [22, 24–26]. The specific purpose of this report, therefore, is to review the information available relating to the mutagenicity, carcinogenicity and general toxicity of BNF.

Carcinogenicity and mutagenicity of BNF

A literature search for information relating to the mutagenicity and carcinogenicity of BNF was conducted on a range of commercially available data sources (BIOSIS, EXCERPTA MEDICA, MEDLINE and the Registry of Toxic Effects of Chemical Substances published by the National Institute of Occupational Safety and Health) using a time period from 1966 to 1988 inclusive. Relevant papers published during the first few months of 1989 have also been included.

BNF showed no frameshift mutagenic activity when screened over a wide range of concentrations in several tester strains (TA1535, TA100, TA1537, TA1538, TA98) in the Salmonella/mammalian microsome assay [27].

The literature search produced no primary reports on the carcinogenicity of BNF, suggesting that no definitive study has been carried out specifically to assess the carcinogenicity of BNF.

Some long-term studies have been conducted, however, to examine the effects of BNF when administered in conjunction with other agents such as chemical carcinogens and synthetic steroids. In two similar studies, the effects of the enzyme inducing properties of BNF on the tumorigenesis of benzo[*a*]pyrene and 3-methylcholanthrene in various strains of mice were examined [28, 29]. As a control, BNF was administered alone at a dose level of 150 mg/kg, i.p., once weekly for 12–14 weeks to groups of mice. The mice were examined for tumours between 6 and 18 months after the first dose; the findings of the first study indicated that BNF, in this dose regimen, had no tumorigenic effect when compared to vehicle control or untreated mice [28]. Since BNF was regarded, therefore, as being non-carcinogenic, the findings in the vehicle control and the BNF control groups were combined in the analyses and reports of these studies. However, in the second study, forestomach papillomas were observed at a pronounced level (over 50% of all animals) in the combined control groups, each consisting of five vehicle control mice and ten BNF control mice, in four of the six mouse strains examined [29]. The relevance of this observation was not addressed, and it was not possible to tell from the paper if the tumour incidence in the BNF-treated animals was different from that in the vehicle control animals in this study.

In another study designed to examine the effects of enzyme induction and inhibition on the incidence of hepatocellular carcinoma following administration of synthetic oestrogens to castrated male hamsters, a control group was maintained on a diet containing 0.2% BNF for up to 10 months; no hepatic tumours were found in any animals in this group [30], although the liver appeared to be the only tissue examined histologically.

Toxicity of BNF

In common with the polycyclic aromatic hydrocarbons, administration of BNF during pregnancy in the rat results in marked foetotoxicity. Extensive foetal mortality was observed following administration of low doses (15 mg/kg/day, i.p.) of BNF for 8 days during mid-gestation [31]. In addition, administration of BNF on days 9–14 of gestation caused a significant impairment of late foetoplacental growth, although no signs of maternal toxicity were observed [32]. While the biochemical mechanism for the retardation of foetal growth remains to be elucidated, a recent study has shown that BNF decreases the high-affinity binding of insulin to rat placental membranes with a concomitant decrease in insulin receptor protein kinase activity; in contrast, BNF produced no effect on the binding of insulin to the maternal liver membranes [33]. A strong correlation has been shown to exist between low birth weight and exposure to various polycyclic aromatic compounds; this has been reported for benzo[*a*]pyrene and 2,3,7,8-tetrachlorodibenzo(*p*)-dioxin (TCDD) in rodents, for polychlorinated biphenyls in primates and women, and for cigarette smoking in women. These effects are thought to be related to the P448 inducing properties of these agents, which selectively alter the enzymes of steroidogenesis and affect normal hormonal regulation [31, 32].

In contrast to TCDD, a very potent teratogen and P448 inducer, BNF produced no malformations in the embryos following administration to pregnant mice, and was considered to exhibit no toxicity [34]. However, when BNF and TCDD were co-administered to pregnant mice, both the teratogenicity and the foetolethality of TCDD were increased significantly, presumably due to a perturbation of their respective interactions with the P448 induction mechanism. It is possible, however, that P448 induction by TCDD results in the formation of a more reactive metabolite of BNF, which is then able to increase the apparent toxicity. This would be analogous to the metabolic activation observed with α-naphthoflavone [35]. When co-incubated with liver microsomes from control or phenobarbitone-treated rats, α-naphthoflavone showed no clastogenic activity in Chinese hamster ovary cells. However, when TCDD-induced microsomes were used for activation, α-naphthoflavone was metabolised to a much greater extent and produced marked increases in sister chromatid exchanges and chromosome aberrations in the Chinese hamster ovary cells [35].

There is little evidence of overt toxicity on administration of BNF to animals, although a modest atrophy of the thymus was observed in mice that had received four daily doses of BNF at 80 mg/kg [19]. Pronounced thymic involution is one of the characteristic toxic responses to the extremely potent P448 inducing polyhalogenated aromatic hydrocarbons. In addition, BNF has been shown to elicit an immunotoxic response in mice; this effect appeared to be related to the ability of BNF and other compounds to initiate the receptor-mediated P448 induction process [36].

As mentioned earlier, BNF is a synthetic analogue

of a large series of naturally occurring flavonoid compounds. The flavonoids are widely distributed among vascular plants, including many edible plants such as citrus fruits, vegetables and tea. It has been estimated that the average American diet includes 1 g of various flavonoids per day [27]. Some of these compounds have shown P448 inducing potential on administration to animals [21], although their specific effects in humans have not been assessed, presumably due to the difficulties in controlling their presence in the normal diet. The polyhydroxylated flavones, such as quercetin, show little P448 inducing potential, although the methylated analogues do produce P448 induction [21]. A number of flavonoid compounds, but not BNF, have shown mutagenic activity [27], while quercetin and kaempferol have also been shown to be carcinogenic in the rat [37].

Effects of BNF on the carcinogenicity, mutagenicity and toxicity of various chemicals

BNF has been used widely as a standard P448 inducer to modify the metabolism of various compounds. Although the enhanced metabolism of foreign chemicals that occurs after exposure of animals to enzyme inducers is often an adaptive and protective response which increases the metabolic clearance of the compound, some chemicals are metabolised to toxic products or reactive intermediates; in these cases, enzyme induction effectively increases the toxic potential of these chemicals [18, 38]. Thus, metabolism may result in both activation and detoxification of xenobiotics.

A number of studies have demonstrated the protective effects of BNF on the toxic, carcinogenic and mutagenic effects of various chemicals. Administration of BNF to mice resulted in almost total inhibition of pulmonary adenoma formation produced in response to benzo[*a*]pyrene [39], while the tumorigenic effect of 3-methylcholanthrene was decreased markedly in various tissues of several responsive strains of mouse when BNF was co-administered [29]. The nephrotoxicity produced in rats by high doses of cephaloridine was decreased by pretreatment with BNF; since piperonyl butoxide (a mixed-function oxidase inhibitor) also decreased the nephrotoxicity, whereas phenobarbitone potentiated the effect, it is probable that BNF acts by inducing a detoxification pathway of cephaloridine metabolism [40]. The inclusion of BNF-treated microsomes in mutagenicity tests resulted in decreased activation of both cyclophosphamide and aflatoxin B_1 to mutagenic products [41, 42]. Consistent with the view that all of these inhibitory effects of BNF are due mainly to induction of P448 are the observations that the carcinogenic P448 inducer, 3-methylcholanthrene, also provides protection against the carcinogenicity of an aminoazo dye, bromobenzene hepatoxicity and zoxazolamine lethality at high dose levels [2].

In contrast, numerous studies have shown that administration of BNF and other P448 inducers results in the metabolic activation of various chemicals particularly polycyclic aromatic hydrocarbons, into ultimate carcinogenic and mutagenic forms [14, 18]. The use of hepatic microsomes from BNF-treated animals as an enzyme source in mutagenicity tests produced marked increases in the activation of 2,4-diaminoanisole, quinoline and benzo[*a*]pyrene to mutagenic products [42–44]. The inclusion of rat colon mucosal cells from BNF-treated animals produced a pronounced increase in the mutagenicity of 2-aminoanthracene and benzo[*a*]pyrene [45]. Administration of BNF to male and female mice significantly increased the incidence of micronuclei observed in bone marrow in response to benzene; a concomitant increase in the production of urinary metabolites of benzene was also observed [46].

It is obvious therefore that induction of drug-metabolising enzymes is neither totally beneficial nor totally detrimental to animals exposed to drugs or environmental chemicals. This property is not unique to P448 induction, since a similar increase in both activation and detoxification has also been observed in response to induction of the P450 isozymes following administration of phenobarbitone [18].

Role of the Ah *receptor in P448 induction and toxicity*

Although it is now firmly established that induction of many P450 forms requires *de novo* protein synthesis, it is only in the past few years that the mechanisms which regulate the induction process have begun to be understood at the molecular level; only in the specific case of P448 induction is the overall mechanism reasonably well understood. Much of this progress was due to the discovery of an allelic variant among inbred mouse strains [47]. This DBA/2 strain of mouse was found to lack the ability to induce P448, a defect shown to be an autosomal recessive trait. Since the chemicals first used to demonstrate this strain difference were Aromatic Hydrocarbons, the gene controlling the P448 induction process was termed the *Ah* locus [3]. Although initially discovered in rodents, the *Ah* receptor has now been shown to exist in several mammalian species including humans [38]. Recent work in the mouse, rat, rabbit and human has demonstrated the existence of two distinct forms of P448, both of which appear to be structural gene products under the control of the regulatory *Ah* locus [3, 48].

A number of studies have shown a strong correlation between the binding affinities of halogenated aromatic hydrocarbons for the *Ah* receptor and their potencies for inducing P448 activity *in vivo* [13]. The term, halogenated aromatic hydrocarbons, covers several series of chemicals which are often classed together since, although structurally different, they are all approximate stereoisomers and produce a similar pattern of biochemical and toxic responses [19]. The potency of TCDD and related compounds in binding to the *Ah* receptor and inducing P448 activity has also been found to correlate very closely with their toxic potency as assessed by thymic involution and teratogenic changes. However, the cytosolic *Ah* receptor appears to regulate not only the induction of P448, but also the coordinate expression of UDP-glucuronyltransferase, NADPH-menadione oxidoreductase and glutathione transferase; a number of other enzymes including aldehyde dehydrogenase and ornithine decarboxylase are also induced by TCDD or polycyclic aromatic hydrocarbons and, therefore, may be under the control of the *Ah* receptor [3, 13]. Since the *Ah* receptor governs a considerable number of biochemical and morphological

effects, the toxicity of the aromatic halogenated hydrocarbons may not be related directly to P448 induction. However, it does provide an obvious marker demonstrating that the initial step (binding to the *Ah* receptor) in the toxic mechanism has been stimulated.

Polycyclic aromatic hydrocarbons, including BNF, also exhibit high binding affinities for the *Ah* receptor (3–30% that of TCDD); in contrast, these compounds have a much lower potency *in vivo* for initiating P448 induction (about 30,000 times less than TCDD). It has been suggested that the low induction potency *in vivo* may be due to the fairly rapid metabolism of the polycyclic aromatic hydrocarbons relative to TCDD and related compounds [13, 20]. Similarly, both 3-methylcholanthrene and BNF have shown very much lower potency in producing a toxic response characteristic of TCDD [19]. It is still uncertain if prolonged exposure of the *Ah* receptor to these and other types of P448 inducers would elicit the spectrum of toxic responses typical of the halogenated aromatic hydrocarbons.

The role of the *Ah* locus has been investigated with regard to several biochemical and morphological aspects of carcinogenesis and cellular toxicity. Preliminary studies have also indicated a relationship between the *Ah* receptor and immunosuppression, atherosclerosis, longevity and fertility [3]. Recent work to examine the effects of polycyclic aromatic hydrocarbons as tumour promoters showed that a number of these compounds, including BNF, could inhibit the cellular binding of epidermal growth factor, an endogenous polypeptide hormone involved in the regulation of cell division. It was thought that this effect may have been mediated by binding of the polycyclic aromatic hydrocarbons to the *Ah* receptor [49]. Further studies have shown that TCDD had only a minimal effect on epidermal growth factor binding, and that the rank order of binding affinities of several P448 inducers to the cytosolic *Ah* receptor did not correlate with their effects on epidermal growth factor binding capacity [50]. While much of the early *Ah* receptor work, indicating a correlation among receptor binding, enzyme inducibility and toxicity, was conducted in responsive and non-responsive strains of mouse, recent studies have suggested that these correlations may not hold in the rat [51]. Using TCDD-susceptible and -resistant strains of rat, it was found that TCDD had similar enzyme inducing ability, there were similar numbers of hepatic *Ah* receptors, but there was a marked difference (over 300 times) in the lethality of TCDD between the two strains. While these findings raise questions about the generalisation of the correlation among P448 induction, *Ah* receptor affinity and toxicity, it may be appropriate to investigate further the relative exposure to TCDD in both rat and mouse strains as well as examining the effects on an index of toxicity less gross than lethality.

It is apparent that there are a number of biochemical and morphological responses that are mediated through the *Ah* receptor and are related to cellular toxicity. While these are probably unrelated to P448 induction itself, this particular response is an easily detectable marker indicating that the largely toxic series of *Ah* receptor-related events has been initiated.

P448 induction in humans

Investigation of the clinical effects of P448 induction is severely limited by the fact that, in contrast to the P450 isozymes, there is no known potent P448 inducer in current therapeutic use. It should be noted, however, that enzyme induction data are not available for a large proportion of the therapeutic drugs presently in clinical use. Nevertheless, there are a few clinically useful drugs which have shown some indications of P448 inducing activity, albeit in animals. Phenothiazine and its derivatives were found to be weak inducers of hepatic microsomal P448 activity in the rat [52, 53], although more recent studies have shown that these compounds also induce a P450 isozyme and produce an isozyme profile more similar to that of isosafrole and Aroclor 1254 than to BNF [54]. Prolonged administration of phenothiazine derivatives was found to decrease antipyrine half-life in chronic schizophrenic patients [55], although this effect could be produced by induction of either P450 or P448 isozymes [56]. Phenothiazine [57] and its derivatives [58] are known to produce a number of toxic side-effects in humans, such as blood dyscrasias, liver pathology, photosensitisation and opacities of corneal and lens tissue.

Dantrolene is a skeletal muscle relaxant used in humans to decrease muscle spasticity [59]. Studies in the rat have indicated that this compound is a P448 inducer of fairly low potency [60]. It is not known if dantrolene causes P448 induction in humans; however, its use is limited by a sporadic incidence of serious liver damage, which has a mortality rate of about 0.3% [61]. Although the anti-helminthic agent, albendazole, was used originally only in veterinary medicine where there was evidence that it produced teratogenicity and embryotoxicity, this compound is now being used increasingly in the clinical treatment of hydatid disease [62]. The effects of albendazole on drug-metabolising enzymes were examined recently in the rat where evidence of P448 induction was produced [63]. It is not known if P448 induction occurs in humans at therapeutic doses of albendazole, which have been found to produce signs of liver toxicity in some patients [62].

Nomifensine, a tetrahydroisoquinoline antidepressant, induces P448 activity in the rat [64]. Although its effects on a typical marker of P448 activity have not been examined in humans, this compound produces a marked induction of its own metabolic clearance during chronic administration [65]. Nomifensine has been found to cause acute haemolytic anaemia in a number of patients; the incidence of this side-effect has now led to the withdrawal of the compound from the market [66].

It is obvious that the few clinically useful drugs with known P448 inducing potential in animals all produce a profile of pronounced toxic side-effects in humans at therapeutic dose levels. It is not clear, however, if these compounds induce P448 in humans at therapeutic doses, or if the spectrum of toxicity is related to the mechanism of P448 induction.

Implications of P448 induction for drug development

In the past few years a number of compounds being developed by various pharmaceutical companies have been shown to have P448 inducing potential in animals. A recent publication has shown that nafimidone, an anticonvulsant agent, produces complex effects on the drug-metabolising enzymes in the rat [67]. Nafimidone and its major metabolite were shown to be potent inhibitors of P450-dependent metabolism both *in vivo* and *in vitro*, while, on chronic administration to mice and rats, a mixed (both P450 and P448) spectrum of enzyme induction was observed. During the initial clinical evaluation of nafimidone, marked inhibition of phenytoin and carbamazepine metabolism was observed with no evidence of enzyme induction [68]. This compound is no longer undergoing clinical evaluation; however, this may be a consequence of the fact that drug regulatory authorities may be reluctant to license any type of enzyme inducer, unless it has exceptional therapeutic value [69].

Within ICI Pharmaceuticals, three compounds have been shown recently to be potent inducers of P448 activity in both rat and dog. These compounds, one a cardiotonic agent, have been withdrawn from development as a result of various toxicological findings. There is evidence that other cardiotonic agents of a similar chemical structure may also have P448 inducing potential [70, 71]. One of these compounds, sulmazole, progressed to clinical evaluation where liver dysfunction was observed [72]. It is unclear whether sulmazole produces P448 induction in humans, but unacceptable side-effects have prevented its therapeutic use [73].

It is widely regarded that the induction of the P450-dependent mixed-function oxidases (including P448) produced by lipophilic compounds is basically a physiological, adaptive response aimed at increasing the metabolic elimination of the inducing agent. However, it is quite probable that the stigma associating P448 induction with various forms of toxicity will continue until a fairly potent P448 inducer has completed successfully a full safety evaluation programme and has been in clinical use for a considerable period.

Conclusions

BNF is not mutagenic, but no data relating to its carcinogenicity following chronic administration have been found. Comments in the literature which imply that BNF is not carcinogenic and is, therefore, an attractive model inducing agent for that reason, would seem to be unsubstantiated. While P448 induction can cause both activation and detoxification of co-administered compounds, virtually all known P448 inducers themselves elicit some form of toxic response. Consequently, there are no known potent P448 inducers in common therapeutic use.

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