HAL
open science

Evaluation of the sensitivity of three sublethal cytotoxicity assays in human HepG2 cell line using water contaminants

Adeline Jondeau, Laurence Dahbi, Marie-Helene Bani-Estivals, Marie-Christine Chagnon

To cite this version:

Adeline Jondeau, Laurence Dahbi, Marie-Helene Bani-Estivals, Marie-Christine Chagnon. Evaluation of the sensitivity of three sublethal cytotoxicity assays in human HepG2 cell line using water contaminants. Toxicology, 2006, 226 (2-3), pp.218-228. 10.1016/j.tox.2006.07.007 . hal-00294427

HAL Id: hal-00294427

https://hal.science/hal-00294427v1

Submitted on 24 Sep 2008

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Evaluation of the sensitivity of three sublethal cytotoxicity assays in human HepG2 cell line using water contaminants

Adeline Jondeau [a,*], Laurence Dahbi [a], Marie-Hélène Bani-Estivals [b], Marie-Christine Chagnon [a]

[a] *ENSBANA, UMR (1234) Toxicologie Alimentaire, 1 Esplanade Erasme, F-21000 Dijon*

[b] *Danone Vitapole, Centre de Sécurité des Aliments, RD 128, F-91767 Palaiseau.*

* Corresponding author. Tel.: +33 3 80 39 66 43; fax: +33 3 80 39 66 41.

E-mail adress: adeline.jondeau@u-bourgogne.fr (A. Jondeau)

Keywords: *water contaminants; cytotoxicity assays; HepG2 cell line; RNA synthesis; alamar blue; ATP*

Abstract

The in vitro toxicological index IC50 (the millimolar concentration of compound which inhibits response assay by 50% compared to the solvent control) of 11 water contaminants (acrylamide, atrazine, B[a]P, BPA, 2,4-DAT, 17-αEE, H_2O_2, 4-OP, sodium bromate, sodium chlorate, sodium nitrate) was evaluated on the human hepatoma (HepG2) cells using three short-term bioassays related to their morbidity status [radiometric RNA synthesis assay (RNA), luminometric ATP assay (ATP), fluorometric Alamar blue assay (AB)]. Among all substances, we were not able to determine atrazine IC50 value whatever the test used. Furthermore, B[a]P was not cytotoxic in the ATP and AB assays. Statistical analysis revealed a correlation between the IC50 values obtained in the three assays. Except with 4-OP, RNA assay was always inhibited at lower concentrations than those required in the other assays, suggesting that this assay is a very sensitive indicator of the presence of toxic compounds. ATP and AB assays responded to a similar pattern. Due to its higher sensitivity and its reliability, RNA synthesis assay using HepG2 cell line provides the most suitable tool for the screening of water contaminants.

1. Introduction

Drinking water partly issued from surface water is exposed to a wide human pollution: agricultural, industrial and urban pollution but also to disinfection treatments. Many studies have reported the presence of a variety of hazardous compounds in treated drinking water (Kraybill, 1981; Peters et al., 1990; Sadiq and Rodriguez, 2004). Furthermore, during the production and the filling of mineral water, chemicals may be present accidentally or due to the migration of compounds or degradation compounds from the food contact materials. To protect the health of consumers, all the potential toxic compounds need to be assessed. Analytical methods such as gas chromatography-mass spectrometry or high pressure liquid chromatography-mass spectrometry have been developed for the identification and characterization of toxic constituents in water. Although these studies have a high sensitivity offering very good information, they are often powerless, (i) particularly in the case of degradation products, impurities, incidental contaminants or Non Intentionally Added Substances (NIAS), (ii) often, chemical analysis requires concentration procedures of the samples to be tested, (iii) the toxicity of different compounds is not comparable at the same concentration and (iiii) it is impossible to give a direct idea of the resulting toxicity of a mixture particularly if a synergistic or potentialization effect occurs. Therefore, it will be of first interest to dispose rapid, sensitive and reliable bioassays to first screen the effects of toxic compounds in water instead of analysing large numbers of compounds, which could be time and cost consuming, particularly if the chemical controlling the toxicity is unknown.

Established cell lines represent useful alternatives test systems for such toxicological studies (Crespi et al., 1995) but they must be carefully chosen as regard to their tissue and species origins. The choice of the human cell line were partly based on Multicenter

Evaluation of In vitro Cytotoxicity (MEIC) (Ekwall and Sandström, 1978 a,b; Ekwall and Johansson, 1980; Clemedson et al., 1996; Clemedson and Ekwall, 1999). In these MEIC studies, among different cell lines, a human hepatoma cell line (HepG2) (Thabrew et al., 1997) was retained. These cells display many of genotypic and phenotypic features of normal liver cells, a wide variety of liver-specific metabolic responses to different kind of drugs (Knowles et al., 1980; Knasmüller et al., 1998) and a well functioning glutathione system (Dierickx, 1989), playing a crucial role in the activation/detoxification of genotoxic pro-carcinogens (Knasmüller et al., 1998). Indeed, human cells expressing human xenobiotic metabolising enzymes are more likely to be predictive of human susceptibility to the biological effects of chemicals (Natarajan and Darroudi, 1991; Darroudi and Natarajan, 1993). HepG2 has also been shown to be slightly more sensitive compared to other cell lines (HeLa, ECC-1 and CHO K1) to cytotoxic compounds (Schoonen et al., 2005).

Numerous assays with various physiological mechanisms and endpoints are available. The most frequently used endpoint in cellular toxicology are principally based on cell mortality state as a consequence of membrane damage or cell detachment: neutral red incorporation (Borenfreund and Puerner, 1985), lactate dehydrogenase release (Decker and Lohmann-Matthes, 1988); protein content (Balls and Bridges, 1984). A way to increase the sensitivity of a cytotoxic assay is to follow early perturbations in the cell homeostasis. Indeed cells, before they die, exhibit biochemical perturbations (morbidity state) induced by the toxic compound. Thus, agents that stimulate or modify cell growth may manifest their actions by perturbations in nutrients uptake rates (Koren, 1980). 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). The

efficiency of this cytotoxic assay which is based on the quantitative inhibition of the RNA synthesis rate of the cells exposed to toxic compounds has been demonstrated by comparison of data with those obtained with the more classical physico-chemical determinations (Fauris et al., 1985; Fauris et Vilagines, 1988). This test is already being applied to the detection of pollutants in water using Hela S3 cell line (AFNOR, Association Française de Normalisation, 1996). More recently, the RNA synthesis assay has been adapted as a screening method to microtitration plates (96 wells) by Valentin-Séverin et al., (2002) using the HepG2 cell line and we have already shown in the laboratory that this assay was reliable to detect cytotoxic effects of unknown compounds in spring water using blind tests (Valentin et al., 2001). Futhermore, two cytotoxicity tests related to morbidity state, ATP and alamar blue assays are often mentioned as very sensitive (Crouch et al., 1993; O'Brien et al., 2000; Hamid et al., 2004). Indeed, ATP has been used to assess the functional integrity of living cells since all cells require ATP to remain alive and carry out their specialised functions (Crouch et al., 1993). Previous work has shown the ATP-based assay to be a reproducible and reliable assay of cell viability (Petty et al., 1995) as well as the characterization of drug effects or cytotoxic agents on human cell lines and tumour samples (Andreotti et al., 1994; Cree and Andreotti, 1997). Alamar blue is used to establish relative cytotoxicity of agents within various chemical classes (Slaughter et al., 1999). It is taken up in cells by passive diffusion and reduced in cytosol, mitochondria and/or microsomes (Gonzalez and Tarloff, 2001). Continued growth maintains a reduced environment and causes the redox indicator to change from oxidized (non-fluorescent, blue) form to reduced (fluorescent, red) form. Moreover, both of these tests can be performed using microplates and are suitable for high-throughput screening (HTS) to the identification of cytotoxic compounds regardless of the underlying mechanism and chemical class.

The objective of this work was then to compare RNA synthesis assay with two other sublethal assays (ATP and alamar blue assays) using the HepG2 cell line. To compare these bioassays, the toxicity of eleven kinds of chemicals was evaluated. The water model toxicants used are environmental pollutants: atrazine (herbicide), nitrate (fertilizer), benzo[a]pyrene (mutagen) and endocrine disrupting chemicals (17-αethinyloestradiol, 4-octylphenol, bisphenolA), water purification process contaminants (bromate, acrylamide, chlorate, H_2O_2) and water contact material (2,4-DAT).

2. Materials and methods

2.1. Chemicals and reagents

1X and 10X Minimum Essential Medium (MEM), 100X non-essential aminoacids, trypan blue 0.5% (w/v), sodium dodecyl sulfate (SDS), dimethylsulfoxyde (DMSO), were provided by Sigma-Aldrich (Saint Quentin Fallavier, France), as well as most of the compounds tested (Fig. 1): Acrylamide (purity ≥99%), Atrazine (purity 99.2%), B[a]P (purity >97%), BPA (purity >99%), 2,4 Diaminotoluene (purity 98%), 17-α ethinylestradiol (purity 98%), 4-octylphenol (purity 99%), sodium bromate (purity >98%), sodium chlorate (purity >99%), sodium nitrate (purity >99.5%), H_2O_2 (30%). Heat-inactivated fetal bovine serum (FBS), L-glutamine, phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS), trypsin (0.05%)-EDTA (0.02%) and sodium bicarbonate ($NaHCO_3$) were obtained from Invitrogen (Cergy-Pontoise, France). [5,6-^{3}H] uridine (1.33 TBq/mmol) was purchased from Amersham (Orsay, France). All other chemicals were all of analytical grade. Alamar Blue™ was purchased from Biosource Europe (Nivelles, Belgique) and ATP Vialight™ was from Cambrex (Verviers, Belgique).

2.2. Cell and culture media

HepG2 human hepatoma cell line was obtained from the ECACC (European Collection of Cell Culture, U.K.). Routine monitoring has shown the HepG2 cells to be mycoplasma free (Mycoalert kit from Cambrex). After 10 passages, HepG2 cells were no longer used for the assays. The cells were grown in monolayer culture in MEM supplemented with 2mM L-glutamine, 1% non-essential aminoacids and 10% FBS (v/v) in an humidified atmosphere of 5% CO_2 and at 37°C. Continuous cultures were maintained by subculturing flasks every 7 days at 2.2×10^6 cells/75 cm^2 flask by trypsination.

2.3. Test plate preparation

HepG2 cells were seeded into 96-well plate (Dutscher, France) in 200 µl of complete culture medium at a final concentration of 5.10^4 cells/well for the Alamar blue (AB), ATP luminescence (ATP) and RNA synthesis (RNA) assays.

2.4. Concentration response curves

After a 28 h incubation (when the cells were in exponential growth phase), the medium was removed and cells were exposed to the test substance for 20 h (excepted for the unstable H_2O_2 molecule, 1h) in 100 µl of fresh complete MEM supplemented with only 0.5% FBS. Eleven compounds were tested in each assay in a dose response manner by direct addition in medium: acrylamide (1.46-50 mM), sodium bromate (1.48-5.66 mM), sodium chlorate (10-300 mM), sodium nitrate (14.6-257 mM), H_2O_2 (0.001-150 mM) or after dissolution in DMSO used at a final non cytotoxic concentration of 0.25% (v/v) in the culture medium: atrazine (0.42-2.89 mM), B[a]P (0.0015-0.060 mM), BPA (0.036-0.38 mM), 17-αEE (0.0045-0.30 mM), 2,4-DAT (0.50-25 mM), 4-OP (0.031-0.121 mM). Control cells were exposed with medium only or with medium containing vehicle. Maximal concentration of tested compounds was systematically established according to their dissolution capacity into the DMSO or the culture medium. Each concentration was tested in replicates of four wells and the reported results are the mean of three independent experiments.

2.5. Polluted mineral water samples

As model polluted water, we used a mineral water (Volvic, screened for toxicants, from Danone, France) spiked with high (acrylamide) or moderate (bisphenol A) or low

(17-αEE) water soluble compounds. Samples have been prepared with drinking mineral water in glass flasks and deliberately polluted with known amounts of acrylamide (0, 84, 210, 420, 840 and 2520 mg/L) or bisphenol A (0, 6.7, 13.5, 27, 40.5, 54, 67.5 and 81 mg/L) or 17-α ethinylestradiol (0, 1.1, 1.7, 2.4, 3.6 and 5.3 mg/L). These samples were tested with the three assays (AB, ATP and RNA) after a 20 h exposure. In order to estimate the performance of the assays, samples were coded for a blind evaluation.

For these assays, a 6.45X concentrated MEM medium was diluted (1 volume of concentrated MEM + 5.45 volume of tested water) just before use with the drinking water (blank) or with polluted water. The concentrated medium was made as following: 100 ml MEM 10X + 30 ml $NaHCO_3$ (7.5%) + 10 ml L-glutamine (100X) + 10 ml of MEM non-essential aminoacids (100X) and 5 ml FBS. The pH of the medium was adjusted when necessary.

2.6. Cytotoxicity assays

RNA synthesis determination. The original assay was first described by Fauris et al., (1985), adapted to HepG2 cells by Valentin et al., (2001) and finally automated by Valentin-Séverin et al., (2002). The latest assay was used here. Briefly, tritiated uridine was added to the culture medium (10 µL, 0.3 µCi/well) and its amount of incorporation into the cellular RNA was measured (after 6, 12, 18, 24 and 30 min uptakes). The RNA synthesis rate was determined by calculating the slope of the straight regression line corresponding to the experimental values. This rate was then plotted against that obtained for the non toxic blank, arbitrarily fixed to 100%. The results are expressed as a percentage of RNA synthesis in relation to the blank (control).

Alamar blue assay. The alamar blue assay was performed with a fluorimetric method according to the procedure described by O'Brien et al., (2000). Three hours before the

end of the treatment incubation, 10 µL AB/well were added yielding a final concentration of 10% AB. Plates were returned to incubator and the fluorescence was read after 3h. The plates were exposed to an excitation wavelenght of 535 nm and the emission at 580 nm was recorded on a Dynex fluorimeter. The percent viability was expressed as fluorescence emitted by treated cells compared to control (medium or vehicle only).

ATP bioluminescence assay. At the end of the treatment incubation, 50 µL of cell lysis reagent (Vialight kit content) were added to each well. Following a 10-min incubation at room temperature, 100 µL of cell lysate were transferred to an opaque white walled plate. A substrate solution (Vialight kit, 100 µl /well) was then added and the microplate was incubated for 2 min at room temperature. Plates were covered by an adhesive seal and counted using a luminometer (TopCountNT, Packard). The percent viability was expressed as luminescence emitted in the presence of test compound as a percentage of that in the blank (medium alone or vehicle).

2.7 IC50 and IC20 values calculation

GraphPad Prism® 4.0 software was used to calculate the concentrations associated with 50% inhibition or 20% inhibition of measured parameter (IC50 or IC20 values) using a Hill function non linear regression analysis.

The IC50s and IC20s were transformed in log values for the assay comparisons. Linear regression analysis was used for that purpose.

3. Results

3.1 In vitro cytotoxicity of the compounds tested.

The sigmoidal concentration-response curves of ten compounds tested in the bioassays are shown in Fig. 2. All curves have an r^2 greater than to 0.80. Among the eleven chemicals tested for this study, only atrazine failed to give a concentration-response curve whatever the bioassays used. Furthermore, alamar blue and ATP assays failed to detect the effects of B[a]P, even at the limit of its solubility.

The toxicological potential of the compounds, expressed by their IC50 values calculated with the three bioassays (when possible), is summarized in Table 1. The levels of toxicity varied not only according to the substances tested, but also to the bioassays (RNA, AB or ATP).

Major differences of cytotoxicity potential exist between the contaminants. In these experimental conditions, the IC50 values allowed the substances to be arbitrarily ranked in three levels of toxicity: high toxicity, IC50 values < 0.1 mM; mild toxicity, 0.1 mM < IC50 values < 10 mM and low toxicity, IC50 values > 10 mM. Whatever the bioassays, a low toxicity was observed for sodium chlorate and sodium nitrate, a mild toxicity for 2,4-DAT, sodium bromate and H_2O_2 and high toxicity for 4-OP. Among the other substances, some of them can be specified as being relatively more toxic for one bioassay than the others. BPA and 17-αEE (especially) were high toxic compounds in RNA assay and mild in AB and ATP assays. The level of toxicity of acrylamide is low in AB and ATP assays and moderate in RNA assay. Furthermore, B[a]P is a highly toxic compound in RNA assay and not cytotoxic in AB and ATP assays. Atrazine revealed a mild toxicity (30% inhibition at 0.6 mM) in RNA assay exclusively.

Cytotoxic effects of the contaminants were as follow using RNA assay: Sodium nitrate < Sodium chlorate < Acrylamide = 2,4 DAT < Sodium bromate < Atrazine < H_2O_2 < BPA < 4-OP < 17-αEE < B[a]P.

3.2 Comparisons of toxicity tests.

The toxicity tests were compared in terms of their responses and in terms of their sensitivities at the two levels IC20 and IC50.

A comparison of the bioassays responses to contaminant substances was performed by mean square root linear regression analysis. The results are shown in Fig. 3. The relationship between the bioassays were expressed by their equations of regression. They showed significant correlations between the IC50 or IC20 calculated with all bioassays. The best correlations were obtained between the IC50 values issued from ATP and AB assays (r^2=0.96) on one hand and between RNA and AB assays (r^2=0.94) on the other hand.

Sensitivity was first measured by ranking the tests according to the IC20 values (Table 2). The range of variation (R) of the IC20 values is summarized in Table 2. Excepted for 4-OP (R=1), all of the lowest IC20 values were determined with the RNA assay which was the most sensitive test. Furthermore, the highest IC20 values were calculated with ATP or AB assays at the same frequency suggesting the same overall sensitivity for these two bioassays. For all of compounds listed in Table 2, the range of variation of the IC20 values was R ≤ 22. As can be seen in Fig.3, AB and ATP assays have the same overall sensitivity at the two levels of toxicity but the difference in sensitivity between RNA assay and the two other tests was more pronounced at the low level of toxicity (3.5 fold more sensitive taking into account the IC50 values compared to 5 fold more sensitive taking into account the IC20 values).

3.3 Toxicity evaluation of spiked water samples.

Fig. 4 presents the concentration-response curves for the three assays employed when HepG2 cells were exposed to mineral water samples spiked with known concentrations of acrylamide or BPA or 17α-EE for 20h. Following blind exposure, significant toxicity is observed with the RNA assay at lower concentrations compared to the ones required for detection with the AB and ATP assays. Furthermore, when 17-αEE is evaluated at relevant environmentally concentration, e.g. at the limit of its solubility, only RNA assay allowed to detect its effects. The results confirmed that AB and ATP assays are the least sensitive when compared to the RNA assay. Then, AB and ATP assays could lead to false negative data.

Table 3 presents the IC50 and IC20 values calculated (when possible) from the concentration response curves for the RNA, AB and ATP assay when HepG2 cells were blind exposed to the spiked water samples for 20h. The IC50 and IC20 values for acrylamide, bisphenol A and 17α-EE were in the same range of the values presented in tables 1 and 2, excepted for the values calculated in the ATP assay for acrylamide which were approximately two fold lower. This results showed that the RNA and AB assays were more reliable that the ATP assay.

4. Discussion

This study investigated cytotoxic effect of potential water contaminants on HepG2 cell line in order to design a sensitive and reliable assay. The RNA synthesis assay was compared to the ATP and alamar blue, two very sensitive cytotoxic assays chosen for their capacity to give information on the sublethal cell damage. However, a direct comparison of the sensitivities of RNA, AB and ATP assays from the literature is difficult because of varying experimental conditions, especially different exposure times to the test chemicals or different cell types.

The 11 compounds tested herein have a wide range of toxicities. B[a]P is known to be a hepatocarcinogen. HepG2 cells have been shown to be sensitive to B[a]P and this sensitivity reflected their ability to metabolise B[a]P to cytotoxic products (Diamond et al., 1980; Sassa et al., 1987; Babich et al., 1988). B[a]P was the most toxic compound in this study with a very low IC50 value (0.006 mM) determined only in the RNA assay. Indeed, alamar blue and ATP assays failed to detect a cytotoxic effect even under microscope, cell blebbings were observed. Unfortunately, in our experimental conditions the limit of solubility of B[a]P in DMSO was reached (20 mM) not allowing to use higher concentrations. We previously showed that RNA assay was more sensitive than neutral red assay which failed to detect a B[a]P cytoxicity in HepG2 cells (Valentin et al., 2001). Our data obtained with the ATP assay are in accordance with Storer et al. (1996). These authors were not able to detect a cytotoxic effect of B[a]P using an ATP assay and primary rat hepatocytes up to 150 µM. Concerning alamar blue, only Schoonen et al. (2005) determined a minimal toxic concentration (0.032 mM) but after a longer time exposure (72h). 17-αEE was also very cytotoxic especially in the RNA assay (IC50 = 0.014 mM) but we lack data about its cytotoxic effect in the litterature. 4-OP was one of the most cytotoxic compound tested in this study (IC50 ~ 0.050 mM)

whatever the assay used, suggesting a non specific toxicity to the endpoints measured in HepG2 cells. BPA was quite cytotoxic for the HepG2 cells (IC50 between 0.074-0.24 mM). Again the RNA assay was more sensitive to detect BPA cytotoxicity compared to ATP or alamar blue assays and compared to Storer et al. (1996) studies using primary rat hepatocytes culture and using several mortality or sublethal cytotoxic assays.

H_2O_2 is a major component of reactive oxygen species (ROS) which causes DNA damage. In our study, H_2O_2 was mildly cytotoxic for the HepG2 cells after 1 hour contact in the RNA assay (IC50 = 0.50 mM) that was again the most sensitive test. Concerning the alamar blue data, our results are correlated with Li et al. (2004) who used HeLa cells. In contrast to the RNA assay, atrazine at 0.6 mM was not detected as cytotoxic in alamar blue and ATP assays. This result is in accordance with Tchounwou et al. (2001) who found that atrazine was not toxic to the HepG2 cells in the MTT assay even at its maximum solubility. The toxicity of bromate was moderate in HepG2 cells whatever the cytotoxicity assay used (IC50= 2-4 mM). In our study, acrylamide and 2,4-DAT were midly cytotoxic compounds for the HepG2 cells. Acrylamide is a probable human carcinogen (IARC, 1994) with genotoxicities including micronuclei (Higashikuni, 1994). 2,4-DAT has recently been shown genotoxic in HepG2 cells in the laboratory by Séverin et al. (2005a). Finally, sodium nitrate and sodium chlorate were the less toxic compounds in this study with low IC50 values (>10mM). This is in accordance with Bharadwaj et al. (2005) studies using HepG2 cells.

In this study, alamar blue, ATP and RNA assays performed with the HepG2 cells intercorrelated significantly demonstrate that these assays are similar tools for overall evaluation of cytotoxicity. Toxic effects can be compared at different levels of toxicity, either at the IC50, the IC20 or the no-observed-effect concentration level. Normally, IC50 values are determined. However, we also found good correlations at the 20%

effect level. This shows that the IC20 values are also valuable for estimation of cytotoxicity, particularly when IC50 can't be evaluated (for low water soluble compound for exemple). In the context of water screening, IC20 values were closer than IC50 values to environmentally relevant concentrations (low concentration). For the nine tested compounds which are given a concentration-response curve simultaneously in the three bioassays used, the range of variation (R) of the IC20 values was moderate (R <22). This result can be related to the non specific toxicological cellular mechanism of the mentionned substances. A bioassay used for screening studies must be sensitive and it must detect all major cellular disturbances. But RNA assay, according to its higher sensitivity, allowed cellular toxic effects to be significantly detected at lower concentration.

This study clearly shows that RNA assay was the most sensitive test because the IC20 and IC50 values determined with the tested compounds were always statistically lower than those obtained with alamar blue and ATP (except for 4-OP). Furthermore, RNA assay was able to detect all the tested compounds suggesting a higher discriminate potency of this assay and was also reliable in the blind test experiments. The high sensitivity of this assay is essentially due to the dynamic nature of the test which does not compare amounts of synthesised RNA but the rapidity of RNA synthesis. Kjeldgaard (1963) has shown that RNA synthesis is proportional to the square of cellular growth rate.

In the context of screening cytotoxic compounds presence, we have to be careful with the suitability of the assay to prevent false negatives. IC50 values determined using spiked water samples were closed to the IC50 obtained by classical way (dissolved in DMSO) excepted for the very low water soluble compound (17-αEE) which is not identified as cytotoxic in AB and ATP assays when realistic cytotoxicological

evaluation is performed. RNA assay data obtained with contaminants spiked in mineral water suggest a high performance of this assay. To assess the presence of potential toxic compounds, we need a global test able to detect a large range of various chemicals classes. In this study, while RNA assay succeeded to detect all the tested, ATP and alamar blue could lead to false-negatives. Furthermore, we previously demonstrated that RNA assay, when used prior to genotoxic tests, was a good tool to avoid false positive data. RNA assay has been retained in a pre-normative research with the objective to establish a set of in vitro cytotoxicity and genotoxicity assays that will be easily performed to assess safety of food-contact paper and board (Séverin et al., 2005b). Experiments are underway in the laboratory to check effects of different contaminants mixtures.

In conclusion, this study shows a high sensitivity of the RNA assay compared with the alamar blue and ATP assays. This test was reliable, it is a global cytotoxic assay as it is able to detect toxic compounds with different action mechanisms making this assay the most appropriate to detect various water contaminants. As human cells are more relevant for human risk evaluation, we think that RNA assay using HepG2 cells could be a promising tool for the screening of water samples in order to evaluate the harmful effects of compounds.

Acknowledgements.

The authors are thankful to Danone Vitapole for the preparation of blind test samples. They also want to express their gratitude to the Regional Council of Burgundy and Danone Vitapole for financial support of this work.

References

AFNOR XP P 41-250-3. (1996). Effet des matériaux sur la qualité des eaux destinées à la consommation courante. Matériaux inorganiques. Part 3 : Méthode de mesure de cytotoxicité. pp.1-19.

Andreotti P.E., Linder D., Hartmann D.M., Cree I.A., Pazzagli M. and Bruckner HW. (1994). TCA-100 tumor chemosensitivity assay: differences in sensitivity between cultured tumor cell lines and clinical studies. J. Biolumin. Chemilumin. 9, 373-378.

Babich H., Sardana M.K. and Borenfreund E. (1988). Acute cytotoxicity of polynuclear aromatic hydrocarbons determinated in vitro with the human liver tumor cell line, HepG2. Cell Biol. Toxicol. 4, 295-309.

Balls M. and Bridge J.W. (1984). The FRAME research program on in vitro toxicology. In: M.A. Liebert (Eds), Acute Toxicology Testing: Alternatives Approaches, A.M. Goldberg, New York, pp.61-80.

Bharadwaj L., Dhami K., Schneberger D., Stevens M., Renaud C. and Ali A. (2005). Altered gene expression in human hepatoma HepG2 cells exposed to low-level 2,4-dichlorophenoxyacetic acid and potassium nitrate. Toxicol. in Vitro 19, 603-619.

Borenfreund E. and Puerner J.A. (1985). Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol. Lett. 24, 119-124.

Clemedson C., Mcfarlane-Abdulla E., Andersson M., Barile B., Calleja M.C., Chesné C., Clothier R., Cottin M., Curren R., Dierickx P.J., Ferro M., Fiskejö G., Garza-Ocañas, Gómez-Lechón M.J., Gülden M., Isomaa B., Janus J., Judge P., Kahru A., Kemp R.B., Kerszman G., Kristen U., Kunimoto M., Kärenlampi S., Lavrijsen K.,

Lewan L., Lilius H., Malmsten A., Ohno T., Persoone G., Pettersson R., Roguet R., Romert L., Sandberg M., Sawyer Th.W., Seibert H., Shrivastava R., Sjöström M., Stammati A., Tanaka N., Torres-Alanis O., Voss J-U., Wakuri Sh., Walum E., Wang X., Zucco F. and Ekwall B. (1996). MEIC evaluation of acute systemic toxicity. Part II. In vitro results from 68 toxicity assays used to test the first 30 reference chemicals and a comparative cytotoxicity analysis. ATLA 24, 273-311.

Clemedson C. and Ekwall B. (1999). Overview of the Final MEIC Results: I. The In Vitro Evaluation. Toxicol. in Vitro 13, 657-663.

Cree I.A. and Andreotti P.E. (1997). Measurement of cytotoxicity by ATP-based luminescence assay in primary cell cultures and cell lines. Toxicol. in Vitro 11, 553-556.

Crespi C.L. (1995). Xenobiotic-metabolizing human cells as tools for pharmacological and toxicological research. Adv. Drug Res. 26, 179-235.

Crouch S.P.M., Kozlowski R., Slater K.J. and Fletcher J. (1993). The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J. Immunol. Methods 160, 81-88.

Darroudi F. and Natarajan A.T. (1993). Metabolic activation of chemicals to mutagenic carcinogens by human hepatoma microsomal extracts in Chinese hamster ovary cells (in vitro). Mutagenesis 8, 11-15.

Decker T. and Lohmann-Matthes M.-L. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J. Immunol. Methods 15, 61-69.

Diamond L., Kruszewski F., Aden D.P., Knowles B.B. and Baird W.M. (1980). Metabolic activation of benzo[a]pyrene by a human hepatoma cell line. Carcinogenesis 1, 871-875.

Dierickx P.J. (1989). Cytotoxicity testing of 114 compounds by the determination of the protein content in HepG2 cell cultures. Toxicol. in Vitro 3, 189-193.

Ekwall B. and Sandström B. (1978a). Combined toxicity to HeLa cells of 30 drug pairs, studied by a two-dimensional microtitre method. Toxicol. Lett. 2, 285-292.

Ekwall B. and Sandström B. (1978b). Improved used of the metabolic inhibition test to screen combined drug toxicity to HeLa cells-preliminary study of 61 drug pairs. Toxicol. Lett. 2, 293-298.

Ekwall B. and Johansson A. (1980). Preliminary studies on the validity of in vitro measurement of drug toxicity using HeLa cells I. Comparative in vitro cytotoxicity of 27 drugs. Toxicol. Lett. 5, 299-307.

Fauris C., Danglot C. and Vilagines R. (1985). Rapidity of RNA synthesis in human cells. Water Res. 19, 677-684.

Fauris C. and Vilagines R. (1988). Surveillance sanitaire par mesure de la cytotoxicité. Annales des Falsifications et de l'Expertise Chimique 91, 145-155.

Gonzalez R.J. and Tarloff J.B. (2001). Evaluation of hepatic subcellular fractions for Alamar Blue and MTT reductase assays. Toxicol. In Vitro 15, 257-259.

Hamid R. Rotshteyn Y., Rabadi L., Parikh R. and Bullock P. (2004). Comparison of alamar blue and MTT assays for high through-put screening. Toxicol. in Vitro 18, 703-710.

Higashikuni N., Hara M., Nakagawa S. and Sutou S. (1994). 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) is a weak in vivo clastogen as revealed by the micronucleus assay. Mutat. Res. 320, 149-156.

IARC (1994). Monographs on the evaluation of carcinogenic risk of chemicals to humans. Some industrial chemicals, Vol. 60, Lyon, pp. 389-433.

Kjeldgaard N.O. (1963). Dynamics of bacterial growth. Thesis. University of Copenhagen.

Knasmuller S., Parzefall W., Sanyal R., Ecker S., Schwab C., Uhl M., Mersch-Sundermann V., Williamson G., Hietsch G., Langer T., Darroudi F. and Natarajan A.T. (1998). Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens. Mutat. Res. 402, 185-202.

Knowles B.B., Howe C.C. and Aden D.P. (1980). Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 209, 497-499.

Koren R. (1980). The relevance of the state of growth and transformation of cells to their patterns of metabolite uptake. Int. Rev. Cytol. 68, 127-172.

Kraybill H.F. (1981). Carcinogenesis of synthetic organic chemicals in drinking water. J. Am. Water Works Association 73, 370-372.

Li J., Gao X., Qian M. and Eaton J.W. (2004). Mitochondrial metabolism underlies hyperoxic cell damage. Free Radical Biol. Med. 36, 1460-1470.

Natarajan A.T. and Darroudi F. (1991). Use of human hepatoma cells for in vitro metabolic activation of chemical mutagens/carcinogens. Mutagenesis 6, 399-403.

O'Brien J., Wilson I., Orton T. and Pognan F. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem. 267, 5421-5426.

Peters R.J.B., De Leer E.W.B. and De Galan L. (1990). Dihaloacetonitriles in Dutch drinking water. Water Res. 24, 797-800.

Petty R.D., Sutherland L.A., Hunter E.M. and Cree I.A. (1995). Comparison of MTT and ATP-based assays for the measurement of viable cell number. J. Biolumin. Chemilumin. 10, 29-34.

Rozengurt E., Stein W.D. and Wigglesworth N.M. (1977). Uptake of nucleosides in density-inhibited cultures of 3T3 cells. Nature 267, 442-444.

Sadiq R. and Rodriguez M.J. (2004). Disinfection by-products (DBPs) in drinking water and predictive models for their occurrence: a review. Sci.Total Environ. 321, 21-46.

Sassa S., Sugita O., Galbraith R.A. and Kappas A. (1987). Drug metabolism by the human hepatoma cell HepG2. Biochem. Biophys. Res. Commun. 143, 52-57.

Schoonen W.G.E.J., De Roos J.A.D.M., Westerink W.M.A. and Debiton E. (2005). Cytotoxic effects of 110 reference compounds on HepG2 cells and for 60 compounds on HeLa, ECC-1 and CHO cells. II Mechanistic assays on NAD(P)H, ATP, and DNA contents. Toxicol. in Vitro 19, 491-503.

Severin I., Jondeau A., Dahbi L. and Chagnon M.C. (2005a). 2,4-Diaminotoluene (2,4-DAT)-induced DNA damage, DNA repair and micronucleus formation in the human hepatoma cell line HepG2. Toxicology 213, 138-146.

Severin I., Dahbi L., Lhuguenot J.C., Andersson M.A., Hoornstra D., Salkinoja-Salonen M., Turco L., Zucco F., Stammati A., Dahlman O., Castle L., Savolainen M., Weber A., Honkalampi-Hämäläinen U. and Von Wright A. (2005b). Safety assessment of food-contact paper and board using a battery of short-term toxicity tests: European union BIOSAFEPAPER project. Food Addit. Contam. 22, 1032-1041.

Shopsis C. and Sathe S. (1984). Uridine uptake inhibition as a cytotoxicity test: correlations with the draize test. Toxicology 29, 195-206.

Slaughter M.R., Bugelski P.J. and O'Brien P.J. (1999). Evaluation of Alamar Blue reduction for the in vitro assay of hepatocyte toxicity. Toxicol. in Vitro 13, 567-569.

Storer R.D., Mckelvey T.W., Kraynak A.R., Elia M.C., Barnum J.E., Harmon L.S., Nichols W.W. and Deluca J.G. (1996). Revalidation of the in vitro alkaline elution/rat hepatocyte assay for DNA damage: improved criteria for assessment of cytotoxicity and genotoxicity and results for 81 compounds. Mutat. Res. 368, 59-101.

Tchounwou P.B., Wilson B.A., Ishaque A.B. and Schneider J. (2001). Atrazine potentiation of arsenic trioxide-induced cytotoxicity and gene expression in human liver carcinoma cells (HepG2). Mol. Cell. Biochem. 222, 49-59.

Thabrew M.I., Hughes R.D. and Mcfarlane I.G. (1997). Screening of hepatoprotective plant components using a HepG2 cell cytotoxicity assay. J. Pharmacol. 49, 1132-1135.

Valentin I., Philippe M., Lhuguenot J.C. and Chagnon M.C. (2001). Uridine uptake inhibition as a cytotoxicity test for a human hepatoma cell line (HepG2 cells): comparison with the neutral red assay. Toxicology 158, 127-139.

Valentin-Séverin I., Laignelet L., Lhuguenot J.C. and Chagnon M.C. (2002). Uridine uptake inhibition assay: an automated micromethod for the screening of cytotoxicity. Toxicology 171, 207-213.

Figure/table legends.

Table 1. IC50 values (mM) for HepG2 cells following exposure (20h, excepted H_2O_2, 1h) to water contaminants based on the concentration-response curves as derived from RNA, alamar blue and ATP assays.

Table 2. Range of variation (R) of the IC20 values according to the bioassays used.

Table 3. IC50 and IC20 values for HepG2 cells following 20h blind exposure to spiked mineral water based on the concentration-response curves as derived from RNA, alamar blue and ATP assays.

Fig. 1. Chemical structures of tested compounds and CAS numbers

Fig.2. Concentration response curves (using GraphPad Prism) of compounds tested in the RNA synthesis (RNA), alamar blue (AB) and ATP assays. Data represent the mean of 3 experiments ± SEM.

Fig. 3. Linear regression analysis. X=logIC50 or logIC20 (first assay) and Y=logIC50 or logIC20 (second assay). $Y=aX+b$, equation of regression. r^2= coefficient of correlation. p=level of significance. 1: 17-αEE, 2: 4-OP, 3: BPA, 4: H_2O_2, 5: Bromate, 6: 2,4-DAT, 7: Acrylamide, 8: Chlorate, 9: Nitrate.

Fig. 4. Response (in % of control) of acrylamide or BPA or 17-αEE spiked mineral water samples according to the RNA synthesis (RNA), alamar blue (AB) and ATP assays in HepG2 cells after 20h exposure. Data (n=2) ± SEM was statistically analysed with the unpaired student's t-test using the Prism software (*$p<0.05$, **$p<0.01$, ***$p<0.001$, different from control).

Table1

Click here to download Table: Table 1.doc

	IC50 (mM) with 95% confidence limits (n=3)		
Compound	RNA assay	AB assay	ATP assay
Acrylamide	3.5 (3.1-3.9)	19.0 (14.1-25.5)	37.1 (34.6-39.8)
Atrazine	30% inhibition at 0.60mM [a]	Non-toxic or toxicity above 0.60mM [a]	Non-toxic or toxicity above 0. 60mM [a]
B[a]P	0.006 (0.0056-0.0068)	Non-toxic or toxicity above 0.05mM [a]	Non-toxic or toxicity above 0.05mM [a]
BPA	0.074 (0.07-0.08)	0.24 (0.23-0.25)	0.22 (0.20-0.24)
2,4 DAT	3.5 (2.2-5.6)	12.1 (10.5-13.9)	8.3 (7.8-8.9)
17-αEE	0.014 (0.013-0.016)	0.17 (0.16-0.19)	0.097 (0.089-0.106)
H_2O_2	0.50 (0.41-0.60)	3.9 (1.7-8.9)	9.7 (7.4-12.7)
4-OP	0.048 (0.044-0.052)	0.055 (0.046-0.065)	0.055 (0.051-0.059)
Bromate	2.2 (2.1-2.4)	4.0 (3.8-4.2)	2.7 (2.6-2.8)
Chlorate	29.2 (24.9-34.2)	152 (145-159)	81.0 (74.6-87.8)
Nitrate	40.6 (34.5-47.9)	160 (153-166)	72.8 (63.5-83.3)

[a] limit of solubility

Table2

Click here to download Table: Table 2.doc

	IC20 (mM) with 95% confidence limits (n=3)			R = highest IC20/lowest IC20	
Compound	RNA assay	AB assay	ATP assay		
Acrylamide	2.2 (1.8-2.6)	8.3 (5.2-13.3)	32 (29-35)	ATP/RNA	15
BPA	0.043 (0.037-0.049)	0.21 (0.19-0.23)	0.16 (0.14-0.19)	AB or ATP/RNA	5
2,4 DAT	1.0 (0.5-2.2)	3.9 (3.1-5.1)	7.1 (6.4-7.8)	ATP/RNA	7
17-αEE	0.007 (0.006-0.009)	0.12 (0.10-0.14)	0.06 (0.05-0.07)	AB/RNA	17
H_2O_2	0.26 (0.17-0.40)	1.7 (0.6-4.9)	5.4 (3.7-7.9)	ATP/RNA	21
4-OP	0.06 (0.05-0.07)	0.06 (0.05-0.08)	0.07 (0.06-0.08)	RNA=AB=ATP	1
Bromate	1.6 (1.5-1.8)	3.5 (3.3-3.7)	2.4 (2.3-2.5)	AB/RNA	2
Chlorate	10.2 (7.5-13.7)	127 (118-136)	52 (44-61)	AB/RNA	12
Nitrate	15.5 (11-21)	132 (123-141)	35 (28-43)	AB/RNA	9

Table 3

Click here to download Table: Table 3.doc

		IC (mM) determined in the blind test					
		Acrylamide		Bisphenol A		17-α EE	
Assays		IC50	IC20	IC50	IC20	IC50	IC20
RNA	mg/L	403	285	23.8	12.5	5.3	2.5
	mM	5.2	3.6	0.10	0.055	0.017	0.008
AB	mg/L	1609	940	66.5	53.6	Non cytotoxic	
	mM	20.6	12.0	0.29	0.23		
ATP	mg/L	1773	1288	54.0	42.5	Non cytotoxic	
	mM	22.7	16.5	0.24	0.19		

Figure1

Click here to download Figure: Figure 1.doc

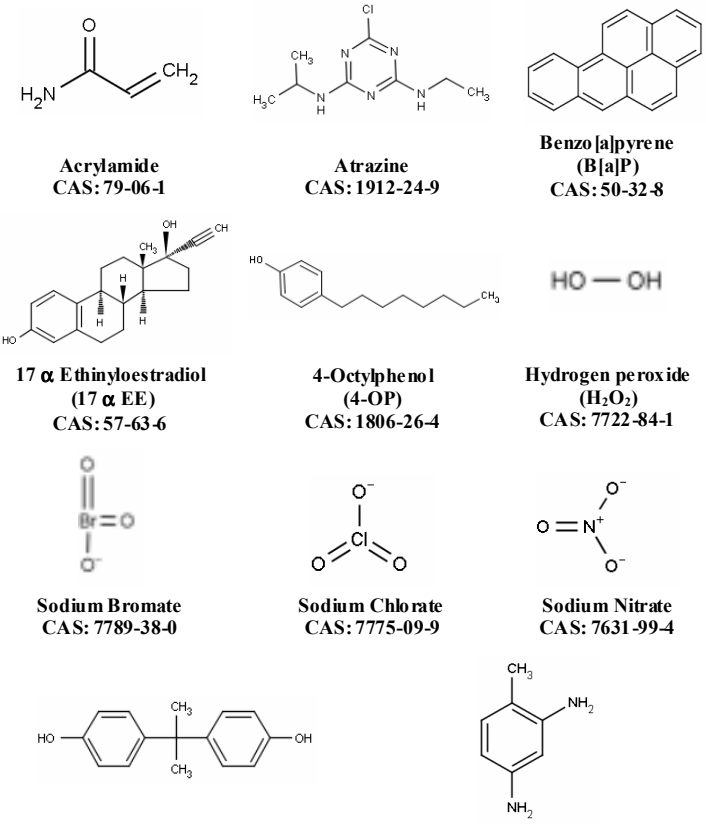


Figure2

Click here to download Figure: Figure 2.doc

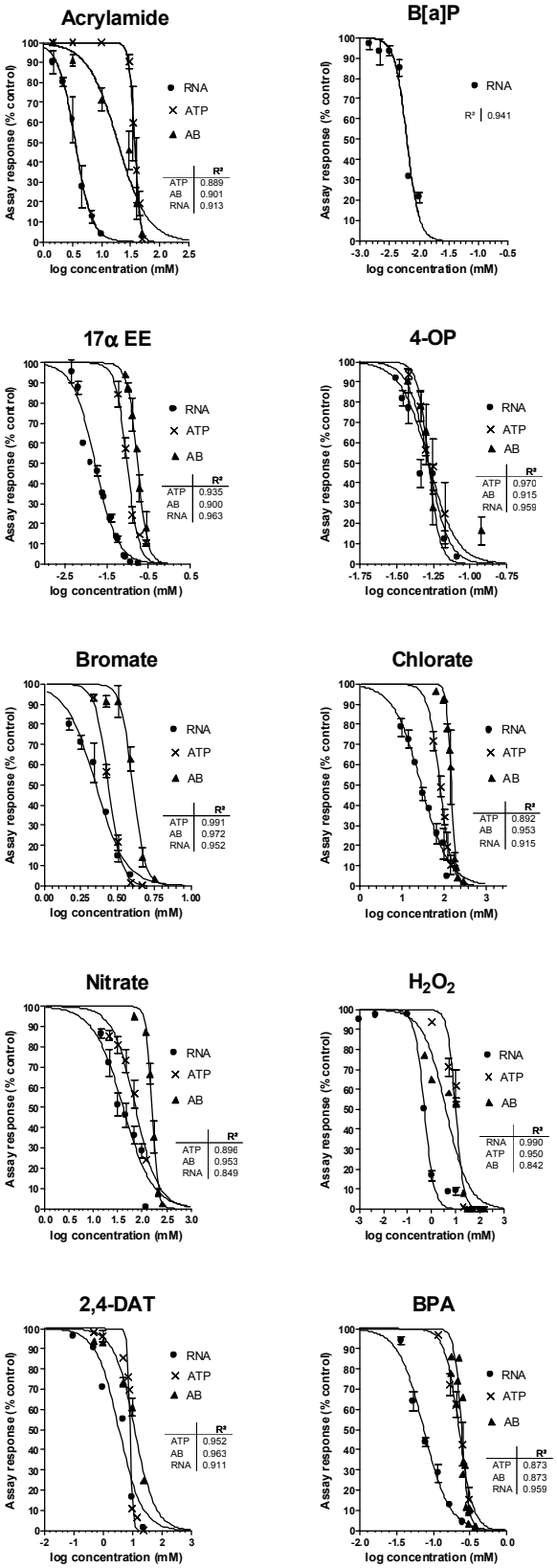


Figure 3

Click here to download Figure: Figure 3.doc

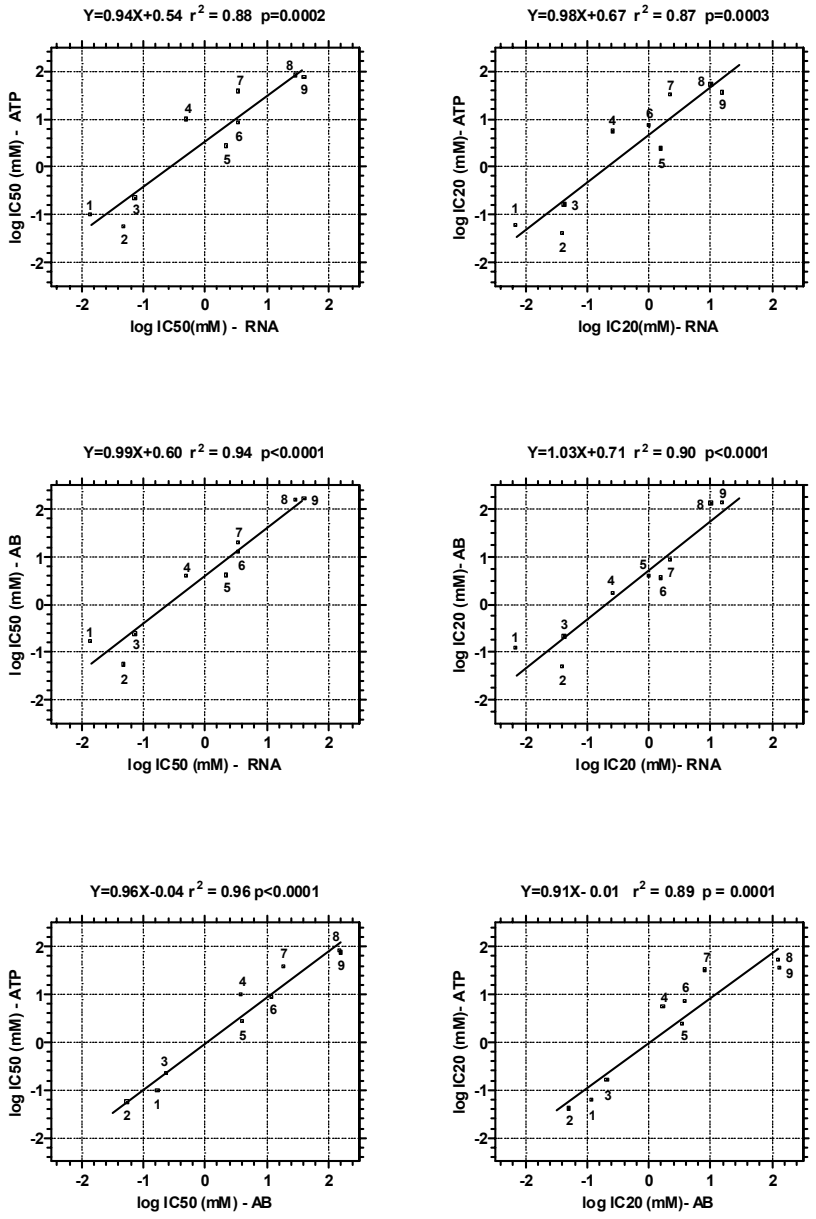
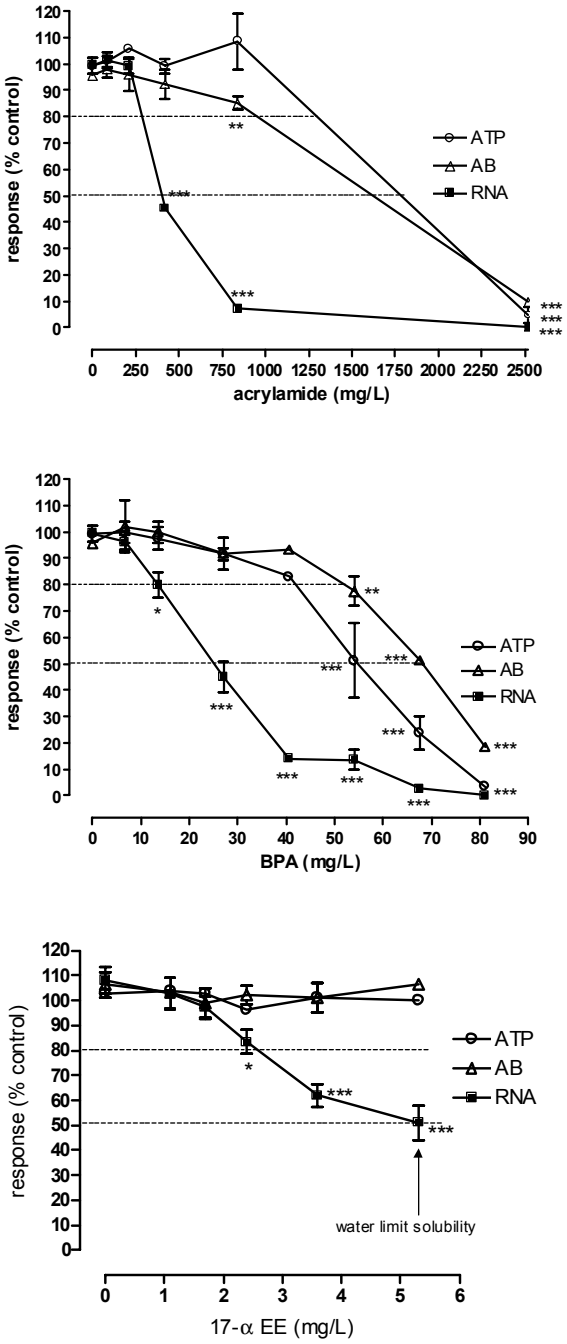


Figure 4

Click here to download Figure: Figure 4.doc