TOXICOLOGICAL SCIENCES **121(2)**, 428–430 (2011)
doi:10.1093/toxsci/kfr053
Advance Access publication March 7, 2011

# LETTER TO THE EDITOR

## Pathophysiological Relevance of Proteomics Investigations of Drug-Induced Hepatotoxicity in HepG2 Cells

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Received January 18, 2011; accepted January 24, 2011

We read with interest a recent paper by Van Summeren *et al.* (2011), who reported protein expression changes in HepG2 cells after exposure to various model drugs for 72 h. According to the authors, the objective of this investigation was to explore "new screening methods that address toxicological hazards early in the drug discovery process" and to use "proteomics techniques to gain further insight into the mechanistic processes of the hepatotoxic compounds." The authors used three compounds (amiodarone, cyclosporine A, and acetaminophen) in their studies. However, we would like to focus our comments on acetaminophen (APAP) because it is a clinically relevant hepatotoxicant and it is the most frequently used model toxin *in vivo* and *in vitro*.

Acetaminophen toxicity has been studied extensively during the last 3 decades, and many mechanistic details have been controversially discussed (Jaeschke and Bajt, 2006). However, one aspect is clearly undisputed: APAP toxicity requires metabolic activation to a reactive metabolite (*N*-acetyl-*p*-benzoquinone imine, NAPQI), which initially depletes glutathione and then binds to proteins (Nelson, 1990). There is no toxicity without protein binding (Nelson, 1990)! Protein adducts have been reported for mouse livers *in vivo* using various methods (Jollow *et al.*, 1973; Muldrew *et al.*, 2002), a human hepatocyte cell line (HepaRG) (McGill *et al.*, 2011b), and human overdose patients (Davern *et al.*, 2006). In addition, APAP-induced mitochondrial dysfunction, which correlates with mitochondrial protein adduct formation, has been observed in mice (Jaeschke and Bajt, 2006), human HepaRG cells (McGill *et al.*, 2011b), and there is emerging evidence for this mechanism in human overdose patients (McGill *et al.*, 2011a). Thus, in order to gain further insight into the mechanisms of APAP toxicity as the authors intended with their study (Van Summeren *et al.*, 2011), the experimental system has to have the capability to reproduce the basic feature of being able to metabolically activate this drug. However, HepG2 cells, as many other hepatoma cells, have very low levels of P450 enzymes, and therefore, APAP cannot induce relevant toxicity in these cells unless P450 levels are transcriptionally induced (Roe *et al.*, 1993) or the cytochrome P4502E1 gene is transfected into the cells (Dai and Cederbaum, 1995). Though the authors mention this caveat, they justify use of the HepG2 cells by quoting studies by Schoonen *et al.* (2005a, 2005b), which "classified 70% of the compounds with known toxicity as cytotoxic when using HepG2 cells." However, it is interesting to note that in both these papers acetaminophen (paracetamol) was classified as "nontoxic" (Schoonen *et al.* 2005a, 2005b), and in fact, APAP did not produce significant changes in either reactive oxygen species production or depletion of glutathione in HepG2 cells (Schoonen *et al.*, 2005b). In support of the limited metabolic activation in HepG2 cells, protein adduct formation is less than 10% of adducts observed in HepaRG cells even after 24 h with APAP (McGill *et al.*, 2011b). In addition, HepG2 cells do not experience the characteristic mitochondrial dysfunction observed in mice *in vivo* (Jaeschke and Bajt, 2006), primary mouse hepatocytes (Bajt *et al.*, 2004) and in HepaRG cells (McGill *et al.*, 2011b). The authors' mention of the expression of Phase II enzymes in HepG2 cells has little relevance here. There are exceptional cases of drug activation or toxification after sulfation, glucuronidation, or glutathionylation (e.g., morphine-6-glucuronide, formaldehyde formation after dichloromethane glutathionylation, etc.). In the case of APAP and most other drugs, however, these mechanisms make up the primary physiological strategy for drug inactivation and elimination. If anything, the expression of these enzymes should further reduce the toxicity of APAP in HepG2 cells.

Nevertheless, hepatoma cells can respond to APAP. Prolonged APAP exposure in these cells leads to caspase-dependent apoptosis (Boulares *et al.*, 2002). In striking contrast, all cells that are capable of metabolically activating APAP do not show evidence of caspase activation or apoptosis

(Gujral *et al*., 2002). Instead, cell death of metabolically competent hepatocytes *in vivo* and *in vitro* occurs by oncotic necrosis as indicated by cell and organelle swelling, massive cell contents release, and karyorrhexis and karyolysis (Bajt *et al*., 2004; Gujral *et al*., 2002; McGill *et al*. 2011b). Together, these data strongly suggest that proteomic, genomic, and other "omic" responses to APAP in HepG2 cells have little or no relevance for signaling mechanisms of APAP-induced cell death in animals or man. This conclusion is also supported by the fact that the basic gene expression profile of cell lines has almost nothing in common with *in vivo* liver gene expression (Boess *et al*., 2003). Thus, HepG2 cells are not a useful model to study mechanisms of APAP-induced liver injury. Moreover, HepG2 cells are a poor experimental system to assess the hepatotoxic potential of unknown drugs as virtually all compounds whose toxicity depends on metabolic activation would be missed.

However, the concerns with the majority of current genomics and proteomics studies including the current one go beyond a poor choice of cell lines. The authors evaluated the toxic response of HepG2 cells with the MTT assay (Van Summeren *et al*., 2011). This assay is not a cell death assay but measures dehydrogenase activity mainly of mitochondria (Huet *et al*., 1992). As such, the authors did not use a true cell death assay as they assumed (Van Summeren *et al*., 2011). Without additional supportive evidence, they may have merely measured a moderate metabolic disturbance of the cell rather than true toxicity. In addition, as recently demonstrated, most cell culture conditions do not reflect the physiological environment of liver cells *in vivo*. This is particularly true of the oxygen content. Hepatocytes *in vivo* experience levels of 3–9% oxygen (Kietzmann and Jungermann, 1997) but most cells in culture are exposed to room air (21% oxygen) or even higher. This can result in exaggerated mitochondrial oxidant stress and cell death (Yan *et al*., 2010) and has the potential to severely affect gene expression changes and the mechanisms of drug toxicity. Furthermore, all drugs including APAP were dissolved in dimethyl sulfoxide (DMSO) (Van Summeren *et al*., 2011). Although DMSO can induce cytochrome P450 enzymes, it is also a competitive CYP inhibitor (Yoon *et al*., 2006) and thereby has the potential to modulate drug toxicity responses, at least in metabolically competent hepatocytes (Jaeschke *et al*., 2006). Thus, solvent effects need to be considered in drug toxicity studies.

The main issues of *in vitro* testing paradigms are not to optimize gene or protein expression changes or toxicity *in vitro* but the critical question is whether the *in vitro* conditions are relevant for the *in vivo* situation (Jaeschke *et al*., 2010). How can we expect to obtain drug toxicity data relevant for animals or humans if we use cell lines that have almost nothing in common with a primary hepatocyte in the intact liver and if we perform experiments under conditions that have little in common with real life? The extreme focus on the latest analytical technology but ignorance of the biology appears to be the main reason why there is little use for data generated by many of these "omics" studies.

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