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

Klaus Gjevreig Jensen¹, Henrik Engelsen Poulsen¹, Johannes Doeherm² and Steffen Loft¹

¹Department of Pharmacology, University of Copenhagen, Denmark and ²Institute of Toxicology and Environmental Hygiene, Technical University, München, Germany

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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 3.7±2.3% (control) to 6.6±3.1% at 20 mM. A significant increase to 13.2±5.5% 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±3% in V79MZH1A2 cells only (P<0.05). The present study demonstrated that paracetamol may disturb the spindle of dividing cells causing a risk of aneuploidy. The spindle disturbing 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-acetylhydrazoquinoneimine, NAPQI (Hinson 1980; Hinson et al. 1990; Pumford et al. 1990), produced by oxidative metabolism catalysed by CYP1A2, CYP2E1 and CYP3A4 (Raucey et al. 1989; Thummel et al. 1993; Thomsen et al. 1995). Despite arylnating 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; Kocksova et al. 1988; Topinka et al. 1989; Anonymous 1990; Hongso 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 (Tatsumi 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 arylnating 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 (Doehner et al. 1992; Wölfel et al. 1992). These cell lines have been used for study of both mutagenesis and aneuploidy (Doehner 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).

Author for correspondence: Steffen Loft, Department of Pharmacology, University of Copenhagen, Præmien Institute, Blegdamsvej 3, DK-2200 Copenhagen, Denmark (fax +4533527619).
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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 (Östfot 1986)).

Materials and Methods

The construction of the V79MZ71A2 (formerly named XEMH-A2MZ) cell line has been described in detail 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 V79MZ71A2 cell line was compared with the native V79MZ cell line which is devoid of CYP activities.

All incubations were performed at 37° in 5% CO₂ in air. Cells (150,000) were seeded on plastic coverslips in plastic petri dishes incubated for 24 hr giving asynchronously 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 Droege, 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 1% BSS 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 (Östfot 1986). Abnormal mitoses (c-mitoses) include disturbances like tripolar spindles, lagging of chromosomes in anaphase, bull mea-

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 resuspended 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 in intact cell cultures incubated with methoxyresorufin 5 μM and dichloromethane 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-mitotic 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±2.6% (P<0.05) at 1 mM. At 5 mM paracetamol the c-mitosis frequency was 19±3.8% in the CYP1A2 expressing cell line, i.e. significantly higher than in the native cell line 14±5.0% (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±10% 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±9% 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 10⁶ 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; Hongso et al. 1994).

In mce 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 (Hongso 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 5 mM but not at 1 mM (Hongso et al. 1988) whereas chro-

mosomal aberrations occurred concentration dependently from 5 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 (Hongso 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 (Hongso et al. 1990). In the present study 1 mM paracetamol increased c-mitosis frequency in V79 cells expressing CYP1A2 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 0.91 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 (Kosesowa et al. 1986; Topinka et al. 1989; Hongso 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 (Hongso 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, Onfeldt (1987) has shown that reduced survival and spindle disturbances can occur independently and that cells surviving this 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 particular 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 (Raey et al. 1993; Thummler 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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