Bull. Environ. Contam. Toxicol. (1991) 46:649–653
© 1991 Springer-Verlag New York Inc.

Environmental Contamination and Toxicology

Correlation of the Neutral Red Uptake Inhibition Assay of Cultured Fathead Minnow Fish Cells with Fish Lethality Tests

Paul J. Dierickx* and Ingrid E. Van De Vyver

Institute of Hygiene and Epidemiology, Wytsmanstraat 14, B-1050 Brussels, Belgium

The continuously increasing production and use of chemicals have led to heavily contaminated fresh-, coastal- and seawater in most of the industrialized regions of the world. These polluting chemicals, both inorganic and organic, are generated by nearly any human activity. The development of appropriate ecotoxicity assays is therefore an indispensable task. Actually the most used one is the short-term lethality test on fish, in which the results are expressed as the concentration of test compound lethal to fish. However, this test is costly, time-consuming and inhuman.

So far cultured fish cells were occasionally used in cytotoxicity testing (Rachlin and Perlmutter 1968; Kocan *et al.* 1979), but were recently used for the ecotoxicity determination of organic and inorganic metal compounds (Babich and Borenfreund 1990; Babich *et al.* 1990; Babich *et al.* 1986), and both aromatic and aliphatic organic pollutants (Babich and Borenfreund, 1987a-c). These authors always made *in vitro-in vivo* comparisons for chemicals belonging to the same chemical group. A good *in vitro* method should ideally be of use for all chemical compounds. Therefore, the cytotoxicity of chemicals belonging to different chemical groups was investigated in cultured fish cells, determined by the neutral red uptake inhibition assay. The results were compared with fish lethality data (LC50) measured on golden orfe (*Leuciscus idus melanotus*) in a 48 hr assay (Juhnke and Lüdemann 1978) as described by Mann (1976). Further analysis of the golden orfe toxicity data was described by Kamlet *et al.* (1987).

MATERIALS AND METHODS

FHM cells are an established fish cell line (American Type Culture Collection N° CCl42, obtained from Flow Laboratories, Brussels, Belgium), derived from tissue posterior to the anus from fat head minnow. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% non-essential amino acids, 100 units/mL penicillin, and 100 µg/mL streptomycin (complete medium) and incubated at 34 °C in a humidified atmosphere of 5% CO_2.

The neutral red uptake inhibition assay was essentially performed as described by Borenfreund and Shopsis (1985). The cells were seeded with an 8-channel pipet into 64 wells of a titer plate (Nunc) (60,000 cells in 0.2 mL complete medium per well). After incubation for 24 hr at 34 °C the medium was removed by inverting the multidish. The cultures were treated with 0.2 mL aliquots of different

*Correspondence and reprint requests

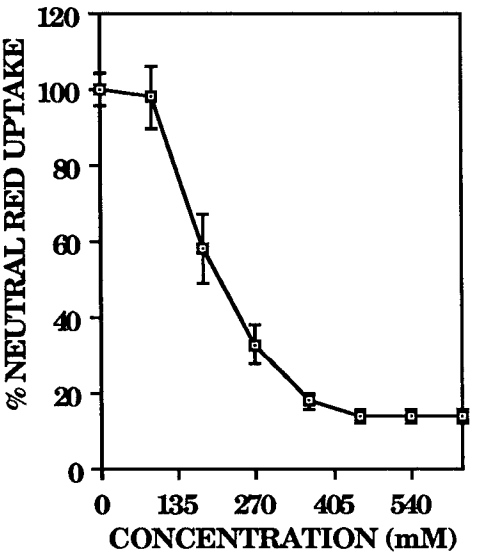

Figure 1. Effect of 2 hr treatment with various concentrations of n-butanone on the neutral red uptake in FHM fish cells.

concentrations of freshly prepared test compounds in complete medium. All test chemicals were soluble in complete medium. Eight wells were used for each concentration. The highest concentration was chosen following preliminary range-finding experiments. After 2 hr the medium with test compounds was removed. The cells were washed with 0.9% NaCl and treated with 0.2 mL neutral red (50 µg/mL) in complete medium for 3 hr. After this the cells were treated for 1 min with 0.2 mL of a solution composed of 1 mL 37% formaldehyde, 10 mL 10% calcium chloride and 89 mL water. This solution was finally replaced by 0.2 mL of 1% acetic acid in 50% ethanol and the titer plate was agitated for 15 min. The absorbance was measured with a microplate reader (Eurogenetics, Tessenderlo, Belgium) at 540 nm. The results are expressed in percentages compared to control cultures. The relative toxicity of the test compounds is established by determination of the NI50. This is the concentration of test compound required to induce a 50% inhibition in neutral red uptake.The endpoint quantitates the amount of dye taken up and retained by viable cells. The absorbance of the dye extracted from cells after exposure to the test chemicals is a reflection of cell survival, since dead or injured cells cannot retain the dye in their lysosomes.

In order to determine the reproducibility of NI50 determinations, the NI50 values of propanol and mercuric chloride were determined in three independent assays. Since a standard deviation of less than 4% was found, the NI50 values of the other compounds were determined in one experiment, each based on 64 different neutral red measurements.

RESULTS AND DISCUSSION

After incubation of the test chemicals with FHM cells for 2 hr, the neutral red uptake inhibition was measured after a neutral red uptake period of 3 hr. The results of a typical NI50 experiment are given for n-butanone in Fig. 1. Interpolation of

Table I. Comparative toxicities of the compounds tested in vitro and in vivo.

Compound	NI50[a]		LC50[b]
	mM	mg/L	mg/L
Mercuric chloride	0.11	30	0.5
Zinc chloride	0.48	65	21
n-Octanol	2.0	260	18
Ethanolamine	2.7	165	375
Barium chloride	2.9	604	870
n-Heptanol	3.9	453	35
Phenol	7.8	734	19
Nickel chloride	9.2	2,187	570
Chloral hydrate	13	2,150	1,720
Citric acid	14	2,942	600
Oxalic acid	17	2,143	242
Diethanolamine	22	2,313	1,640
Benzoic acid	23	2,809	460
Trichloroacetic acid	37	151	>10,000
n-Pentanol	43	3,790	485
Triethanolamine	47	7,012	>10,000
Acetic acid	49	2942	410
Butyric acid	52	4,582	365
Ethyl acetate	86	7,577	301
n-Butanol	120	8,894	1,485
n-Butanone	198	14,278	4,740
Tetrahydrofuran	212	15,287	2,875
n-Propanol	300	18,030	4,440
Isopropanol	418	25,120	9,125
Acetamide	522	30,835	>10,000
Acetone	592	34,383	9,402
n-Ethanol	880	40,542	8,140
Acetonitrile	903	37,068	6,450
Methanol	1,950	62,478	>10,000

[a]*The concentration of test compound required to induce a 50% reduction in neutral red uptake in cultured FHM fish cells.*
[b]*Midpoint toxicity in a 48 hr LC50 assay on golden orfe; data from Juhnke and Lüdemann (1978).*

these curves gives the NI50 values, by computing the concentration needed to reduce the absorbance by 50%. The NI50 values of the 29 investigated compounds are summarized in Table 1, together with their lethality data on golden orfe as measured by Juhnke and Lüdemann (1978). The chemicals are arranged according to their increasing cytotoxic potency. They were selected in order to include chemicals of different chemical groups (inorganic compounds, alcohols, acids, ketones, and others), covering a toxicity range from very toxic to nearly non-toxic, but also in order that some chemicals belonging to the same chemical group were included.

A good linear relationship between chemical structure and cytotoxicity is obtained when the NI50 of compounds belonging to the same chemical group is considered.

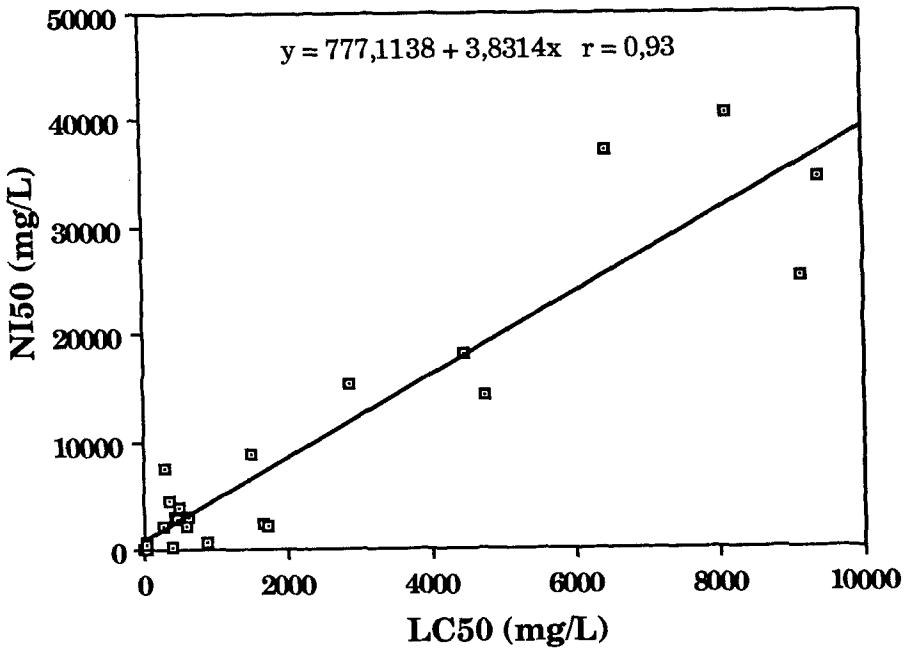

Figure 2. Linear regression analysis of the relationship between neutral red uptake inhibition in FHM cells and the lethality in golden orfe.

This is illustrated by the regression equation obtained for n-alcohols as a function of their C-number x [logNI50 (mM) = 3.76 - 0.44x, r^2 = 1.00, n = 7], for acetic acids as a function of their number x of substituted chloro-atoms [NI50 (mM) = 48.57 - 4.14x, r^2 = 0.96, n = 3], and for amines as a function of their number x of substituted ethanol-groups [NI50 (mM) = 20.40 + 22.15x, r^2 = 1.00, n = 3]. A linear relationship with the n-alcohol chain length was previously also found when the glutamic acid uptake inhibition (Dierickx 1989a) or the total protein content (Dierickx 1989b) was measured in Hep G2 cell cultures.

The relationship between the NI50 and the LC50 (x) values of the n-alcohols [NI50 (mg/L) = 488.46 + 4.73x, r^2 = 0.98, n = 6] and the ethanol-substituted amines [NI50 (mg/L) = 514.63 + 0.67x, r^2 = 0.96, n = 3] was also linear. These values are equal to those obtained for the correlation between the NI50 of organomercurials in BG/F cells and the LC50 in rainbow trout (Babich *et al.* 1990). They are better than those obtained for heavy metals (Babich *et al.* 1986) and the total set of nitro- and chlorophenols (Babich and Borenfreund 1987c). A linear correlation coefficient r^2 of 0.86 was obtained between the LC50 and the NI50 values for the total series of 25 chemicals which were tested in this study and for which quantitative *in vivo* data are available (Fig. 2). Since these chemicals belong to very different chemical classes, this correlation can be considered as very valuable. The neutral red uptake inhibition in cultured fish cells correlates very well with lethality data determined on fish. Nevertheless, the described *in vitro* test can be performed easier, more quickly and is less expensive than the *in vivo* test, and does not require high numbers of fishes. Therefore, the neutral red uptake inhibition assay in cultured fish cells can be considered as a valuable tool for *in vitro* ecotoxicity testing.

REFERENCES

Babich H, Borenfreund E (1987a) Structure-activity relationship (SAR) models established *in vitro* with the neutral red cytotoxicity assay. Toxic In Vitro 1:3-9

Babich H, Borenfreund E (1987b) Polycyclic aromatic hydrocarbon *in vitro* cytotoxicity to bluegill BF-2 cells : mediation by S-9 microsomal fraction and temperature. Toxicol Lett 36:107-116

Babich H, Borenfreund E (1987c) *In vitro* cytotoxicity of organic pollutants to bluegill sunfish (BF-2) cells. Environ Res 42:229-237

Babich H, Borenfreund E (1990) *In vitro* cytotoxicities of inorganic lead and di- and trialkyl lead compounds to fish cells. Bull Environ Contam Toxicol 44:456-460

Babich H, Goldstein SH, Borenfreund E (1990) *In vitro* cyto- and genotoxicity of organomercurials to cells in culture. Toxicol Lett 50:143-149

Babich H, Puerner JA, Borenfreund E (1986) *In vitro* cytotoxicities of metals to bluegill (BF-2) cells. Arch Environ Contam Toxicol 15:31-37

Borenfreund E, Shopsis C (1985) Toxicity monitored with a correlated set of cell-culture assays. Xenobiotica 15:705-711

Dierickx PJ (1989a) Glutamic acid uptake inhibition assay in cultured Hep G2 cells as an alternative method for evaluating potential *in vivo* eye irritation. Alt Lab Animals 16:344-352

Dierickx PJ (1989b) Cytotoxicity testing of 114 compounds by the determination of the protein content in Hep G2 cell cultures. Toxic In Vitro 3:189-193

Juhnke I, Lüdemann D (1978) Ergebnisse der Untersuchung von 200 chemischen Verbindungen auf akute Fischtoxizität mit dem Goldorfentest. Z Wasser Abwasser-Forschung 11:161-164

Kamlet MJ, Doherty, RM, Taft, RW, Abraham, MH, Veith, GD, Abraham, DJ (1987) Solubility properties of polymers and biological media. 8.An analysis of the factors that influence toxicities of organic nonelectrolytes to the golden orfe fish (*Leuciscus idus melanotus*). Environ Sci Technol 21:149-155

Kocan RM, Landolt ML, Sabo KM (1979) In vitro toxicity of eight mutagens/carcinogens for three fish cell lines. Bull Environ Contam Toxicol 23:269-274

Mann H (1976) Fischtest mit Goldorfen zur vergeleichenden Prüfung der akuten Toxizität von Wasserinhaltstoffen und Abwässern. Praktische Erfahrungen aus drei Ringtesten. Z Wasser Abwasser-Forschung 9:103-109

Rachlin JW, Perlmutter A (1968) Fish cells in culture for study of aquatic toxicants. Water Res 2:409-414

Received May 21, 1990; accepted December 15, 1990.