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Paracetamol-Induced Spindle Disturbances in V79 Cells with and without Expression of Human CYP1A2

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Abstract: Spindle disturbing effects in terms of c-mitosis and cytotoxicity of paracetamol were investigated in two Chinese hamster V79 cell lines, one of which (V79MZh1A2) was transfected with human CYP1A2. This enzyme catalyses the oxidative formation of the reactive paracetamol metabolite, NAPQI, believed to initiate hepatotoxicity by covalent binding to proteins after overdose. In the native V79 cell line paracetamol increased c-mitosis frequency in a concentration dependent manner from 8.7±3.5% (control) to 66±18% at 20 mM. A significant increase to 13.3±3.5% was first seen at 2.5 mM in the native cell line ($P<0.05$). In the V79MZh1A2 cells the concentration-effect curve was slightly shifted to the left ($P<0.05$) with c-mitosis frequency increased to 12.1±2.6% ($P<0.05$) at 1 mM paracetamol. At 5 mM paracetamol the c-mitosis frequency was 14.4±5.0% and 19.0±3.8% in the native and CYP1A2 expressing cell lines, respectively ($P<0.05$). At 20 mM paracetamol the c-mitosis frequency was 61±10% in the V79MZh1A2 cells. Cell survival was reduced to approximately 50% at 5–10 mM paracetamol in both cell lines. At 20 mM paracetamol survival was further decreased to 39±9% in V79MZh1A2 cells only ($P<0.05$). The present study demonstrated that paracetamol may disturb the spindle of dividing cells conveying a risk of aneuploidy. The spindle disturbind effect was only slightly enhanced by expression of CYP1A2, suggesting that metabolic activation plays only a minor role in this genotoxic effect. The reduction of survival mirrored the increase in c-mitosis frequency.

Paracetamol is a widely used mild analgesic. In overdose, paracetamol causes severe hepatotoxicity, probably due to a reactive intermediate, N-acetylbenzoquinoneimine, NAPQI (Hinson 1980; Hinson *et al.* 1990; Pumford et al. 1990), produced by oxidative metabolism catalysed by CYP1A2, CYP2E1 and CYP3A4 (Raucy *et al.* 1989; Thummel *et al.* 1993; Thomsen *et al.* 1995). Despite arylating and DNA binding capabilities of this oxidative metabolite epidemiological and experimental studies of paracetamol have generally been negative with respect to carcinogenicity in humans and animals and mutagenicity in relevant test systems, respectively (Dybing *et al.* 1984; Anonymous 1990). However, other signs of genotoxicity in terms of DNA and/or chromosome damage have been shown *in vitro*, in experimental animals treated with paracetamol and even in humans taking therapeutic doses (Dybing *et al.* 1984; Kocisova *et al.* 1988; Topinka *et al.* 1989; Anonymous 1990; Hongslo *et al.* 1991 & 1994; Giri *et al.* 1992).

The fidelity of chromosome segregation and the maintenance of the karyotype is dependent upon the synthesis and functioning of the spindle apparatus of the dividing cell. Disturbance can lead to chromosomal malsegregation, giving rise to aneuploid daughter cells (Tsutsui *et al.* 1983; Önfelt 1986 & 1987). Aneuploidy has severe consequences for reproduction (Hook 1983) and is involved in progression of malignancy (Nowell 1976). Neoplastic transformation is often accompanied by large variation in chromosome numbers. Chemically induced spindle dysfunction and aneuploidy show many similarities to tumour promotion (Önfelt 1986). Repeated treatment is usually required in tumour promotion corresponding to selection against aneuploid/polyploid cells after induction. Aneuploidy can lead to loss of heterozygosity and quantitatively altered gene expression (Sinet *et al.* 1975). Pronounced aneuploidy in early stages of tumour development *in vivo* has been observed (Conti *et al.* 1986; Sudilovsky & Hei 1991), and altered properties of transformation and altered ploidy have been found to coincide in SV40 transformed cells (Hoffmann *et al.* 1979).

So far, it is not known whether paracetamol may disturb the spindle causing aneuploidy besides its other genotoxic effects. Since NAPQI is an arylating species preferentially binding to thiol groups, it has the potential for binding to tubulin leading to disturbances in spindle function.

Study of genotoxicity related to oxidative formation of reactive metabolites requires expression of the relevant CYP forms in the test system. A series of V79 cell lines devoid of spontaneous CYP activity has been constructed for stable expression of c-DNA's encoding at number of rat and human CYP forms (Doehmer *et al.* 1992; Wölfel *et al.* 1992). These cell lines have been used for study of both mutagenesis and aneuploidy (Doehmer 1993; Jensen *et al.* 1993b; Schmalix *et al.* 1993). Recently, a V79 cell line transfected with human CYP1A2 has been shown to correspond to human liver microsomes in terms of the kinetics of phenacetin metabolism and its specific and potent inhibition by fluvoxamine (Wölfel *et al.* 1992; Jensen *et al.* 1995).

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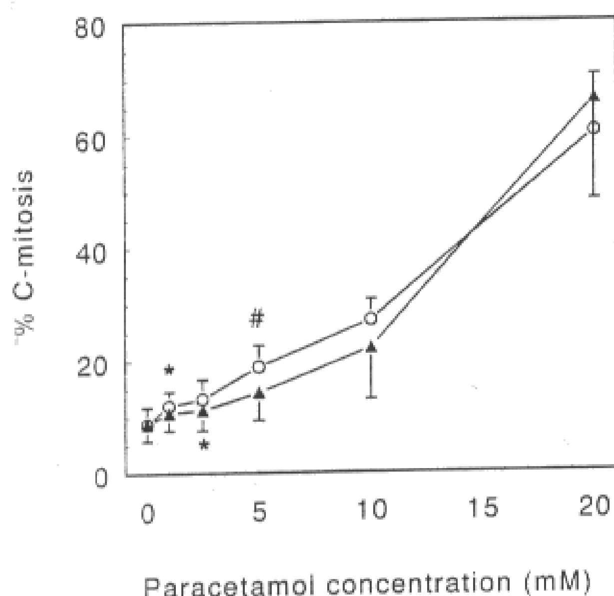

Fig. 1. Relationship between the concentration of paracetamol in 2 hr exposure and genotoxic effects in terms of spindle disturbances measured by c-mitosis frequency in a native V79 cell line (▲) and a V79 cell line expressing human CYP1A2 (○). Values are means with S.D. of 5–8 experiments. * denotes P<0.05 versus control; # denotes P<0.05 between the two cell lines.

The aim of the present study was to investigate spindle disturbing effects of paracetamol in relation to the metabolism by human CYP1A2 expressed in a V79 cell line. The spindle disturbances were evaluated by a highly sensitive and specific method, i.e., scoring of aberrant mitotic figures (partial and full c-mitosis – see (Önfelt 1986)).

Materials and Methods

The construction of the V79MZh1A2 (formerly named XEMhI-A2MZ) cell line has been described in details elsewhere (Wölfel *et al.* 1992). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Uxbridge, U.K.) supplemented with 7.5% heat-inactivated mycoplasma- and virus-screened foetal calf serum (Gibco), penicillin (100 U/ml), and streptomycin (100 µg/ml). Selection of plasmid-containing cells was maintained by addition of G418 400 µg/ml (Gibco). Cells were kept from reaching confluence at any time for maximal expression of enzyme activity. The V79MZh1A2 cell line was compared with the native V79MZ cell line which is devoid of CYP activities.

All incubations were performed at 37° in 5% CO_2 in air. Cells (150,000) were seeded on plastic coverslips in plastic petri dishes incubated for 24 hr giving asychronously growing populations. After being rinsed twice with 2.5 ml Hanks balanced salt solution the cultures were incubated for 2 hr in full medium with paracetamol (Nordisk Droge, Copenhagen, Denmark) 0, 1, 2.5, 5, 10 and 20 mM.

Immediately after exposure, the cells were fixed *in situ*. The fixative, methanol/acetic acid (3:1), was used briefly in a 1:1 mixture with HBSS followed by undiluted fixation for 1–2 hr. The cells were stained with 3% Giemsa (Gurr) in phosphate buffer. Normal and abnormal metaphases, anaphases and telophases were counted according to cytological standards of spindle disturbances in plant cells but expanded in order to be useful in mammalian cells (Önfelt 1986). Abnormal mitoses (c-mitoses) include disturbances like tripolar spindles, lagging of chromosomes in anaphase, ball metaphase, poor alignment of chromosomes in metaphase as well as total scattering of chromosomes in the cytoplasm. All abnormal mitoses, both partial and complete c-mitoses, were counted as c-mitoses. Two hundred mitotic cells per slide and two slides per concentration were examined in each experimental sets. Five to eight experimental sets were performed. All slides were coded before scoring and all slides were scored blindly in random order by the same person.

The effect of the paracetamol treatment on survival of the cells was investigated in parallel experiments. Immediately after the 2 hr exposure the cells were rinsed, trypsinised and resuspended in medium. An aliquot of the suspension was counted in a Coulter counter. One hundred cells were reseeded per Petri dish. Three dishes were used per concentration and the survival study was performed twice for each cell line. After one week the cells were fixed and colonies were counted.

In parallel experiments the CYP1A2 activity of the cells was estimated as phenacetin-O-deethylase activity by measuring the appearance of the metabolite paracetamol in the incubation medium as previously described (Jensen *et al.* 1993a & 1995). In addition CYP1A2 activity was estimated by the methoxyresorufin-O-deethylase activity (MROD) measured by the appearance of resorufin in the medium of intact cell cultures incubated with methoxyresorufin 5 µM and dicoumarol 10 µM for inhibition of diaphorase activity (Wortelboer *et al.* 1990; Burke *et al.* 1994).

Two factorial analysis of variance was used for statistical analysis. For homogeneity of variances log transformed values were used in the test of c-mitosis data. Duncan's multiple range test was used for *post hoc* comparison of means. Probability values less than 5% were considered statistically significant.

Results

In the native V79 cell line paracetamol increased c-mitosis frequency in a concentration dependent manner from 8.7±3.5% (control) to 66±18% at 20 mM (fig. 1). A significant increase to 13.3±3.5% was first seen at 2.5 mM in the native cell line (P<0.05). The expression of CYP1A2 shifted

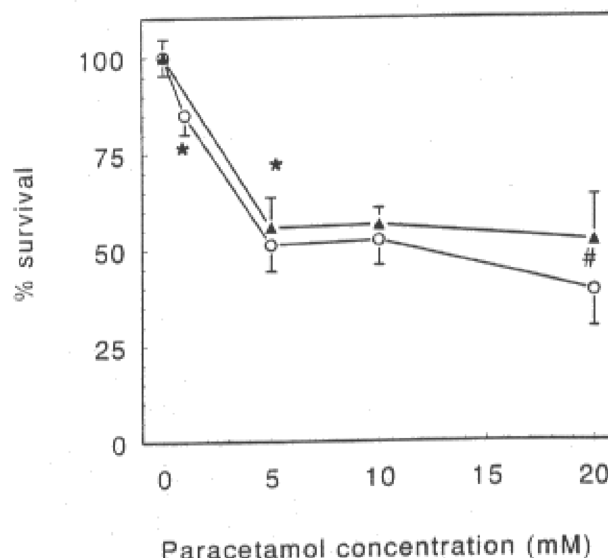

Fig. 2. Relationship between the concentration of paracetamol and cytotoxicity in terms of survival after exposure for 2 hr in a native V79 cell line (▲) and a V79 cell line expressing human CYP1A2 (○). Values are means with S.D. of 6 observations. * denotes P<0.05 versus control; # denotes P<0.05 between the two cell lines.

the concentration-effect curve slightly to the left ($P<0.05$) with the c-mitosis frequency significantly increased to $12.1\pm2.6\%$ ($P<0.05$) at 1 mM. At 5 mM paracetamol the c-mitosis frequency was $19.0\pm3.8\%$ in the CYP1A2 expressing cell line, i.e. significantly higher than in the native cell line ($14.4\pm5.0\%$; $P<0.05$). At other concentrations there were no significant differences between the cell lines. At 20 mM paracetamol the c-mitosis frequency was $61\pm10\%$ in the CYP1A2 expressing cell line.

Paracetamol was also cytotoxic (fig. 2). Cell survival was reduced at 1 mM and further to approximately 50% at 5 mM in both cell lines. In V79MZh1a2 cells only, survival was further decreased to $39\pm9\%$ at 20 mM ($P<0.05$). The phenacetin-O-deethylase and MROD activities of the CYP1A2 expressing cell lines were 20–60 and 1–4 pmol $min.^{-1}$ per 10^6 cells, respectively, whereas no metabolites could be detected in incubations with the native cell line.

Discussion

In the present study paracetamol exposure for 2 hr induced c-mitosis in V79 cells in a concentration-dependent manner. In cells expressing human CYP1A2 the concentration-effect curve was slightly shifted to the left suggesting that the oxidative metabolism of paracetamol to the reactive metabolite, NAPQI, plays only a minor role in the spindle disturbing effect.

Despite the potent arylating capabilities and covalent binding to DNA neither NAPQI nor paracetamol are mutagenic in the usual test systems (Dybing *et al.* 1984; Anonymous 1990) and the bulk of evidence do not support carcinogenicity (Anonymous 1990). Nevertheless, a number of genotoxic effects have been demonstrated *in vivo* and *in vitro* although the doses or concentrations required are rather high and may also cause other signs of toxicity (Dybing *et al.* 1984; Anonymous 1990; Giri *et al.* 1992; Hongslo *et al.* 1994).

In mice dosed with paracetamol *in vivo* chromosome aberrations in bone marrow cells were demonstrated from a single dose of 200 mg/kg body weight and sister chromatid exchange from 50 mg/kg (Giri *et al.* 1992), whereas 450 mg/kg failed to induce micronuclei (King *et al.* 1979). In agreement, prior glutathione depletion and 3–600 mg paracetamol per kg body weight were required to demonstrate covalent binding of NAPQI to DNA and strand breaks in mouse liver (Honglo *et al.* 1994). In rat dams 500 mg paracetamol per kg body weight caused aneuploidy in their 12-day embryos (Tsuruzaki *et al.* 1982).

In vitro paracetamol 5 mM was required to induce unscheduled DNA synthesis in isolated hepatocytes, whereas NAPQI added to the medium bound covalently to DNA and induced strand breaks in hepatoma cells at concentrations which later caused cytotoxicity (Dybing *et al.* 1984). For demonstration of micronuclei in a rat kidney cell line 10 mM paracetamol was required (Dunn *et al.* 1987). In V79 cells paracetamol caused DNA strand breaks at 3 mM but not at 1 mM (Hongslo *et al.* 1988) whereas chromosomal aberrations occurred concentration dependently from 3 mM (Muller *et al.* 1991). In a mouse mammary tumour cell line, sister chromatid exchanges and chromosomal aberrations have been shown at 1 mM of paracetamol (Hongslo *et al.* 1990). In that study the effect was suggested to be related to inhibition of ribonucleotide reductase and reduced DNA synthesis which could be demonstrated already at 0.1 mM (Hongslo *et al.* 1990). In the present study 1 mM paracetamol increased c-mitosis frequency in V79 cells expressing CY1A2 whereas 2.5 mM was required in the cells without this enzyme. These effects were mirrored by a reduction in cell survival at those concentrations. Thus, with the exception of reduction of DNA synthesis genotoxic effects of paracetamol is seen at concentrations exceeding plasma levels after therapeutic doses but certainly attainable in overdose. The volume of distribution of paracetamol is 0.9 l per kg body weight and a therapeutic dose of 1 g and an overdose of 50 g may thus result in peak concentrations of approximately 0.1 and 5 mM, respectively (Forrest *et al.* 1982). Nevertheless, even in human subjects taking therapeutic doses of paracetamol genotoxicity has been demonstrated in circulating lymphocytes in terms of chromosomal aberration, unscheduled DNA synthesis and sister chromatid exchange (Kocisova *et al.* 1988; Topinka *et al.* 1989; Hongslo *et al.* 1991), although the most recent and the only randomized double-blind controlled trial was unable to demonstrate chromosomal aberrations in this context (Kirkland *et al.* 1992). In addition, paracetamol may inhibit DNA synthesis and repair (Hongslo *et al.* 1990, 1993 & 1994). Moreover, paracetamol is massively used and abused and human exposure is frequent. Accordingly, even small signs of genotoxicity requiring rather high concentrations may have some as yet undetermined relevance for human risk.

The present spindle disturbing effects of paracetamol mirrored a cytotoxic effect as determined by the reduced survival of the cells. Thus, it cannot be excluded that the two effects are related and that some disturbed cells are bound for death without risk of aneuploid progeny. However, Önfelt (1987) has shown that reduced survival and spindle disturbances can occur independently and that cells surviving similar challenge can indeed form aneuploid daughter cells. So far, actual documentation of aneuploidy induced *in vivo* has required extremely labourious manual counting of chromosomes. Recently, new tools have emerged in molecular cytogenetics, in particularly related to multicolour fluorescence *in situ* hybridization (FISH) for analysis of male gametes (Abruzzo & Hassold 1995). With such assays it should be possible to study the risk of aneuploidy from compounds such as paracetamol *in vivo*.

The present slight enhancement of paracetamol-induced c-mitosis frequency by expression of CYP1A2 suggests that the reactive metabolite NAPQI plays only a minor role. Indeed, NAPQI has the properties to bind covalently to proteins, including tubulin. The effect of 20 mM paracetamol on survival was also increased by CYP1A2. However, the actual differences between the cell lines with and without

CYP1A2 expression were small and NAPQI may just have acted on top of a direct effect of paracetamol. Similarly, the clastogenic effect of paracetamol in V79 cells was only slightly enhanced by external activation by primary hepatocytes (Muller *et al.* 1991). In human liver microsomes CYP2E1 and CYP3A4 in addition to CYP1A2 are involved in the formation of NAPQI (Raucy *et al.* 1989; Thummel *et al.* 1993), but this fact is not likely to invalidate the inference from the present study regarding the limited role of paracetamol bioactivation in the possible spindle disturbing effects.

In conclusion the present study demonstrated that paracetamol at high concentrations may disturb the spindle of dividing cells conveying a risk of aneuploidy. The spindle disturbing effect was slightly enhanced by CYP1A2 which catalyse the formation of the reactive paracetamol metabolite, NAPQI. The increases in c-mitosis frequency were mirrored by reduced survival of the cells.

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