Uridine uptake inhibition as a cytotoxicity test for a human hepatoma cell line (HepG2 cells): comparison with the neutral red assay

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Abstract

This study describes a sensitive microassay for measuring cytotoxicity based on the degree of inhibition of RNA synthesis in HepG2 cells. RNA synthesis is measured by the kinetic uptake of radiolabeled uridine. A large number of compounds were tested in a wide range of concentrations. The concentration required to induce 50% inhibition of HepG2 uridine uptake rates (IC_{50}) was determined for each compound and used to rank its potency. These IC_{50}s were compared with IC_{50}s measured with the neutral red assay. 2-acetylaminofluorene, benzo[a]pyrene and methylnitrosourea were not cytotoxic in the neutral red assay. Uridine uptake was always inhibited at lower concentrations than those required in the neutral red assay, suggesting that the uridine uptake assay is a more sensitive indicator of toxic action than the neutral red inclusion. Uridine uptake assay provides a rapid and quantitative method for assessing toxicity in a human cell line. Application of this method to bottled spring waters are described. Due to its high sensitivity and reproducibility, this method provides a suitable tool for screening a great number of samples and will be a helpful test for evaluating food safety and controlling the recycling process of wrapping materials. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Cytotoxicity-HepG2 cell line; Uridine uptake; Neutral red

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1. Introduction

The use of in vitro assays as short term tests or as screening tests for predicting genotoxicity of chemicals and for studing cellular and subcellular mechanisms is receiving increasing attention. People who work with in vitro genotoxicity tests usually check the compound's cytotoxicity first to establish the concentration range they will use in genotoxicity assays. It is generally agreed that the maximum concentration of the tested substance should produce viability $> 75\%$ in order to avoid false negative or positive responses due to cytotoxicity in genotoxicity test such as the comet assay (Henderson et al., 1998). Some cytotoxic endpoints consist in measuring a specific biochemical parameter like the tetrazolium MTT test (Mosmann, 1983) or the neutral red assay. These assays are sensitive but sometimes too selective. The most frequently used endpoints in cellular toxicology are the breakdown of the cellular permeability barrier, measured by dye exclusion (trypan blue) or by the release of intracellular enzymes like lactate dehydrogenase (Decker and Lohmann-Matthes, 1988); the cellular protein content (Balls and Bridge, 1984) and plating efficiency (Strom et al., 1983). But some of these tests are principally based on cell mortality state as a consequence of membrane damage or cell detachment (Fauris and Vilagines, 1998). However, before they die, cells exhibit biochemical perturbations (morbidity state) induced by the toxic compound under study. Thus, agents that alter the integrity of plasma membranes, either directly or as a secondary result of damage to some other cell component, will alter the net rates of nutrient uptake in the cells. Furthermore, the rates of some uptake processes are well regulated aspects of the metabolism of cultured cells changing in reproducible ways in response to alterations in cellular growth states. Thus, agents that stimulate or modify cell growth may manifest their actions by perturbations in nutrients uptake rates (Koren, 1980). Kjeldgaard (1963) has shown that DNA and protein synthesis are directly proportional to cellular growth rate while RNA synthesis is proportional to the square of cellular growth rate. In fact, this high sensitivity is essentially due to the dynamic nature of the test which does not compare amounts of synthesised RNA but the rapidity of RNA synthesis. Uridine is phosphorylated before integrated into the RNA. The uptake of nucleosides by mammalian cells seems to proceed in two steps; (1) nucleosides are transferred rapidly across the plasma membrane by a facilitated diffusion mechanism; and (2) intracellular nucleosides are phosphorylated by specific nucleoside kinases (Rozengurt et al., 1977). Therefore, both the loss of membrane activity and alterations in phosphorylation caused by altered growth status or changes in concentrations of intracellular high energy metabolites should be revealed by changes in uridine uptake.

These perturbations can be detected in the cell; rapidity of RNA synthesis of viable Hela S3, or Balb/c 3T3 cells is a cellular parameter which has been shown to be very sensitive (Shopsis and Sathe, 1984; Fauris et al., 1985). This cytotoxicity test is based on the quantitative inhibition of the RNA synthesis rate of cells in the presence of toxic compounds. Its efficiency has been demonstrated by comparison of data with those obtained with the more classical physico-chemical determinations (Fauris et al., 1985). This test is already being applied to the detection of pollutants in all types of water (Afnor, 1996).

In order to further analyse mechanisms involved during DNA lesions, we are interested in a global, sensitive and quantitative cytotoxic assay measuring a morbidity state rather than a mortality state for detecting the chemosensitivities of HepG2. HepG2 is a human hepatoma cell line with a wide variety of liver-specific metabolic responses to different kind of drugs (Knowles et al., 1980; Knasmuller et al., 1998) and a well functioning glutathione system (Dierickx, 1989). Indeed, human hepatoma line (HepG2) plays a crucial role in the activation/detoxification of genotoxic pro-carcinogens (Knasmuller et al., 1998) and reflects metabolism of such compounds in vivo better than experimental models with metabolically incompetent cells and exogenous activation mixtures (Natarajan and Darroudi, 1991; Darroudi and Natarajan, 1993).

Thus, we proposed to set up the uridine uptake assay which is normally used with cells living in

suspension, with HepG2 cells, an adherent cell line. As we intend to use further this test as a screening method, we have adapted this protocol to microtitration plates (96-wells). For this study, we have chosen toxic compounds (H_2O_2, SO, $K_2Cr_2O_7$), genotoxic compounds (MMS, MNU, MNNG, 4-NQO, HMPA) and hepato-carcinogens (DMN, 2-AAF and B[a]P) with different action mechanisms. To establish the uridine uptake assay sensitivity, concentration of toxic compounds required to induce 50% of the inhibition (IC_{50}) in uridine uptake rates has been determined and compared with IC_{50}s obtained with an other cytotoxic assay, the neutral red assay. Neutral red is a supravital dye taken up in the lysosomes of viable cells (Borenfreund and Puerner, 1984), this assay is often used to measure relative cytotoxicities of a spectrum of agents including surfactants, pharmaceutical, industrial chemicals and aquatic pollutants (Borenfreund et al., 1988).

Some examples of application of this new method are described.

2. Material and methods

2.1. Chemicals

Dulbecco's Modified Eagle's Medium (DMEM) with glutamax®, heat-inactivated fetal bovine serum (FBS), phosphate buffered saline (PBS), trypsin (0.05%)-EDTA (0.02%), folic acid and sodium bicarbonate ($NaHCO_3$) were obtained from Gibco laboratories (Cergy-Pontoise, France). Trypan Blue 0.5% (w/v), sodium dodecyl sulfate (SDS), dimethylsulfoxyde (DMSO), uridine were provided by Sigma Chemicals (La Verpillière, France), as well as the toxic compounds (MMS (purity > 98%), DMN, 2-AAF (purity 97%), B[a]P (purity 97%), MNNG (purity > 97%), MNU, H_2O_2, $K_2Cr_2O_7$ (purity > 99.5%), HMPA (purity > 95%), SO (purity > 97%)). 4-NQO (purity > 97%) and trichloroacetic acid (TCA) were from ICN Biochemicals (Orsay, France). [5,6-^{3}H] uridine (1.33 TBq/mmol) was purchased from Amersham Pharmacia Biotech (Les Ulis, France). All other chemicals were all of analytical grade.

2.2. Cell and culture media

2.2.1. HepG2 cells were a gift of Fournier Laboratories Research Center (Daix, France)

Routine monitoring has shown the HepG2 cells to be mycoplasma free. The cells were grown in monolayer culture in DMEM with the addition of 10% FBS and subcultured every 5–7 days at 1:3 split ratios. Medium was changed every 2 days and the cells were maintained at 37°C in a humidified atmosphere containing 6% of CO_2. Stocks of cells were routinely frozen and stored in liquid N_2.

2.3. Treatment

2.3.1. All experiments were performed with cells at passage levels between 88 and 93

Individual wells of a 96-well tissue culture plate (Dutscher, France) were inoculated with 0.2 ml of the culture medium containing 5×10^4 HepG2 cells by using an electronic Biohit Proline multi-channel pipettor (Prolabo, France). Plates were prepared in parallel for the uridine uptake assay and the neutral red assay.

After 28 or 44 h incubation, cells were treated for 20 or 4 h depending on the compound tested (Fig. 1), as some have a quick action (4 h contact) and others need more time to exert their cytotoxicity (20 h contact). For the treatment, 0.2 ml of culture medium supplemented with 0.5% FBS with or without (control) various concentrations of toxic compounds dissolved in DMSO (0.1%) were added into the wells.
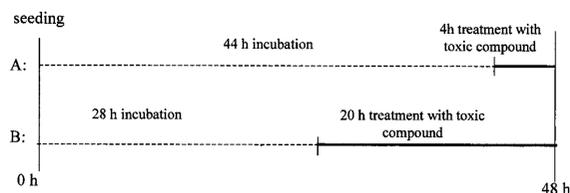

Fig. 1. HepG2 treatment with toxic compounds. (A) Toxic compound with 4 h treatment. (B) Toxic compound with 20 h treatment. HepG2 cells were incubated at 37°C in a humidified atmosphere containing 6% CO_2. - - - - HepG2 incubation time before treatment with toxic compound. — Treatment time with toxic compound.

All experiments were performed with four wells per concentration arranged in geometric series. After treatment, cytotoxicity tests were performed.

2.4. Experience of reversibility

After cell treatment with different concentrations of toxic compounds, the medium was removed and the cells washed with PBS, microwells were refilled with fresh culture medium supplemented with 10% of FBS. Then, cells were incubated 4 or 7 h at 37°C with high humidity and 6% CO_2 and ^3H uridine uptake kinetics were determined.

2.5. Polluted samples

Samples have been prepared with mineral water in glass flasks and deliberately polluted with two different concentrations of diazinon (Sophyc, Levallois Perret, France), SDS or limonen (Sigma, La Verpillère, France). Cocktails were also prepared in order to identify potential synergic effects between agents. All samples were made by ADRIAC laboratories (Reims, France) following a blind procedure. For these assays, 10X Dulbecco's Modified Eagle's Medium (Gibco laboratories, Cergy-Pontoise, France) was used and diluted just before use with sterile water sample. The medium was supplemented with 3.7 g/l $NaHCO_3$, 4 mg/l folic acid, 29.2 mg/ml glutamin and 0.5% FBS. The pH of the medium was adjusted to 7.4 with HCl 1M.

2.6. Cytotoxicity assays

2.6.1. ^3H uridine uptake kinetic (RNA test)

Labelling of RNA was initiated by addition of 10 μl tritiated uridine (0.8 M and 0.3 μCi/well) to each well every 5 s. Uridine incorporation was stopped by addition of 3% (w/v) SDS (30 μl) in each well every 5 min and until 30 min. Every 5 min, samples were substracted and spotted on whatman 3 MM paper (Polylabo, Strasbourg, France). After drying, the paper sheet was chromatographed in 5% (w/v) TCA. Radioactivity was determined by liquid scintillation counting using
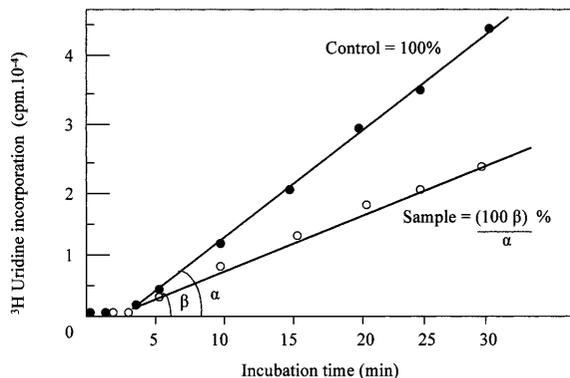

Fig. 2. Determination of HepG2 viability in the uridine uptake assay. ●, uridine incorporation of control cells. ○, uridine incorporation of sample. α and β indicate, respectively, control and sample slopes.

Optiscint Hisafe Wallac (EG and G Instruments, Evry, France).

RNA synthesis rate was determined by the slope of the regression straight line obtained from the experimental values. It was then expressed in relation to that obtained with the non-toxic blank sample (DMSO) which is maximal and considered to be 100% (Fig. 2).

Results are expressed as RNA synthesis percentage of the results obtained with the control cells. At and under 70% of control viability, the assay is considered positive and the compound toxic for the cells.

2.6.2. Neutral red assay

The neutral red assay (NR) was performed with microtitration plates and with a fluorimetric method, according to the procedure described by Rat et al. (1994). At the end of the treatment incubation, the medium was replaced with 0.2 ml of DMEM per well containing 50 μg/ml of neutral red, and the plate was returned to the incubator for 3 h. All incubation steps were maintained at 37°C in a humidified 6% CO_2 and 94% air atmosphere allowing the lysosomes of viable cells to take up the dye. Thereafter, the cells were carefully washed twice with 0.2 ml of PBS to eliminate extracellular NR. The incorporated dye was eluted from the cells by adding 0.2 ml elution medium (50% ethanol supplemented with 1% ace-

tic acid, v/v) into each well followed by gentle shaking of the microplate for 10 min (Titramax, Polylabo, France). The plate was then transferred to a microplate counter equipped with a 535 nm excitation wavelength and 580 nm emission wavelength to measure the fluorescence of the extracted dye on a Dynex spectrofluorimeter (France) at 7 volts sensibility. Results are expressed as percent of fluorescence of neutral red extracted from control cultures.

2.7. Toxicity ranking

The relative toxicities of the test compounds were established by determination of the toxic concentration required to induce 50% inhibition of uridine uptake or neutral red incorporation (IC_{50}). IC_{50}s were calculated by linear regression analysis of cytotoxicity assays data induced by a range of test compound concentrations.

3. Results

3.1. Uridine uptake kinetic assay conditions

3.1.1. Uridine uptake kinetic with different concentrations of HepG2 cells

The procedure originally described by Fauris et al. (1985) has been set up especially for cells living in suspension like Hela S3 cell line. However, these conditions are not suitable with an adherent cell line like HepG2. We decided to use microtitration plates in order to further screen great numbers of samples at different concentrations. As cells are incubated with the toxic compound in our experimental conditions in a maximal time of 48 h, increased dilutions of cells have been performed in 96-wells microtitration plates in order to study the cell density effect on RNA synthesis kinetics during 48 h. Slopes of the different straight lines have been calculated and linear regression coefficient determined for each kinetic point and expressed in relation with cells number added into the well (Fig. 3).

After 48 h incubation, data show higher slope values when cells concentrations per microwell are at least 40 000 cells. Linear regression coefficient
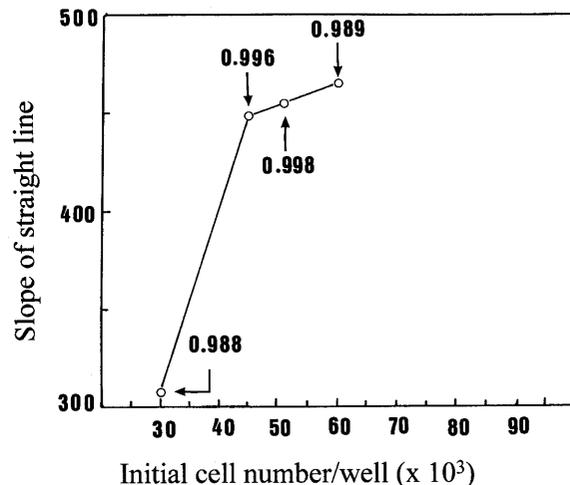
Fig. 3. Uridine uptake kinetic with different HepG2 seedings. Kinetics were determined starting at 5 min and for the next 25 min. ○, slope of regression lines obtained with different concentrations of cells. Arrows point out linear regression coefficient values.

were correct as well (around 0.998). At higher density (60 000 cells), even if the slope was a bit higher (465), microscopic observations of the microtitration wells indicated a cell density too high after 48 h leading to cell blebbing and cell detachment from their support suggesting a lack of space for the cells in the well.

3.1.2. Uridine uptake kinetic of HepG2 cells with the time

Uridine uptake kinetic was determined at different incubation times (Fig. 4). The data show
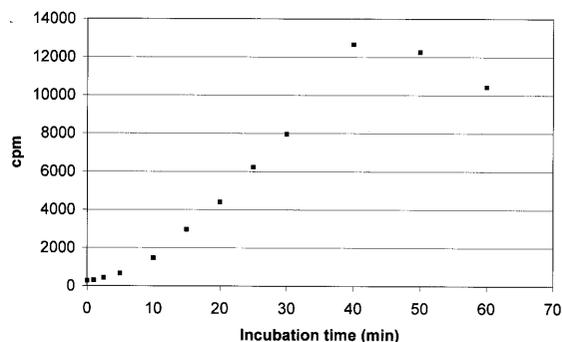
Fig. 4. Kinetic of ³H uridine incorporation into the HepG2 cells.

the presence of a lag phase of 5 min corresponding to the incorporation of ^3H uridine into the cells. After that, incorporation was linear with incubation time and then reached a plateau after 40 min.

Assay conditions of the uridine uptake into HepG2 cell line were then fixed with a seeding of 50 000 cells to get the highest sensitivity with a good linear regression coefficient and the kinetics were determined starting at 5 min and for the next 25 min.

3.2. Cytotoxic effect of xenobiotics on the uridine uptake kinetic in HepG2 cells

Subsequent experiments with 11 different chemicals were performed using a 4 or 20 h treatment according to the compound tested (Fig. 5). In these experiments, a wide range of concentrations of the test compounds has been used, maximal concentrations were systematically established according to their dissolution capacity into the culture medium. Only HMPA was not cytotoxic in our experimental conditions as the slopes obtained with the concentrations tested were not different from the control cell value. For all other compounds, data are represented on Fig. 5. When HepG2 cells are in contact with 4-NQO, MNNG, $K_2Cr_2O_7$, H_2O_2, SO, MMS, MNU, 2-AAF, DMN or B[a]P, slopes of uridine uptake kinetic assay decreased (88–0.4%) following a concentration dependent pattern suggesting a cytotoxic effect of the compound when the concentration increased. Linear regression coefficients were up to 0.98 for each experiment (data not shown).

Expressed in relation to the control slope value (Fig. 5), microscopic observations of the microtitration plates show cell blebbings, which is a sign of an advanced toxicity at % superior or equal to 70. Thus, we considered that 70% of control cell viability was a cytotoxic concentration in the uridine uptake assay. At very low percent (10% and above), cells were totally detached from the wells after 48 h incubation. In order to compare further the cytotoxic potency of all the xenobiotics tested, we determined for all compounds the IC_{50} values (Table 1). For example, the IC_{50} determination is described in Fig. 6 for the MMS,

when data are plotted as probit of control versus logarithmic chemical concentration, IC_{50} value is 0.9 ± 0.07 mM. IC_{50} values of the Table 1 show that in the order of increasing cytotoxicity, DMN is the less cytotoxic compound for HepG2 cell line following by MNU, H_2O_2, MMS, SO, 2-AAF, MNNG, $K_2Cr_2O_7$, B[a]P and 4-NQO.

3.3. Reversibility experiment

Reversibility is an important requisite of a toxicity test indicating the possible recovery from toxic insult. In this experiment, cells were in contact with 0.75 or 1 mM of MMS for 4 h. After washing the cells and replacing the culture medium, cells were able to recover after 4 h most of their capacity of uridine uptake kinetic (58% after treatment and 88% after medium change and washing) suggesting that the cells viability was intact (Fig. 7a). However, when the cells were in contact with 1 mM MMS, (Fig. 7b) the concentration leading to 32% viability, they did not recover after 4 h (54%) or even longer (data not presented).

3.4. Validation of the uridine uptake assay

IC_{50}s obtained with the uridine uptake assay have been compared with IC_{50}s obtained in the red neutral assay, an other cytotoxicity assay which can be used with microtitration plates (Rat et al., 1994) (Table 1). Except the HMPA which was never cytotoxic at concentration tested whatever the assay used, all compounds tested showed a cytotoxic effect at least in the uridine uptake assay. Indeed, 2-AAF, B[a]P and MNU were only cytotoxic in the uridine uptake assay. For all the other compounds, IC_{50}s determined with uridine uptake assay were always lower than those obtained with the neutral red test suggesting a higher sensibility of the uridine uptake assay. Discrepancies between both IC_{50}s changed with the compound tested. The smallest difference was observed with MMS (1.5-fold). It was more important with DMN (3.2-fold), $K_2Cr_2O_7$, SO and H_2O_2 (4.5-fold) and difference in IC_{50} values was very high when 4-NQO or MNNG (145-fold) were tested.
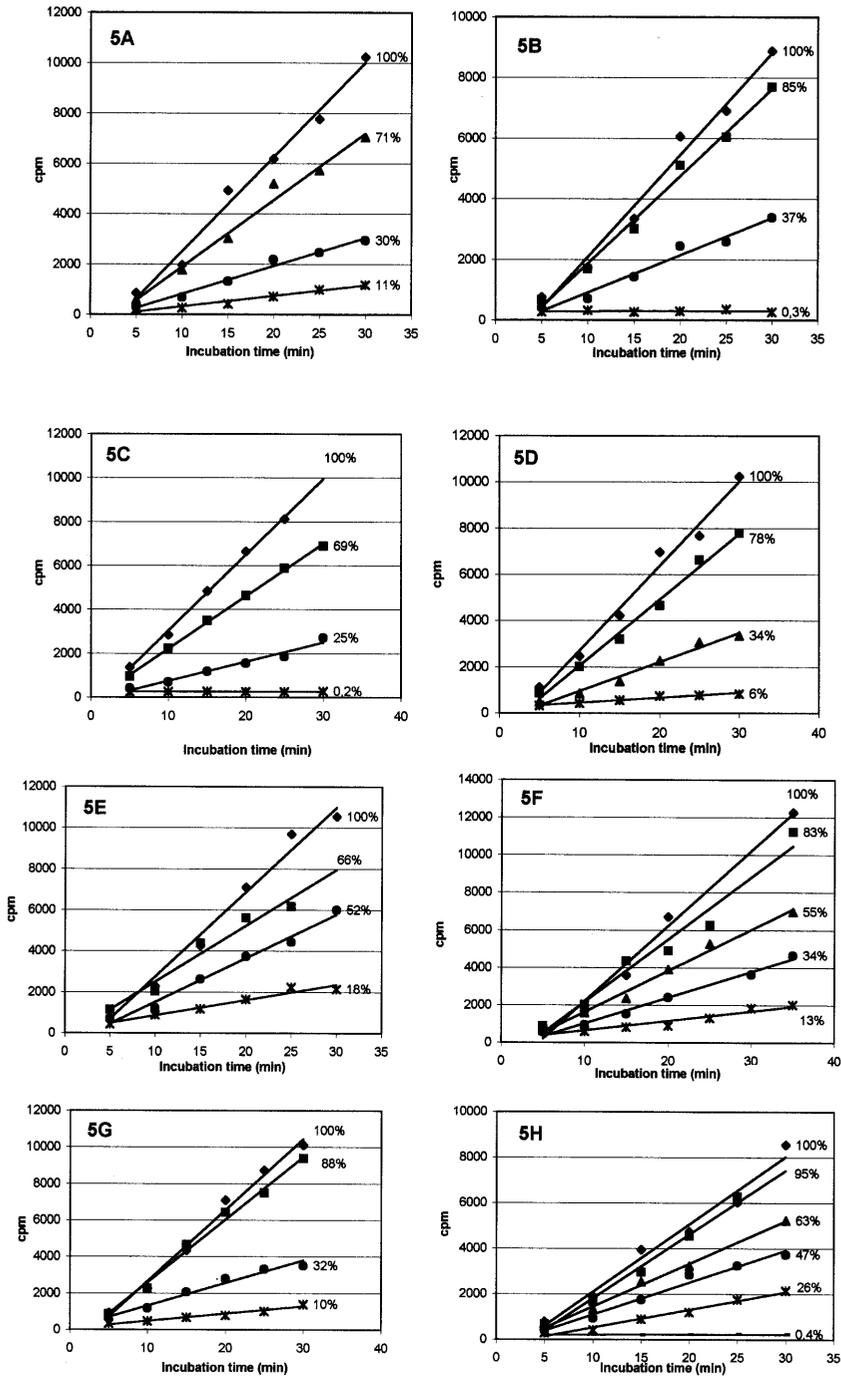
Fig. 5. (*Continued*)

3.5. Polluted samples screening

Uridine uptake assay was efficient for the detection of contaminants present in water issued from spring water bottles (Table 2). Limonen was cytotoxic in the uridine uptake assay only at 10 mg/l (63% of the control viability).

SDS was the less cytotoxic compound (70% at 20 mg/l). Diazinon was more potent; 63% of the control viability at 5 mg/l and 51% at 10 mg/l. All the mixtures tested were strongly cytotoxic (0% of the control cells viability). All contained a high concentration of SDS (40 mg/l) suggesting that combinations of agents effects were the same as those obtained with individual components.

4. Discussion

This study was investigated in order to design a sensitive method for the screening of toxic compounds in a human hepatoma cell line. In genotoxicity, testing the selection of an adequate test substance concentration range is an essential prerequisite. In general, guidelines recommend that with increasing concentrations, cytotoxicity effects should become detectable. Compared with the neutral red assay (NR), the RNA synthesis kinetics presented a higher reproducibility and sensitivity. This assay is capable of detecting a large range of toxic compounds. This study demonstrates that IC_{50}s determined in the RNA synthesis assay are always lower than those calculated with the NR assay. Differences in sensitivity between both assays could be high as we have shown with 4-NQO or MNNG. 2-AAF, B[a]P and MNU were not detected as cytotoxic compounds in the NR assay whatever the concentrations used. Even if both assays are measuring different cytotoxic endpoints, the higher sensitivity of the uridine uptake test could be due also to the morbidity state of the cells. Thus, we have shown that when the culture medium was changed after treatment with a cytotoxic compound, HepG2 cells were able to recover a good viability. In this study, each of the tested compounds has its own action mechanism.

H_2O_2 is a major component of reactive oxygen species (ROS) produced intracellularly during many physiological and pathological processes in
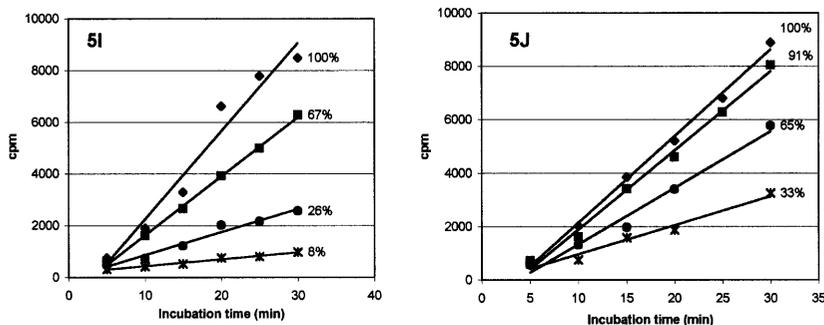

Fig. 5. Kinetic of ^{3}H uridine incorporation into the HepG2 cells in presence of different xenobiotics. Percentages represent sample cell viability compared with control cells (100%). 5A, potassium dichromate, ◆ control, ▲ 2.5 μM, ● 5 μM, ★ 10 μM. Control regression line value, $y = 374.58x - 1229.5$, 5B, hydrogen peroxide, ◆ control, ■ 0.1 mM, ● 1 mM, ★ 10 mM. Control regression line value, $y = 336.26x - 1267.5$, 5C, styrene oxide, ◆ control, ■ 0.1 mM, ● 1 mM, ★ 10 mM. Control regression line value, $y = 345.46x - 417.12$, 5D, methyl-methane-sulfonate, ◆ control, ■ 0.5 mM, ▲ 1 mM, ★ 2 mM. Control regression line value, $y = 365.76x - 954.53$, 5E, methylnitrosourea, ◆ control, ■ 5 mM, ● 7.5 mM, ★ 10 mM. Control regression line value, $y = 411.99x - 1372.2$, 5F, 2-acetylaminofluorene, ◆ control, ■ 10 μM, ▲ 50 μM, ● 100 μM, ★ 250 μM. Control regression line value, $y = 400.31x - 1793.4$, 5G, benzo[a]pyrene, ◆ control, ■ 1 μM, ● 5 μM, ★ 15 μM. Control regression line value, $y = 389.65x - 1242.2$, 5H, dimethylnitrosamine ◆ control, ■ 10 mM, ▲ 50 mM, ● 100 mM, ★ 250 mM, − 500 mM. Control regression line value, $y = 297.14x - 874.08$, 5I, 4-nitroquinoline-N-oxide, ◆ control, ■ 1.5 μM, ● 3 μM, ★ 7.5 μM. Control regression line value, $y = 340.84x - 1159.7$, 5J, methyl-nitro-nitroso-guanidine, ◆ control, ■ 1 μM, ● 5 μM, ★ 10 μM. Control regression line value, $y = 324.46x - 1098.3$.

Table 1
IC_{50} results in the uridine uptake assay (RNA) or the neutral red assay (NR)[a]

Molecule	$n°$ CAS	contact (h)	IC_{50} RNA	IC_{50} NR
2-AAF	53-96-3	20	122 ± 20 μM	No cytotoxicity
B[a]P	50-32,8	20	3.4 ± 0.4 μM	No cytotoxicity
DMN	62-75-9	4	110.2 ± 24.1 mM	355.4 ± 1.9 mM
H_2O_2	7772-84-1	4	0.7 ± 0.2 mM	3.3 ± 0.6 mM
HMPA	680-31-9	20	No cytotoxicity	No cytotoxicity
$K_2Cr_2O_7$	7778-50-9	20	4.2 ± 1.2 μM	19.1 ± 6.5 μM
MMS	66-27-3	4	0.9 ± 0.07 mM	1.5 ± 0.02 mM
MNNG	70-25-7	4	5.5 ± 1.2 μM	796.6 ± 0.1 μM
MNU	684-93-5	4	6.7 ± 0.4 mM	No cytotoxicity
4-NQO	56-57-5	4	1.9 ± 0.3 μM	204.1 ± 11.2 μM
SO	96-09-3	4	0.3 ± 0.04 mM	1.5 ± 0.4 mM

[a] Values are mean $\pm$ S.D. of three independent experiments.

the presence of transition metal ions or via other mechanisms (Halliwell et al., 1992). The formation of hydroxyl radical and other ROS initiates lipid peroxidation and causes damage including DNA damage. Recently, regulation of cellular toxicity induced by H_2O_2 over a wide concentration range was assessed (Gardner et al., 1997). These authors detected three distinct patterns. The highest concentration (> 10 mM) rapidly induced a necrotic form of death and between 5 and 10 mM, concurrently with cytotoxicity, target cell death was associated with evidence of apoptosis. Lowest concentration of H_2O_2 (0.5 and 0.1 mM) induced delayed cytotoxicity with no morphologic evidence of apoptosis. H_2O_2 has been shown to be cytotoxic (70% plating efficiency) after 1 h on a murine tumor model (MN-11) at 20 μM (Sandhu and Birnboim, 1997) and IC_{50} was determined at 60 μM in V79 cells after 1 h treatment by measuring the same parameter (Ziegler-Skylakakis and Andrae, 1987). In our study, H_2O_2 was one of the less cytotoxic compound for the HepG2 cells after 4 h contact (IC_{50} 0.71 mM). As about SO, it is a labile metabolite of styrene accepted generally to be responsible for any genotoxicity associated with styrene (Barale, 1991) but there are toxicological significant doses and species dependent differences in the metabolism and pharmacokinetic of styrene and SO (Mendrala et al., 1993). SO was not very cytotoxic compared with 4-NQO for the HepG2 cells as its IC_{50} was high (0.3 mM) in the RNA test.

Hexavalent chromium (Cr^{6+}) of $K_2Cr_2O_7$ is suspected as being the more potent toxic compound compared with the trivalent form of chromium (Cr^{3+}). Soluble Cr^{6+} compounds are taken up quickly by cells by sulfate transport systems and after entering cells, Cr^{6+} undergoes non enzymatic reductive metabolism yielding stable Cr^{3+} (Norseth, 1986). Although mechanism of Cr^{6+} induced DNA damage as well as the ultimate genotoxic species are still unknown, chromium genotoxicity manifests as gene mutation, several types of DNA lesions and inhibition of the synthesis of macromolecules (Stearns et al., 1995; Singh et al., 1998). $K_2Cr_2O_7$ IP_{50} determined by Dierickx (1989) was 0.345 mM when HepG2 cells were in contact with $K_2Cr_2O_7$ for 24 h. In our study, IC_{50} in the RNA assay was lower, only 4,2 μM with a contact time of 4 h. DMN is one of the most widely occurring carcinogenic compound in the environment and requires metabolic activation for its cytotoxic and carcinogenic actions (Doolittle and Goodman, 1984). Although at high concentration, DMN is cytotoxic in the absence of metabolic activation due to its high protein denaturing ability which may result in cell membrane destruction (Argus and Arcos, 1978). The major activation step is believed to be the oxygenation of the α-carbon catalysed by a cytochrome P_{450} dependent enzyme system commonly known as DMN demethylase and different forms of P_{450} isoenzymes can be involved

depending on DMN concentrations (Fournier, 1990). Studies showed that DMN was not cytotoxic after 24 h contact with rat hepatocytes by using the LDH assay (Olson et al., 1984). Doolittle and Goodman (1984) have evaluated DMN cytotoxicity expressed as cloning efficiency relative to control and concluded that up to 100 mM DMN was not cytotoxic for V79 cells but concentration of S15 fraction in the reaction mixture can markedly influence the degree of DMN induced cytotoxicity. In our study, DMN was a weak cytotoxic compound for the HepG2 cells (IC_{50} at 110 mM after 4 h contact). Cytotoxic effect of DMN was examined also by NR assay on rat and human hepatocyte cultures and was dependent on the concentration and time of exposures (IC_{50} was 20 mM in rat hepatocytes after 24 h) but these authors worked without serum in the medium, this could explain a higher sensitivity of the cells as chemicals or metals can bind to serum components and in turn become unavailable for uptake into the cells. B[a]P is a carcinogenic compound metabolised by phase 1 and 2 enzymes by isolated rat hepatocytes, but after 100 min, 80 µM was not a cytotoxic concentration in the trypan blue assay (Jones et al., 1977). HepG2 have been shown to be the most sensitive cells to B[a]P and this sensitivity reflected their ability to metabolise B[a]P to cytotoxic products (Babich et al., 1988). These authors showed a toxicity starting with the NR assay after 24 h at 16 µM and after 48 h at 4 µM. In our study, 15 µM was very cytotoxic after 20 h

Fig. 7. Reversibility experiment by using MMS as toxic compound. Percentages represent sample cell viability compared with control cells (100%). Control regression line value, $y = 208.29x - 858.62$, 6A, treatment with 0.75 mM MMS, ■ = control, ▲ = treatment. — = 4 h contact, - - - = 4 h recovery. 6B, treatment with 1 mM MMS, ■ = control, ▲ = treatment. — = 4 h contact, - - - = 4 h recovery.

and B[a]P IC_{50} was low (3.4 µM). 2-AAF is a potent rat liver carcinogen and is another example of a compound that is carcinogenic and mutagenic only after activation by cellular metabolic pathways to a reactive electrophile (Miller, 1970). Cytotoxicity has been identified in coculture (rat hepatocytes and human fibroblasts) by Strom et al. (1983) by measuring the plating efficiency. In this model, authors showed a cytotoxic effect after 45 h and only after activation with hepatocytes above 10^{-4} M. The 2-AAF IC_{50} in the RNA assay was 0,122 mM after 20 h of contact with HepG2 cells.

Genotoxic compounds as MNU, 4-NQO, MNNG and MMS are often used as positive markers in the unscheduled DNA synthesis assay (Priyardashini et al., 1987; Naji-Ali et al., 1994), the latter noted a toxicity starting for HepG2 cells
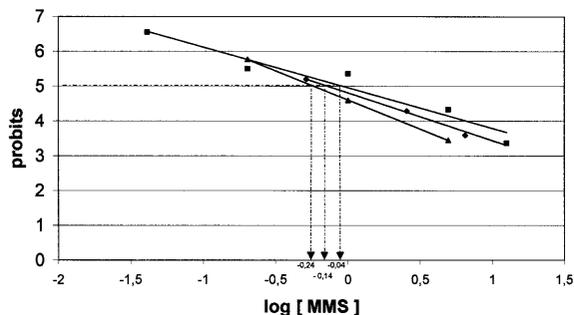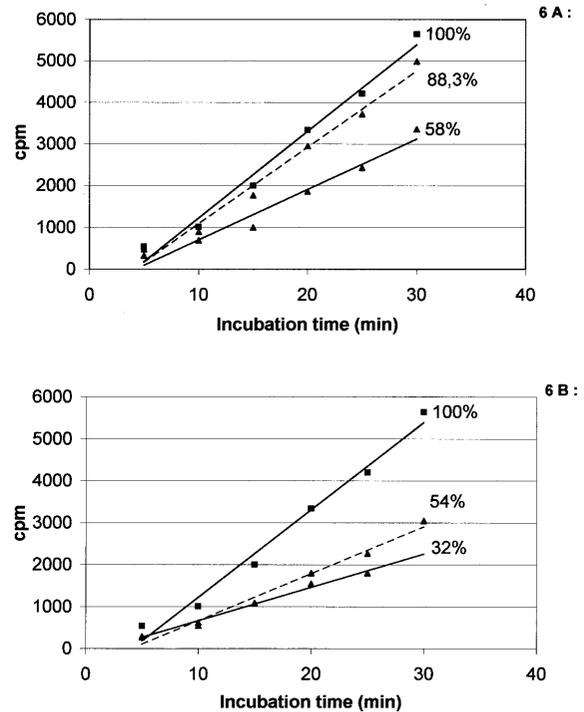

Fig. 6. Graphic determination of MMS IC_{50}. Data are plotted as probit of control versus logarithmic chemical concentration. ■, ▲, ♦ represent three independent experiments, arrows point out the respective IC_{50} values.

at 50 μM (24 h contact) with the MMS. MMS has been also identified as cytotoxic for rat hepatocytes after 18 h contact at 100 μM; 37% of control viability in the NR assay (Fautz et al., 1991). In our experiments, MMS IC_{50} was 0.9 mM in the RNA assay but the contact time was only 4 h and the sensibility of both in vitro models can be totally different. MNNG was toxic towards isolated rat hepatocytes in a concentration dependent manner and 50% cytotoxicity, as determined by the trypan blue inclusion occurred 2 h after incubating the hepatocytes with 350 μM MNNG (Niknahad and O'Brien, 1995). Cytotoxicity of MNNG has also been evaluated on the basis of growth rate of treated cells as well as by colony forming ability on V79 cells after 30 min. Cytotoxicity was starting at 6×10^{-6} M with the trypan blue test but plating efficiency was affected at 10^{-6} M (Slamenova et al., 1998). In our study, IC_{50} was established at 5.5 μM in the RNA assay after 4 h contact.

As about 4-NQO, toxicity was starting for HepG2 cells at 2.5 μM after 24 h contact using the NR assay (Naji-Ali et al., 1994), we deter-

mined an IC_{50} at 1.9 μM after 4 h contact in the RNA assay.

Discrepancies observed among the literature results depend on the cellular toxicity endpoint measured, the cells, the origin of the cells, the cell density, the cell passage and the contact time used. However, trypan blue and LDH assays have several disadvantages; trypan blue requires microscopically scoring and is, therefore, laborious, time consuming and subjective. LDH amounts need to be determined by enzyme kinetics and the method requires monitoring of the increase in UV absorbance due to the reduction of NAD^+ (Jauregui et al., 1981). Therefore, with several concentrations, the assay is time consuming and needs a precise time schedule. NR assay has been used to measure the relative cytotoxicities of a spectrum of agents (Borenfreund and Puerner, 1985). An interesting observation in Zhang et al. (1990) is that in vitro monolayer aging plays a role in NR uptake. The NR accumulated in hepatocyte lysosomes increased 3–4-fold in 4 days old cultures as compared with 1-day-old cultures. However, as estimated by total cellular LDH and protein con-

Table 2
Application of the uridine uptake assay to detect unknown toxic molecules in bottled spring water, Control 1, $y = 590.34x–1500$, Control 2, $y = 570.5x–2873.2$

Molecules	Concentration (mg/l)	Samples number	Contact (h)	Cell viability (% of control)
Control 1		0	20	100
SDS	20	8	20	70
	40	6	20	0
	60	2	20	0
Diazinon	5	4	20	63
	10	9	20	51
Control 2		0	20	100
Limonen	5	7	20	75
	10	10	20	63
Mixture				
Limonen	8	1	20	0
SDS	40			
Diazinon	5	3	20	0
Limonen	5			
SDS	60			
Diazinon	5	5	20	0
Limonen	8			
SDS	60			
Diazinon	10	11	20	0
SDS	40			

tent, cell density was gradually decreasing during the same culture medium due to cell aging with subsequent detachment from the culture surface. Thus, the enhanced cellular NR accumulation might be due to the lysosomal proliferation in aged hepatocytes.

This study shows a high sensitivity of the RNA assay. This test is a global cytotoxic assay as it is capable of detecting a large range of toxic compounds with different action mechanisms which remain undetected by conventional methods. Furthermore, cells are in a morbidity state and not a mortality state as it is the case in current cytotoxic assays. We set up this test for an adherent cell line such as HepG2 cells. It makes this RNA assay suitable for studies involving toxic compound biotransformations as these cells are able to activate pro-cytotoxic compounds to cytotoxic compounds. This test gives only comparative results showing that the test sample is more toxic for the cells that the reference sample but this method provides a powerful tool to compare relative toxicity levels and sample of known absolute toxicity as potassium dichromate can be used as positive marker. Thus, this test as a global and sensitive assay is also totally suitable for recycling materials which can contain unknown pollutants able to migrate from wrap into the food; data obtained in the blind experiment with polluted samples clearly showed a good correlation between cytotoxicity and the level of water pollution.

This RNA assay is actually in the laboratory on the way to be automated by using a microplate radioactivity counter.

Acknowledgements

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