An improved resazurin-based cytotoxicity assay for hepatic cells

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**Abstract**

A simple resazurin-based cytotoxicity assay is presented for screening of cytotoxicity in hepatocytes and liver cell lines. Human hepatoma (HepG2) cells in 96-well culture plates were exposed to known toxic (cisplatin, 5-fluorouracil, ethionine, flufenamic acid, and diflunisal) and control (transplatin, 5-chlorouracil, methionine, and acetylsalicylic acid) compounds for 1–3 days, and resazurin (5 µmol/L) was added. A conventional short-term (1 h) assay was first performed, where cytotoxicity is indicated by decreased reduction of resazurin to its fluorescent product resorufin. Our improved assay consists of additionally measuring fluorescence 2–4 days later, when cytotoxicity is indicated by a striking increase in the concentration of resorufin, resulting from two distinct processes. First, viable liver-derived cells slowly convert resorufin to nonfluorescent metabolites. Fluorescence of control cell wells decreased to background during a 2- to 4-day exposure to resazurin. This metabolism of resorufin was largely blocked by dicumarol and to lesser extents by disulfiram and SKF525a. Second, dead or dying cells slowly convert resazurin to resorufin but do not further metabolize resorufin; thus this fluorescent metabolite accumulates to high levels in wells with dead cells by 2 to 4 days. A similar increase in fluorescence associated with cytotoxicity was observed in primary cultures of rat hepatocytes using the long-term resazurin-based assay. In addition to an improved signal relative to the short-term assay, the inversion of the fluorescent signal from high = alive short-term to high = dead long-term allows determination of two independent cytotoxicity endpoints after addition of one innocuous vital dye.

**Abbreviations:** FLU, relative fluorescence units; 5FU, 5-fluorouracil; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

**Introduction**

Cytotoxicity assays provide convenient in vitro screens for comparing relative toxicities of compounds (reviewed by Harbell et al., 1997; Skehan, 1999; Wilson, 2000). Since the liver is a common and serious target of most orally administered drug toxicities, primary liver cells
and human hepatic cell lines are frequently used as targets in these screening assays. Cytotoxicity assays are based on cellular functions that are particularly sensitive to and irreversibly inhibited by toxins. The best cytotoxicity assays are those that are easiest to perform. A battery of dissimilar cytotoxicity assays is usually run, giving complementary data that are easier to interpret. Among the more commonly used are cellular permeability assays; some measure live cell function (for example, the ability to accumulate acetoxyethyl ester dyes), others reflect loss of the ability to exclude dyes such as trypan blue and propidium iodine. Assay of the release of cytosolic lactate dehydrogenase into the media is time-honored but cumbersome. Cytotoxicity assays dependent on the metabolic state of treated cells provide a different approach. Cellular ATP levels can be easily measured in a luminescent assay. Reductase-based assays (such as MTT, XTT, WST-1, and Alamar Blue assays) require live cells or at least functioning mitochondria to convert a precursor dye into a measurable fluorescent or colored product. Reductase-based assays are heavily used as they are sensitive, easy to use, and inexpensive.

Resazurin, a redox-sensitive dye long used for detecting bacteria and yeast, is the primary reporter dye in Alamar Blue, a proprietary mixture with other compounds (poising agents) added to optimize mitochondrial reduction and inhibit nonspecific reduction of resazurin (Lancaster and Fields, 1996). According to the package insert, Alamar Blue (resazurin) is reduced by respiring mitochondria, and the reduced form (resorufin) is a more sensitive reporter than other commonly used mitochondrial reductase dyes such as MTT and XTT. MTT and XTT are reduced by components early in the respiratory chain and ultimately block electron flow and respiration; these indicator dyes are thus inherently toxic. Alamar Blue substitutes for molecular oxygen as an electron acceptor for oxidoreduc-
tases, such as cytochrome oxidase, the last cytochrome in the respiratory chain; accepting electrons at this last step does not interfere with respiration, and thus resazurin is nontoxic. Recent reports have questioned the mitochondrial location of Alamar Blue (as well as MTT and XTT) reduction, as well as the need for poising agents (Rasmussen, 1999). Although the details of its cellular metabolism remain unclear, resazurin is a useful short-term general indicator of cellular metabolic activity; in particular, healthy cells reduce resazurin more effectively than do dead or dying cells. Resazurin reduction leads to a loss of oxygen and a gain of hydrogen in the molecule, and its reduced product, resorufin, can be detected both colorimetrically and fluorometrically. Resorufin is a highly fluorescent molecule, and also serves as an indicator product of several cytochrome P450 isoform-sensitive substrates. Unlike other reductase indicator dyes, resazurin is nonmutagenic and relatively nontoxic, and can be washed free from the cells so that other assays can be performed. The resazurin-based cytotoxicity assay is also relatively insensitive to interference from drugs, serum, and phenol red (Page et al., 1993; Mershon et al., 1994; Ramu et al., 1996; Gazzano-Santoro et al., 1997; Pitt et al., 1997; Zhi-Jun et al., 1997; Nociari et al., 1998; Mathew et al., 1999).

Since resazurin is relatively nontoxic, it is possible to add resazurin and follow resorufin production over a period of days to continually assess effects of agonists and growth factors on cellular proliferation (Ahmed et al., 1994). This approach may work well for many types of cells, and is more convenient and easier to interpret than performing a series of short-term viability/proliferation assays on similarly treated “sister” plates over a period of days. Fortuitously, we found that this long-term resazurin exposure approach does not work well for liver-derived cells, but rather yields an improved cytotoxicity assay. After examining a