Comparison of Yeast Resazurin Versus MTT Assay in vitro Methods For Determining Acute Toxicity of Halogenated Alkanes

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ABSTRACT

Halogenated alkanes may have potentially human health effect as a result of their persistence, bioaccumulation and toxicity after their release from environment into the food chain and water products. It is leading to increase attention for legislation aimed at prevention and great pressure to reduce the production and emission rate of halogenated alkanes. Besides many research efforts to understand the fate and (eco)toxicological effects of the halogenated alkanes. Several investigators have used animal in vivo in conventional toxicity studies of halogenated alkanes. Nevertheless, experimental by using animal testing is always time and resource demanding. Thus, it is not deemed suitable for screening of large number of potential toxicants. The main objective of this work was to investigate the comparability of yeast resazurin assay versus MTT assay for determining in vitro acute toxicity (EC_{50}) of halogenated alkanes. The MTT assay was conducted using Chinese Hamster Ovary (CHO cell), whilst yeast strains were used in yeast resazurin assay. The study demonstrates a comparability result to which halogenated alkanes is more toxic to CHO cell than to yeast cell.

Keywords: Halogenated alkanes, in vitro, yeast resazurin, MTT, acute toxicity

INTRODUCTION

The halogenated alkanes (HA's) are an important class of halogenated aliphatic hydrocarbon that is produced in large quantity and the worldwide annual production accounts for billion of pounds (Crebelli *et al*. 1995). HA's compounds have been widely used for many years in household, agriculture and chemical industry. They are used as pesticides, soil fumigants, disinfectants, solvents in the dry-cleaning process, or chemical reagents (Holloway *et al.* 1998, Glatz *et al.* 2000, Olaniran, *et al*. 2004]. However it is notable that their widespread production and application have an ecological effect.

For long time ago, it has been assumed that HA's mainly entering the environment from anthropogenic sources as consequence of their use (Crebelli *et al*. 1995). Once HA's enter the environment, many of these chemicals are naturally non-degradable and persist in the environment to become major pollutant of the biosphere and groundwater (Kulakova *et al.* 1995). Environment contaminations of HA's have been reported in several literatures and were found at high level of concentration. Nicholls *et al.* (2001), reported the concentration of HA's in soil, ranged from 0.2–65.1 mg/kg dry weight. Similar to Nicholls *et al.* (2001), Guo *et al.* (2004) found HA's concentration ranged from 10 to 20 $\mu g/m^3$ in air samples.

Moreover, HA's may have potentially human health effect as a result of their persistence, bioaccumulation and toxicity after their release from environment into the food chain and water products (Trohalaki *et al.* 2000). Toxicity studies show that HA's were potentially carcinogenic in humans based on mammalian and organism experiments (IARC 1987). HA's induced respiratory tract tumour through inhalation exposure to rodent and produced *c*DNA-damage in nasal mucosa cells in male mice (Eckert *et al*. 1997). Oral administration of male Wistar rats caused mild-to-moderate toxic injury of the lung (Salovsky *et al.* 2002). Other studies demonstrated that HA's induced apoptosis retinal cell cultures (Malchiodi-Albedi *et al.* 2003) and exhibited reproductive toxicity in rats (Ichihara *et al.* 2004).

Now, it is clear that anthropogenic introduction of HA's in nature may have important due to their consequences to human health and its environment. As result there has been increasing attention for legislation aimed at prevention and great pressure to reduce the production and emission rate of HA's. There has also been research effort to understand the fate and (eco)toxicological effects of the HA's. Several investigators have used animal *in vivo*

in conventional toxicity studies of HA's (Fisk *et al*. 1998, Fisk *et al*. 1999, Yen *et al.* 2002). It is argued that providing chemicals toxicity information which is using animal experiment, gives the more reliable data about the effect of chemicals. Nonetheless, the experimental by using animal testing is always time and resource demanding. Thus, it is not deemed suitable for screening of large number of potential toxicants (Netzeva & Schutz 2005, EU 2001). So an alternative approach, that is called in vitro techniques, could be useful. The in vitro technique is offer a higher speed and is not always resource demanding compare to the animal testing. Thus, the aim of the present work was to investigate the comparability two *in vitro* methods (yeast resazurin versus MTT assay) for determining the acute toxicity of HA's.

METHODS

Materials

Test chemicals of the highest purity and commercially available were used. 1,8-dichlorooctane, 1-chlorooctane, 1,9-dichlorononane, 1-chlorononane, 1,10-dichlorodecane, 1-chlorodecane, 1-chlorotetradecane, perfluorooctane, 1-fluorooctane, 1-fluorodecane, 1-fluorododece, 1-fluorotetradecane, 1,8-dibromooctane, 1-bromodecane, 2-bromododecane, 1-bromododecane, 1,10-dibromodecane, 1,11-dibromoundecane, 1,12-dibromododecane, 1-bromopentadecane, 1-chlorodo decane, 1-bromooctane, 1,9-dibromononane, 1-bromononane, 1-bromoundecane, 1-bromotridecane, 1-bromotetradecane, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra zolium bromide (MTT), Glucose, Sodium chloride and Copper II Sulfate-pentahydrate (CuSO4.5H20) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany); Dimethyl sulfoxide (DMSO) from Acros Organic (New Jersey, USA); 7-Hydroxy-3H-phenoxazin-3-one 10-oxide, sodium salt (Resazurin), Fetal calf serum (FCS), Hank's basic salt solution (HBSS), Dulbecco's minimum essential medium (D-MEM), phosphate buffer saline pH 7.4 solution, yeast extract, and trypsin were purchased from Gibco Invitrogen (Scotland, UK); Phosphate buffer saline (Dulbecco A) tablet from OXOID (Hampshire, England); and peptone were purchased from DIFCO Laboratories (Michigan, USA).

Instruments

The following instruments were used in present work i.e. Fluorescence Spectrophotometer (Varian Instrument California, USA), Spectrophotometer Spectra Max 340 (Molecular Device Corporations,Sunnyvale, USA) and Spectrophotometer Multiskan Spectrum QRG (ThermoLabsystem Vantaa, Finland).

Assays

The acute toxicity assay of HA's was conducted using two different methods i.e., yeast resazurin and MTT. These methods were performed as described in the next following section. The obtained data of test compound, which is represented as median effect concentration (EC50), were then calculated using LSW Analysis Toolbox (MDL 2004). The statistical significance different (P) values of EC50 of tested compound were calculated using a two-tailed Student's t-test, assuming unequal variance, within the MS Excel 2000 software (Microsoft 2004). In general, yeast resazurin assay was performed by using a method generally adopted from TOX 20306 (WUR 2005), with slightly modification.

Yeast strain culture and growth condition

The yeast culture was grown on agar-containing tube. One loop of yeast colonies were cultured in yeast/peptone/glucose growth medium (20 ml) followed by incubation overnight at 30oC and 175 rpm. The optical density at 620 nm of the cultured cell suspension was adjusted to 1.9. The cultured cell suspension was centrifuged for 5 minutes at 1500 rpm and 30oC. The supernatant was discarded and the cells were resupended with the same volume using phosphate buffer saline (PBS)-enriched glucose solution. PBS-enriched glucose solution containing yeast cells were stored on the ice for the assay.

Assay procedure

Serial dilutions of concentration stock of HA's (20-1000 mM) were made to achieve the desired final concentration in PBS-enriched glucose solution with the maximum final concentration of DMSO in the solution was 1%. PBS-enriched glucose solution containing yeast cells (100 µl) was transferred to 96-wells plate followed by transferring of PBS-enriched glucose containing test compound (100 µl) to the same plate. Each compound was tested in six replications of well per each concentration used. The plates were incubated on a shaking incubator for 24 hours at 30^{o}C. After 24 hours, the culture was taken out and resazurin (20 µl) was added to each well. The plate was returned to incubator and the fluorescence was read at 530 nm ($\lambda_{excitation}$) and 590 nm ($\lambda_{emission}$) after 3 hours. The cell activity as percentage of non-exposed cell was used to determine the EC_{50} values.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay

The MTT assay was conducted using a modification of the method used by Labieniec *et al*. (2003) and Lapshina *et al.* (2005). Chinese Hamster Ovary (CHO) cell lines were used in this assay.

Cell cultivation
The CHO cells were cultivated in Dulbecco's minimum essential medium (D-MEM) supplemented with 10% fetal calf. The cells were grown in incubator at 37^{o}C and 5% CO_2.

Seeding of CHO cells
Confluent cells (70–80%) in Corning flasks were detached using a trypsin solution followed by removing the trypsin. The cells were re-suspended in D-MEM growth medium and counted using a hemacytometer. Cell densities were adjusted to $1x10^5$ cells/ml. The suspension of cells (100 µl) was seeded into a 96-wells plate. Six replications of well for each concentration of compound used were made. The plate was incubated at 37°C and 5% CO_2 for 24 hours.

Assay procedure
After seeding the cells for 24 hours, the plate was taken out from the incubator and the cells were exposed with D-MEM growth medium containing test compound and incubated for a further 24 hours at 37°C and 5% CO_2. Each compound was tested in ten different concentrations. Thereafter 10 µl of MTT solution (5 mg/ml final concentration) was added to the wells and incubated for 1 hour at 37°C and 5% CO_2. At the end of the treatments, the medium was removed prior to the addition of DMSO (200 µl) into each well. The 96-wells plate was allowed to stand for 10 minutes followed by shaking for 15 seconds. The color was measured at wavelength 562 nm. The absorbance obtained was used to determine the EC_{50} values.

RESULTS AND DISCUSSION

Yeast resazurin assay
Viable yeast cells possess mitochondrial enzymes, which are capable of metabolizing compounds. The viability of the cells determines the activity of those enzymes. The assay is based on the ability of living yeast cells to convert resazurine (blue) into resorufin (pink) through a reduction-oxidation reaction. The resorufin (pink) reaction-product can therefore be determined by measuring the generated fluorescent signal at $\lambda_{emission}$ = 590 nm and $\lambda_{excitation}$ = 530 nm (Labieniec & Gabryelak 2003).

The first experimental acute toxicity testing of HA's was done by using the method adopted from TOX-20306 (WUR 2005). In this method, the resazurin was added to the exposed yeast cell immediately after exposing the yeast cell to HA's followed by 1 hour incubation (*un-modified method*). In brief, result shows that HA's were not toxic at all HA's (Figure 1). Since the HA's were not toxic, two modifications were introduced (*modified method*). First, the exposure time was 24 hours. Second, after the addition of the resazurin, the yeast cells were incubated for 3 hours.

Figure 1 shows a comparison between un-modified and modified method applied to 1,10-dichlorodecane as an example of test compound. By using un-modified method, 1,10-dichlorodecane was not revealed toxicity effect to yeast cell (non detectable of the EC_{50}) and the relative fluorescence units (RFU) were not statistically significant (P<0.05) compared with control (RFU < 100). Furthermore, by using modified method, 1,10-dichlorodecane showed a toxic effect to the yeast cell (EC_{50} = 0.358) and increasing the RFU values were achieved (RFU > 100).

Additionally, The RFU values of 1,10-dichlorodecane is decreasing with concentration dependent manner (statistically significant at P<0.05), in which increasing the concentration of 1,10-dichlorodecane led to decreasing its RFU values. Moreover, the modified method was used in the acute toxicity experiment of all HA's. The acute toxicity ranges of all HA's attained, expressed in EC_{50} (Table 1), were 0.212-0.988 mM, 0.152-1.413 mM, and 0.139-0.793 mM for chlorinated, fluorinated, and brominated group, respectively. Two important results were found using modified method. Firstly, 1,10-dichlorodecane represent all tested HA's showed toxicity effect to the yeast cells (EC_{50} = 0.358 mM). The reason might be 1,10-dichlorodecane with high level of hydrophobicity (log K_{ow}) has low capability to enter the cell membrane of the yeast cells. In other words, 1,10-dichlorodecane need longer time to enter the yeast cell membranes. Secondly, RFU were significantly increasing with the RFU values is higher than 100 (Figure 1). This might be the yeast cells needs longer time to convert the resazurin to resorufin through redox reaction. This result is comparable to work performed by Riss *et al.*, (2006). Their study showed that prolongs the time of resazurin reduction by Jurkat cells led to higher fluorescence signal of resorufin.

MTT assay
Compounds can be toxic to cells in different ways for example resulting in: diminished cell growth, lowered biological activity (e.g. changed enzyme activity), and decreased cell membrane integrity, followed by the starting of programmed cell death (apoptosis). To be able

to quantify cell metabolic activity and proliferation the MTT (3-[4.5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay is used. The yellow tetrazolium salt MTT is converted to purple formazan crystals by metabolically active cells. The amount of purple staining is a measure for the activity of this enzyme in the cells. The purple reaction-product can be determined spectrophotometrically at a wavelength of 562nm (Labieniec & Gabryelak 2003, Lapshina *et al.* 2005).

Many MTT studies have been performed which were related to viability of different cells (Ribiero-Dias *et al*. 2000, Sjogren *et al*. 2000, Labieniec & Gabryelak 2003, Lapshina *et al.* 2005). The first attempt was to find a suitable density of CHO cell for the assay. The cell density should give approximately 75-85% of cell confluences after 24 hours incubation. $1.5x10^4$ of CHO cells/ml, referred to by Labieniec & Gabryelak, (2003), were grown into 96-wells plate followed by 24 hours incubation. After the incubation the confluence of cells was found < 50%. Since the confluence of CHO cells was not enough to reach cell confluence as required, the density of CHO cells was modified to 1×10^5 cells/ml.

The modification of cell density led to increase the CHO cells confluence up to approximately 75-85%. Thereafter the cell density of 1×10^5 cells/ml was applied to investigate the acute toxicity of all HA's. The acute toxicity ranges of all HA's obtained (Table 1) were 0.134-1.080 mM, 0.208-1.270 mM, and 0.073-0.493 mM for chlorinated, fluorinated, and brominated group, respectively. Figure 2 shows the viability of the yeast and CHO cells after 24 hours exposure to 1.10-dichlorodecane as an example, where the viability of yeast and CHO cells decreased with concentration dependent manner. 1.10-dichlorodecane significantly ($P<0.05$) decreased viability of yeast and CHO cells at all tested concentrations. Additionally, the viability of yeast cells obtained from resazurin assay was higher than CHO cells using MTT assay. It might be the capability of 1,10-dichlorodecane to enter the CHO cells was easier than to yeast cell due to the yeast cell membranes is thicker than CHO cells. The acute toxicity (EC_{50}) of 1.10-dichlorodecane to yeast and CHO cell was 0.358 mM and 0.204 mM, respectively.

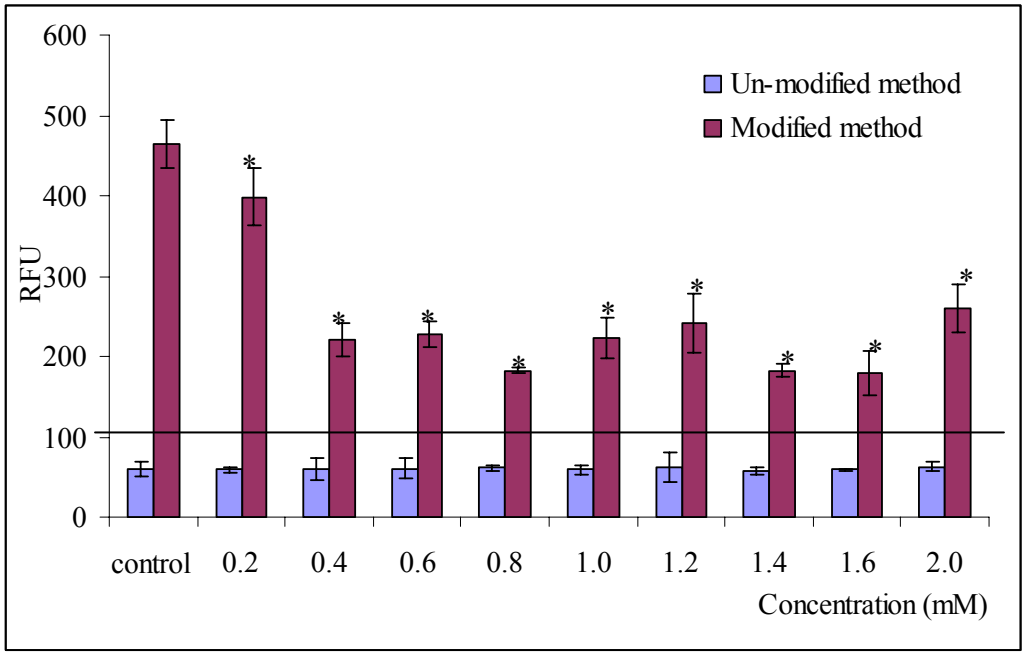

Figure 1. Yeast cells treated with 1-chlorodecane using un-modified and modified method. Each point on the graph represents relative fluorescence ± standard deviation (n=6). * = statistically significant at $P<0.05$ compared with control, two-tailed Student's *t*-test, assuming unequal variance.

A similar comparison study using resazurin and MTT assay has been conducted by O'Brien *et al*., (2000) using the HeLa cell lines. The result showed that the EC_{50} obtained from MTT assay is lower that from yeast resazurin assay. However, the result from O'Brien *et al*., (2000) could not be compared to this study. The reason is that the present study used different cells i.e. yeast and CHO cells in resazurin and MTT assay, respectively. Therefore, further study to investigate the acute toxicity of HA's by using the same cell lines in both of assays may be helpful and would be more comparable.

Generally, in comparison of the acute toxicity between yeast resazurin and MTT assay was found that HA's were more toxic to CHO cells (i.e. twenty HA's) than yeast cells (i.e. seven HA's) (Table 1). A number of factors may contribute to the relative toxicity of HA's to these two species organisms. Zhao *et al.*, (1998) considered that there are two main factors that can affect to the species differences for acute toxicity properties of many compounds. The first factor is the effect of bio-concentration and the second is biotransformation including metabolism and the kinetic of clearance and accumulation. Therefore, it would be important to take into account the characteristic cell membranes of test species related to the toxicity study of HA's. Since HA's with high level of hydrophobicity (log K_{ow}) will probably affect to the interaction processes of these chemicals to the cell membranes of test species.

CONCLUSION

From those two different assay, it might be concluded that the acute toxicity (EC_{50}) of HA's against CHO cells (MTT assay) is lower than against yeast (yeast resazurin assay). However, a comparative study between resazurin and MTT assay using the same type of animal cell lines is likely preferable so that a better comparability of these two assays can be obtained.

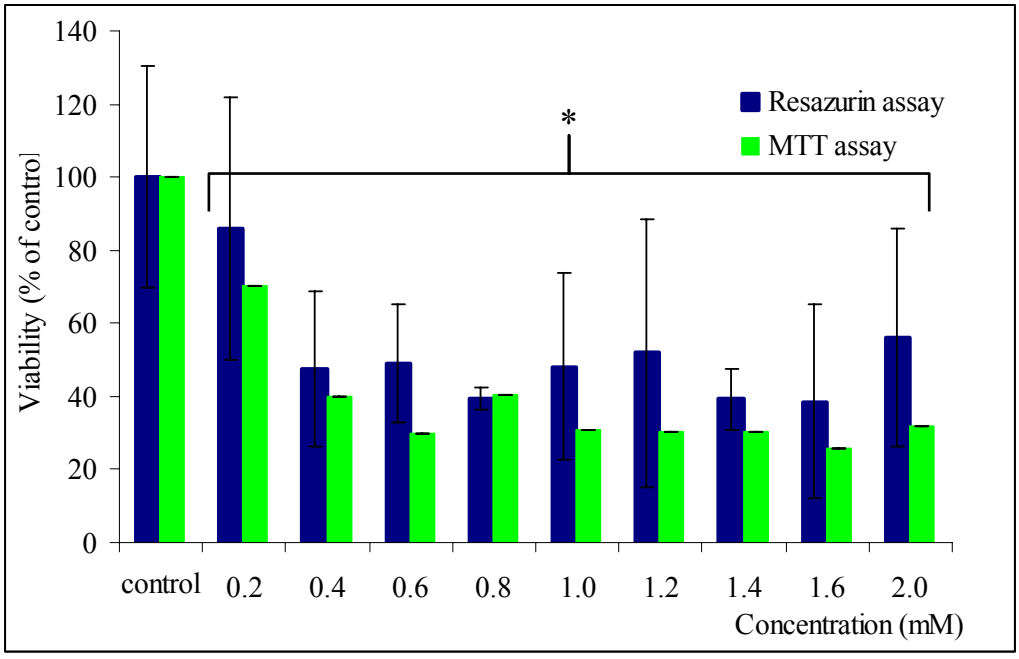

Figure 2. Cytotoxicity effects of 1,10-dichlorodecane to yeast and CHO cells treated for 24 hours. Each point on the graph represent ± standard deviation (n=6). * = statistically significant (P<0.05) compared with control, two-tailed Student's *t*-test, assuming unequal variance.

Table 1. Median effect concentration (EC_{50}) of HA's studied

No	CAS Number	Name of compound	Molecular formula	Log K_{ow}	Assay (EC_{50})	
					Yeast (mM)	MTT (mM)
1	2162-99-4	1,8-dichlorooctane	$C_8H_{16}C_{l2}$	4.352	0.494	0.353*
2	111-85-3	1-chlorooctane	$C_8H_{17}Cl$	4.639	0.512	0.482*
3	821-99-8	1,9-dichlorononane	$C_9H_{18}C_{l2}$	4.881	0.786	0.333*
4	2473-01-0	1-chlorononane	$C_9H_{19}Cl$	5.168	0.394*	0.407
5	2162-98-3	1,10-dichlorodecane	$C_{10}H_{20}Cl_2$	5.410	0.358	0.204*
6	1002-69-3	1-chlorodecane	$C_{10}H_{21}Cl$	5.697	0.212	0.134*
7	112-52-7	1-chlorododecane	$C_{12}H_{25}Cl$	6.755	0.988	0.268*
8	2425-54-9	1-chlorotetradecane	$C_{14}H_{29}Cl$	7.813	0.407*	1.080
9	307-34-6	perfluorooctane	C_8F_{18}	4.150	1.413	1.270*
10	463-11-6	1-fluorooctane	$C_8H_{17}F$	4.200	0.332	0.243*
11	334-56-5	1-fluorodecane	$C_{10}H_{21}F$	5.260	0.394	0.208*
12	334-68-9	1-fluorododecane	$C_{12}H_{25}F$	6.320	0.152*	0.551
13	593-33-9	1-fluorotetradecane	$C_{14}H_{29}F$	7.370	0.285*	0.897
14	4549-32-0	1,8-dibromooctane	$C_8H_{16}Br_2$	4.632	0.364	0.161*
15	111-83-1	1-bromooctane	$C_8H_{17}Br$	4.779	0.171*	0.493
16	4549-33-1	1,9-dibrononanone	$C_9H_{18}Br_2$	5.161	0.706	0.397*
17	693-58-3	1-bromononane	$C_9H_{19}Br$	5.308	0.316*	0.411
18	112-29-8	1-bromodecane	$C_{10}H_{21}Br$	5.837	0.304	0.285*
19	4101-68-2	1,10-dibromodecane	$C_{10}H_{20}Br_2$	5.690	0.701	0.326*
20	693-67-4	1-bromoundecane	$C_{11}H_{23}Br$	6.366	0.139*	0.192
21	16696-65-4	1,11-dibromoundecane	$C_{11}H_{22}Br_2$	6.219	0.764	0.476*
22	3344-70-5	1,12-dibromododecane	$C_{12}H_{24}Br_2$	6.748	0.736	0.193*
23	13187-99-0	2-bromododecane	$C_{12}H_{25}Br$	6.895	0.793	0.240*
24	143-15-7	1-bromododecane	$C_{12}H_{25}Br$	6.895	0.214	0.162*
25	765-09-3	1-bromotridecane	$C_{13}H_{27}Br$	7.424	0.532	0.128*
26	112-71-0	1-bromotetradecane	$C_{14}H_{29}Br$	7.953	0.738	0.073*
27	629-72-1	1-bromopentadecane	$C_{15}H_{31}Br$	8.482	0.421	0.115*

*More toxic than other assay

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