Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity

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We show here the identity of Alamar Blue as resazurin. The ‘resazurin reduction test’ has been used for about 50 years to monitor bacterial and yeast contamination of milk, and also for assessing semen quality. Resazurin (blue and nonfluorescent) is reduced to resorufin (pink and highly fluorescent) which is further reduced to hydroresorufin (uncoloured and nonfluorescent). It is still not known how this reduction occurs, intracellularly via enzyme activity or in the medium as a chemical reaction, although the reduced fluorescent form of Alamar Blue was found in the cytoplasm and of living cells nucleus of dead cells. Recently, the dye has gained popularity as a very simple and versatile way of measuring cell proliferation and cytotoxicity. This dye presents numerous advantages over other cytotoxicity or proliferation tests but we observed several drawbacks to the routine use of Alamar Blue. Tests with several toxicants in different cell lines and rat primary hepatocytes have shown accumulation of the fluorescent product of Alamar Blue in the medium which could lead to an overestimation of cell population. Also, the extensive reduction of Alamar Blue by metabolically active cells led to a final nonfluorescent product, and hence an underestimation of cellular activity.

Keywords: Alamar Blue; cytotoxicity; fluorescence; resazurin; resorufin.

The ideal test for in vitro cell proliferation and cytotoxicity is a simple, rapid, efficient, reliable, sensitive, safe and cost-effective measurement of cell viability. Obviously, it should not interfere with the compound to be tested. Moreover, if the same method is applicable to high throughput screening techniques and the cells can be used for further experiments, then biologists have the perfect tools. Since 1993, a new dye fulfilling this description has been available, Alamar Blue (AB) [1]. It is the most simple and rapid test, whereby 10% of the commercially available solution is added to the cell medium and measured either by colourimetry or fluorimetry. However, greater sensitivity is achieved using the fluorescent property. This can be done either as an endpoint reading or in a kinetic fashion, providing an excellent estimation of cell proliferation, as well as the potency of toxicants. As few as 80 cells give a reproducible and sensitive signal, which is in the range of radioactive methods, but without all the exposure and waste disposal issues. Alamar Blue is nontoxic to cells and does not necessitate killing the cells to obtain measurements, as with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT). This allows precious cells to be reused for further investigations, saving time and money, especially in the case of primary tissue cultures. Automation is easily set-up in microplates and analysed using microplate readers. Double wavelength fluorescent readers allow correlation, for example, between the toxicity assessed by Alamar Blue with the flux of Ca\(^{2+}\) using Fluo-3 on the same samples, minimising the variations.

However, full knowledge of how this dye works, is more than useful to avoid hidden pitfalls. The oxidized, blue, nonfluorescent Alamar Blue is reduced to a pink fluorescent dye in the medium by cell activity (likely to be by oxygen consumption through metabolism). Although it has been arbitrarily postulated as being reduced by mitochondrial enzymes [2], it is not known whether this occurs intracellularly, at the plasma membrane surface or just in the medium as a chemical reaction. The ‘resazurin reduction test’, whereby resazurin is reduced to resorufin, works in a very similar way and has been used since the 1950s to assess bacterial or yeast contamination in biological fluids and milk [3], it is also used to measure the viability of sperm by colorimetry [4]. Resazurin seems to be the original name for Alamar Blue, before it had been used for in vitro mammalian cell proliferation. There is a direct correlation between the reduction of Alamar Blue (or resazurin) in the growth media and the quantity/proliferation of living organisms, ranging from bacteria to mammalian cells. It has been assessed several times on different types of cell for its cytotoxicity reliability, i.e. fibroblasts [5], immortalized and cancer cell lines [6,7], and for its cell proliferation reliability on mouse and human lymphocytes [2,8], and primary neuronal cell culture [9]. Although it compares favourably with many other cell proliferation techniques, we have uncovered some pitfalls to avoid and have also explored its mechanism of action.

MATERIALS AND METHODS

Materials

Alamar Blue obtained from Serotec Limited was used as a 10% solution unless otherwise stated. MTT obtained from Sigma Chemical Co., was prepared as a stock solution of 5 mg·mL\(^{-1}\) in NaCl/P, and used at a final concentration of 500 µg·mL\(^{-1}\). All other reagents and chemicals, including resazurin, were obtained from Sigma.

Abbreviation: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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A Fluoroskan Ascent (Labsystems International) was used to measure fluorescence in 96-well plates at wavelengths of 530 nm excitation and 590 nm emission. A Titertek Multiskan MC (Flow Laboratories) was used to measure absorbance for the MTT assay at 550 and 690 nm. A DU7000 spectrophotometer (Beckman) was used to measure the absorbance of Alamar Blue, resazurin and resorufin.

Isolation of Alamar Blue

In order to obtain a definitive identification of Alamar Blue, the dye present in the reagent was isolated using solid-phase extraction on to a C18 bonded cartridge containing 500 mg of sorbent (Mega Bond Elut, Varian, Jones Chromatography Ltd, Hengoed, UK). The cartridge was conditioned by the sequential application of methanol (5 mL) and water (5 mL), followed by 10 mL of reagent. The cartridge was then washed with a further 5 mL of water and the dye, which was retained completely under these conditions, was then recovered by elution with methanol (10 mL). The methanol was then removed using a stream of nitrogen gas at ambient temperature prior to spectroscopic analysis.

NMR spectroscopy

Single pulse 1H NMR spectroscopy was acquired on a Bruker DRX 500 spectrometer operating at 500.13 MHz. Typically, 128 scans were collected into 65 536 data points over a spectral width of 9980 Hz. Solvent suppression was achieved using the NOESY-PRESAT pulse sequence (Bruker Spectrospin, Germany) with presaturation of the water peak during the relaxation delay of 1 s and the mixing time of 150 ms. The extracted sample was then redissolved in D2O (99.9% purity, Fluorochem Ltd, UK) or 10% D2O (v/v) was added to medium (Gibco) supplemented with 2 m3-trimethylsilyl-(2,2,3,3-D4)propionate (Aldrich, UK) at a field frequency lock. Spectra were referenced to sodium 31P 0.0.

Mass spectrometry

Positive ion LSIMS mass spectra were obtained using a Quattro II tandem mass spectrometer (Micromass UK) fitted with a caesium ion gun. The liquid matrix material was 3-nitrobenzyl alcohol, and daughter ion spectra were produced by collision induced dissociation in argon gas.

Cells

Primary rat hepatocytes were obtained from the liver of Alderley Park Wistar-derived male rats. Livers were perfused following the method of Seglen [10]. Following collection, cells were checked for viability using Trypan Blue and seeded at a density of 1.5 x 10^6 viable cells per well in 200 mL of William's E medium containing 2 mm glutamax I (Gibco) and supplemented with 170 nM insulin, 30 nM dexamethasone, 100 U·mL⁻¹ penicillin, 100 mg·mL⁻¹ streptomycin and 10% heat-inactivated fetal bovine serum (Gibco), in 96-well collagen I-coated plates (Biocoat, Becton-Dickinson). HeLa and HepG2 cells were obtained from the American Tissue Culture Collection (Maryland, USA). The cells were seeded in 175-cm² tissue culture flasks (Falcon), in minimum essential medium (Gibco) supplemented with 2 mm glutamax I (Gibco), 100 U·mL⁻¹ penicillin, 100 mg·mL⁻¹ streptomycin, with 10% fetal bovine serum (Gibco) until nearly confluent. Cells were harvested by trypsinization and an assessment made of their density, via haemocytometer and viability by Trypan Blue.

Cells were diluted in medium to a density of 7.5 x 10^4 viable cells per mL and 200 µL (1.5 x 10^5 cells) added to each well of a 96-well tissue culture-treated plate (Costar). All cells were incubated overnight and maintained in a humidified atmosphere at 37 °C and 5% CO₂. Cells were treated the following day. Serum-free medium was used during treatment with test compound.

MTT was used to assess the viability of cells following treatment. Aliquots of 20 µL of stock MTT solution (5 mg·mL⁻¹) were added to each well containing 200 µL of medium (10% final solution) and incubated with the cells for 2 h. Following incubation the medium was removed and 200 µL of dimethylsulfoxide added to solubilize the formazan crystals. The absorbance was read on the plate reader at 550 and 690 nm. A subtraction analysis of the dual wavelength was performed (D550 to D690) to increase accuracy of the measurement.

Alamar Blue and resazurin

Solutions of resazurin and dilution of Alamar Blue (10%) were prepared in NaCl/Pi, (Gibco). Each compound was wavelength scanned using the spectrophotometer and lambda max values determined. A series of dilution of resazurin was prepared in NaCl/Pi, and standard curves were produced, against a NaCl/Pi, blank. Samples of Alamar Blue were read at the lambda max (lambda max was the same for resazurin and AB) and the concentration of Alamar Blue calculated from the standard curve of resazurin.

Accumulation of reduced Alamar Blue into cell culture media

HepG2 cells were seeded at several densities from 40 to 8000 cells per well in a 96-well plate in minimum essential medium with 10% (20 µL) Alamar Blue added. The plate was incubated under standard conditions of 37 °C and 5% CO₂ in a humidified atmosphere and fluorescent measurements read 19 and 45 h later.

Confocal microscopy

Alamar Blue stained cells were examined using the Bio-Rad MRC600 Laser Scanning Confocal Microscope. A yellow high sensitivity filter at 568 nm excitation wavelength was utilized with the resultant emission at 585 nm.

R E S U L T S

Alamar Blue and resazurin are the same compound

Confirmation of the identity of Alamar Blue as resazurin was obtained via high-field 1H NMR spectroscopy and tandem mass spectrometry of material isolated from the Alamar Blue reagent by solid-phase extraction (as described in Materials and methods). The spectra obtained for Alamar Blue were compared with those for the standard material and shown to be identical.

The mass spectra recorded from standard resazurin and also material extracted from Alamar Blue reagent both showed ions at 214 and 230 amu; the latter ion corresponding to the expected protonated molecular ion, [M + H]⁺, formed from resazurin (C13H22NO4, molecular mass 229 Da). The daughter ion spectra of the ion at 230 amu derived from both resazurin and Alamar Blue showed very comparable representations of the ions at 185, 202, 213 and 214 amu.

The NMR spectra of both Alamar Blue and resazurin showed the presence of three aromatic protons at 6.3, 6.7 and 7.9 p.p.m.
The identity of the Alamar Blue sample isolated from the reagent was further confirmed by over-spiking with authentic resazurin, which demonstrated that the chemical shifts of the aromatic protons for both compounds were identical.

The concentration of the commercial Alamar Blue stock solution has been determined as being 440 μM against a resazurin standard curve.

**Accumulation of reduced Alamar Blue into cell culture media**

It has been said that Alamar Blue has no toxic effects on cells, even after a long period of incubation and gives a linear curve over a wide range of cell concentrations [1].

![Fig. 1. Alamar Blue fluorescence as a function of cell number. HepG2 cells were incubated with Alamar Blue as described in Materials and methods. For both panels, values were deducted from the autofluorescence of Alamar Blue without cells. The number of cells per well were 40, 80, 400, 800, 4000 and 8000. Incubation was for (A) 19 h, (B) 45 h. Dotted line: linear regression (n = 8).](image1)

![Fig. 2. HepG2 cells treated with increasing concentration of nickel chloride. HepG2 cells were dosed for 24 h with NiCl₂ at the same time as Alamar Blue. Although 1 mM treated cells were dead, as seen by light microscopy, the fluorescent signal was ≈ 60% of negative control due to the maintenance of reduced Alamar Blue (n = 8).](image2)

![Fig. 3. Primary rat hepatocytes treated with 20 mM sodium azide and different percentages of Alamar Blue. Rat hepatocytes were treated for (A) 2 h and (B) 20 h with 20 mM NaN₃ and either 20, 10 or 2% of Alamar Blue. Over-reduction of Alamar Blue produced an uncoloured and nonfluorescent product (hydroresorufin) leading to artefactual results in which treatment with a toxic compound led to an apparent boost of the treated cells (n = 8).](image3)
A low number of HepG2 cells were seeded into 96-well plates and incubated in the presence of 10% Alamar Blue. Cell number was in the range 40±8000 cells per well which represent a cell density of 125±25,000 cells per cm². Two measurements of fluorescence were performed at 19 and 45 h. Figure 1 shows that there was a very good correlation between cell number and reduction of Alamar Blue (fluorescent product) at both time points. The autofluorescence of 10% Alamar Blue in the culture medium alone was, for all experiments, about five or six relative fluorescent units (RFU) at 0 min. In this experiment, the autofluorescence at 19 h incubation was <8.25±0.11 RFU and at 45 h incubation 13.4±0.10 RFU. Although there was a slight auto-reduction of Alamar Blue over the period of the experiment, this was very small in comparison with the fluorescence generated by the cells. The lag time between the two measurements was 26 h, which is less than the HepG2 doubling time (<33 h). Therefore, a strict correlation between cell growth and measured RFU should have been around 80 RFU at 45 h for 8000 cells per well, instead of 114 RFU. This indicated a build-up of the fluorescent reduced Alamar Blue in the medium. We then checked the half-life of reduced Alamar Blue in the same medium in the absence of cells. Reduced Alamar Blue was very stable with a half-life of <10 days under these conditions.

Additional reductions of reduced Alamar Blue into colourless and nonfluorescent product

The reduction rate of Alamar Blue has been shown to vary according to the cell line tested [6,7]. In the case of primary rat hepatocytes, which exhibit a fairly high rate of metabolism, the fluorescence value often reached >300 RFU (data not shown). However, the media, although originally turning from blue to pink, eventually became colourless. This phenomenon has been known for a long time as being due to the extensive reduction of resazurin (blue) into resorufin (pink) and then hydroresorufin (colourless) [3]. This can lead to artefactual results as shown in Fig. 3. Primary rat hepatocytes have been treated with 20 mM sodium azide (NaN₃) and with either 20, 10 or 2% of Alamar Blue over 20 h. The less Alamar Blue in the medium, the more rapidly it was completely reduced by the cells and became colourless and nonfluorescent. After 2 h as expected, NaN₃ treated cells reduced less Alamar Blue than nontreated cells. However, at 20 h, control cells gave a lower value than treated cells, especially where Alamar Blue was less concentrated (10 and 2%). We have checked that NaN₃ was not reacting chemically with Alamar Blue giving a fluorescent product. The same event is presented in Fig. 4A and B, where primary rat hepatocytes have been treated with copper sulfate (CuSO₄). Cells were dosed for 4 h with CuSO₄ at different concentrations ranging from 0.1 to 5 mM, then 10% Alamar Blue was added.

Further reduction of reduced Alamar Blue into colourless and nonfluorescent product

Therefore, to avoid overestimation of survival, it is highly recommended that Alamar Blue be added after the compound incubation period rather than at the same time, especially where the drug uptake is unknown. This means that kinetic experiments may be unreliable unless strictly controlled, although Alamar Blue is nontoxic and readings may be performed on living cells.
Measurements were taken at 6 and 24 h (equivalent to 2 and 20 h incubation with AB). At 24 h, 0.1 mM CuSO₄ was very toxic as shown by MTT results, but the very high value with Alamar Blue (Fig. 4B, arrow) compared with nontreated cells, indicated cell growth, not toxicity. In Fig. 4C and D, Alamar Blue indicated a toxic effect in HepG2 cells at lower doses of CuSO₄ than MTT at both 6 and 24 h of treatment. Also, Alamar Blue was able to detect a toxicity earlier than MTT, as 5 mM CuSO₄ showed a clear reduction in fluorescence when MTT gave the same answer as the control at 6 h. However, using HeLa cells (Fig. 4E and F) the contrary occurred for an unknown reason. Extensive reduction of Alamar Blue within 2 h seemed unlikely with HeLa cells and the medium was still blue, indicating the presence of nonreduced AB.

Alamar Blue/resazurin fluoresces intracellularly

It has been said that Alamar Blue is reduced in response to ‘chemical reduction’ of the medium resulting from cell growth [1] or by mitochondrial enzymes [2]. We have removed some ‘highly reduced’ medium from an old cell culture (24–48 h incubation) and added Alamar Blue to it and monitored the reduction of Alamar Blue up to 24 h. The Alamar Blue was stable and did not give rise to any fluorescent product, whereas the same medium with cells gave a very rapid and high fluorescent value (47 RFU). Therefore, the presence of cells was essential for reduction of AB. We then removed the medium from the cell culture incubated with Alamar Blue and measured the fluorescence from both the medium alone and the cells alone. The cells alone gave = 5 RFU, which was within the control value of spontaneously reduced Alamar Blue in absence of cells, whereas the medium contained the whole reduced Alamar Blue with 42 RFU. To confirm that there was no fluorescence in the cells, we cultivated HepG2 and rat primary hepatocytes on glass coverslips in the presence of 10% Alamar Blue and examined the cells with a confocal microscope. Figure 5 shows that fluorescence was primarily in the cytoplasm, however, not in a quantity large enough to be detected by the fluorescent microplate reader. It is therefore likely that Alamar Blue entered into the cytoplasm where it was reduced to the fluorescent product, and then excreted into the medium. This is supported by increasing fluorescent background (i.e. media) observed in confocal microscopy when the cells were cultivated for longer, whereas the cytoplasmic fluorescence remained fairly constant. The shining spots seen in the cytoplasm were too large to be mitochondria. In some cases, fluorescent nuclei were observed, but only in dying or dead cells (Fig. 5). Some reduced Alamar Blue may have diffused into the nucleus via the damaged nuclear membrane and due to its planar structure (Fig. 6), intercalated into the DNA. Nevertheless, it could not be ruled out that Alamar Blue could have been reduced outside of the cell and then diffused into the cytoplasm and nucleus.

Fig. 5. Confocal microscopy examination of Alamar Blue-treated rat primary hepatocytes. (A) Phase contrast, (B) confocal microscopy. Plain arrows indicate nonfluorescent nuclei and empty arrows indicate dying cells.
DISCUSSION

The identity of Alamar Blue as resazurin has helped us to understand the transient fluorescence in the presence of very active cells such as primary hepatocytes. Reduction of the blue resazurin yields pink resorufin, but reduction of resorufin produces colourless hydroresorufin [3]. It appeared that hydroresorufin was also nonfluorescent. Reduction of the fluorescent product into a further reduced nonfluorescent product may lead to aberrant results in which living cells produced a weak signal and dying cells, which could not sustain further reduction, yielded a high fluorescent signal. This phenomenon was enhanced by the long half-life of reduced Alamar Blue which was very stable in the medium without cells. Resazurin is a chemical indicator used in the dairy industry to monitor microbial contamination in milk [3]. Reduction of the milk by bacteria or yeast changes the redox status leading to the two-step colour change. We have shown that other fluorescent dyes may chemically reduce Alamar Blue to the fluorescent product (data not shown). Therefore, there is no doubt that Alamar Blue reacts to the redox status of its environment. However, in already ‘reduced’ CO2 buffered-medium, Alamar Blue appeared very stable and therefore needs to be present when reduction is occurring to capture electrons. The presence of living cells allowed reduction of the dye. The cells may either induce a reduction of the medium or enzymatically reduce the Alamar Blue intracellularly, as some fluorescence was found in the cytoplasm and nucleus. Using a confocal microscope, we were not able to find any evidence of Alamar Blue mitochondrial reduction. Of all the reductases, diaphorase has been identified as the enzyme most likely to be responsible for the reduction of resazurin in semen [12]. The term diaphorase has been applied to different enzymes, such as dihydrodipicolinate dehydrogenase (EC 1.8.1.4.) [13], NAD(P)H:quinone oxidoreductase (EC 1.6.99.2) [14] and flavin reductase (EC 1.6.99.1) [15]. It has been shown in vitro that diaphorase (EC 1.8.1.4) was able to reduce resazurin into resorufin [13]. Diaphorases are widely distributed, being found in many species from bacteria to mammals where they can be either mitochondrial (EC 1.6.99.2) or cytoplasmic (EC 1.6.99.1). Therefore, diaphorases are good candidates to be responsible for the reduction of resazurin into resorufin and possibly from resorufin into hydroresorufin. However, any other reductase, such as NADH dehydrogenase (EC 1.6.99.3), could also use resazurin as an electron acceptor, as shown in vitro [16].

Whichever is responsible for the Alamar Blue or resazurin reduction, this test remains a very simple, fast, sensitive and accurate cell viability measurement, providing that certain precautions are taken. First, it is worth checking the cross-reactivity of Alamar Blue with any compound to be tested, without any cells in the medium. Second, the reduction rate by cells in culture should also be checked so that conditions for Alamar Blue concentration and incubation time are optimized in order to avoid over-reduction of Alamar Blue into the colourless and nonfluorescent hydroresorufin. Third, Alamar Blue is more valuable as an endpoint measurement for cytotoxicity rather than as a kinetic measure for monitoring cell growth. All these initial steps may be seen as cumbersome and lengthening a process which is supposed to be quick and simple. However, once established for routine screening, the test will be an invaluable tool. High throughput screening is probably the most obvious application for this test particularly since resazurin, as opposed to Alamar Blue, is very cheap with 5 g of powder making 400 L of solution to be used at 10% in cell culture medium.

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REFERENCES


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