Comparison of 4 Cytotoxicity Assays Performed Sequentially on the Same Cell Sample

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Introduction and Summary:

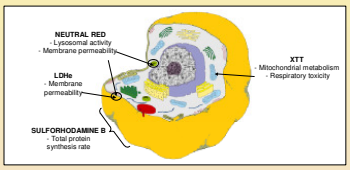
Novel compounds need to be evaluated for unwanted cytotoxic effects early in development. At these stages the amount of available test compound is usually limited, yet as much information as possible on unwanted side-effects should be obtained in order to determine which compounds warrant further development.

The evaluation of cytotoxic effects on cell lines or primary cells is a well established and widely accepted method. Several different cellular targets using various read-outs and different exposure protocols are being used. Some assays measure effects on overall survival or proliferation, while others can give more specific insights on toxic mechanisms.

Toxicity testing at early stages of development should have a high sensitivity, i.e. effectively toxic substances should have a very high probability of being identified. Moreover, it should be reasonably simple, reproducible, and cost-effective. The PAN I cytotoxicity kit by Xenometrix allows to apply 4 commonly used cytotoxicity assays sequentially on 1 cellular sample. Such an approach has the following main advantages:

- 4 x less test compound consumption than with individual assays
- Enhanced probability to identify toxic compounds
- Information on possible mechanisms of action
- Reduced consumption of valuable primary cells
- No inter-assay variability due to variations in cell composition, cellular activity, cell density or other external factors
- Quality controlled reagents and straightforward, highly reproducible protocols.

Here we present a comparative evaluation of 6 cytotoxic compounds in the newly available PAN I Cytotoxicity assay kit. This kit combines assays for **extracellular Lactate Dehydrogenase (LDHe**) to measure membrane integrity, the **XTT** tetrazolium salt to evaluate mitochondrial activity, **Neutral Red (NR)** for the evaluation of lysosomal activity, and the **Sulforhodamine stain (SRB)** for total protein content.



The 4 assays are performed sequentially on the same cell samples in triplicates. This experimental set-up eliminates intra-test variability due to potential variables such as passage number, time-since-plating, cell density, medium composition (medium age) and others. Differences in IC50 between assays reflect therefore more accurately the differential response of cellular functions and compartments to toxicants because all other sources of variability due to external factors are eliminated. Compounds were tested for their cytotoxic activity on L929 mouse fibroblasts after 4 different exposure times.

The results demonstrate the feasibility of performing 4 different assays sequentially, and time-dependent examples of differential responses in the assays are presented. The PAN I assay can identify compound toxicity with a higher probability than a single assay, and valuable information on cellular target structures may be obtained.

Methods:

The tests were performed on L929 mouse fibroblasts. Cells were harvested with Trypsin/EDTA and seeded at 20'000 (2 hr and overnight exposure) or 10'000 (24 and 48 hr exposure) cells per well into 96-well microtiter plates in DMEM/Ham's F12 medium supplemented with 10% FCS, Glutamine and Penicillin/Streptomycin.

The cells were allowed to adhere for 6 hrs before addition of test compounds for 2, 16 (overnight), 24, or 48 hrs.

The following compounds were tested:

Compound	Highest conc.	Solvent
5-Fluorouracil	1 mM	DMSO
Isophorone	100 mM	TC Medium
Chlorpromazine	500 µM	Water
Chloroquine	5 mM	Water
p-Phenylenediamine	1,5 mM	DMSO
Propranolol	5 mM	DMSO

From each compound 8 concentrations, serially diluted in half-log steps were tested in triplicates. Each test was repeated once.

Assays for LDH, XTT, NR and SRB were performed sequentially on the same cells according to the 'Instructions for Use' of the InCytotox PAN I kit from Xenometrix.

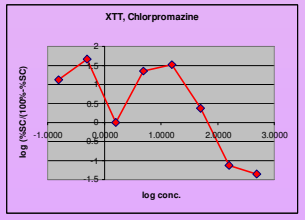
IC50 values were determined as follows:

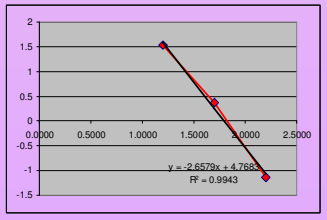
A linearized graph was obtained by plotting

log (%SC/(100%-%SC) vs. log concentration

where

%SC is the OD (measured at an assay-specific wavelength and corrected for the blank value) expressed as a percentage of the Solvent Control OD obtained. From such linearized graphs those data clustering around "0" (= 50% of SC) were used to calculate a linear regression and from this equation the corresponding IC50 was determined. An example is shown below.



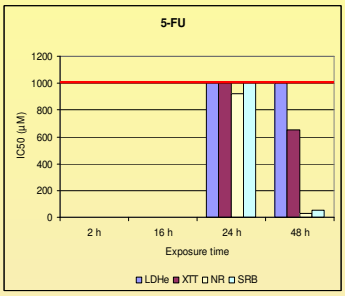


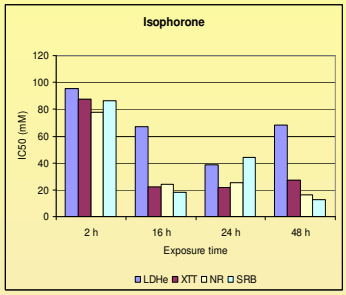
In this example the calculated IC50 is 62 µM

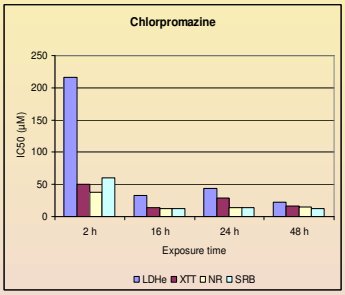
All IC50 values obtained by this method were averaged over two independently conducted experiments.

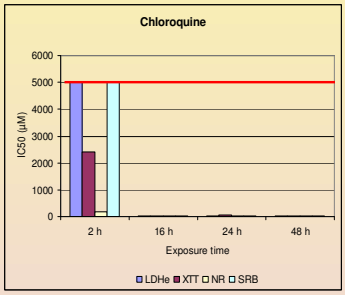
Results:

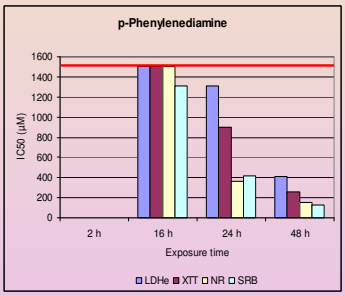
Figure 1 shows IC50 values of 6 toxicants tested sequentially for LDH, XTT, NR and SRB inhibition. Four different exposure times are shown. The red bar indicates the highest concentration tested; the real IC50 may be higher. Note that 5-FU was not tested after 2 hrs and overnight exposure due to absence of toxicity at 24 hrs. p-Phenylenediamine was not tested after 2 hrs exposure.

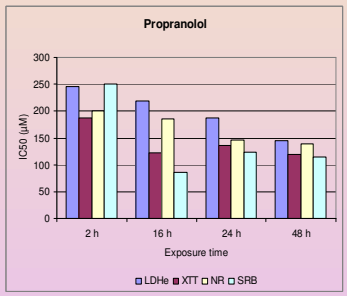












Notable findings:

5-FU: Note the strong difference of IC50 between assays at 48 hrs. For toxicants like 5-FU, LDH and XTT are poor indicators of toxicity.

Chloroquine: In a short time exposure (2 hrs) the Neutral Red assay is very sensitive and suggests a primary toxic effect on the lysosomal compartment. The other assays show little or now response at 2 hrs.

Chlorpromazine: is a fast acting toxicant with 3 of the 4 assays. LDH toxicity is measurable only after longer exposure times.

Conclusions:

- **The PAN I assay kit allows the sequential measurement of 4 cytotoxicity parameters on 1 cellular sample.**
- **For some compounds (e.g. Propranolol) any of the 4 cytotoxicity tests yields comparable IC50 values.**
- **For other compounds the apparent toxicity depends strongly on the assay chosen, and the exposure time.**
- **Using 4 sequential cytotoxicity tests greatly enhances chances to detect toxicants which might go unnoticed if only 1 assay were used. This becomes even more pronounced in experimental set-ups where only 1 exposure time is used.**
- **The comparison of the kinetic behaviour of the IC50's can provide information on the possible mechanisms of action of test compounds.**