

Development of Respiratory Mucosal Irritation and Toxicity Screening Methods: Comparison of MTT and Colorimetric Resazurin-Based Assay

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Abstract

Availability of reliable and reproducible assay methods that use simple tools will facilitate research especially in resource-poor settings. The purpose of this study is to evaluate colorimetric resazurin reduction assay (CRA) as a possible tool in respiratory mucosal cell irritation and toxicity screening. It compared CRA with another well-accepted colorimetric cell viability assay method (3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide {MTT} assay). Cell viability results of treated cells were obtained using both assay methods and used to determine Z' factor, signal-to-background (S/B) ratio and IC₅₀ values. The results show all the Z' values obtained were positive except values from CRA for cells treated with triton-X 100 incubated for 30min and 2h. All the Z' values for MTT shows the method is optimized (0.5 < Z' < 1.0) while most values for CRA were outside the acceptable range (Z' < 0, Z' > 1). MTT demonstrated large S/B ratio at both 30min and 2h for all the compounds. The range of values obtained for the S/B ratio is 12-57 for MTT and 1-3 for CRA. Furthermore, CRA failed to estimate IC₅₀ for some of the compounds (BKC, 2h; SDS, 2h; BKC, 30min). The values obtained for Z' factor, S/B ratio and IC₅₀ for both assays were at variance. This result demonstrates that CRA performed below acceptable assay standard and therefore may not be an appropriate method for human respiratory cell viability studies. Consequently, we conclude that CRA is not useful in human respiratory mucosal irritation and toxicity studies.

Keywords: Calu-3 cells; Colorimetric resazurin assay; Assay performance measures; Respiratory mucosal irritation; Toxicity screening; MTT

Abbreviations: CRA: Colorimetric Resazurin Reduction Assay; DST: Drug Susceptibility Testing; REMA: Resazurin Microtiter Assay; FRA: Fluorimetric Resazurin-Based Assays; RA: Resazurin-Based Assay; APM: Assay Performance Measures; HTS: High-Throughput Screening

Introduction

There seems to be a conflict between the reported sensitivity of colorimetric resazurin-based assays (CRA) and the wide clinical use of Resazurin Microtiter Assay (REMA). REMA, a liquid culture-based CRA has been endorsed by the World Health Organization for drug susceptibility testing (DST) for *Mycobacterium tuberculosis* [1]. Though CRA is not popular in respiratory cell culture as fluorimetric resazurin-based assays (FRA) [2,3], its use in DST of *M. tuberculosis* has been compared with similar assay methods and reported to be accurate [4,5]. Furthermore, the method has been used as a reference method in the evaluation of other assay methods [5]. The inherent advantage of resazurin-

based assay (RA) that cells may be re-used (since the dye is not lethal) makes the method more suitable in resource-poor settings where acquisition and sustenance of some cell types poses a challenge. There is need to assess the extent of deficiency of the method as well as determine its relative usefulness especially in resource-poor countries.

Though, CRA have previously been judged less sensitive than FRA, supporting data is scarce [2,3,6]. Outcome measure of resazurin-based assay (RA) is taken either by colorimetric or fluorimetric determination [7-11]. Fluorimetric reduction assay have earlier been compared with other assay methods [7-9]. One

of such studies demonstrated that FRA is comparable with MTT [12]. Beyond DST of *M. tuberculosis*, few studies use CRA [13-15]. Consequent upon the successes recorded with CRA, as well as the understanding that the rate at which resazurin are reduced changes from cell to cell [3,16,17], there arises a need to explore the usefulness of CRA in respiratory mucosal cell viability.

Assay performance measures (APM) may quantify the amount of separation in measured signals between positive and negative controls in an assay, while accounting for the observed variability [18]. APMs are used in the evaluation of assay methods. Commonly used APMs include Z' factor, signal-to-background ratio, and assay variability ratio. The Z' factor is a dimensionless, simple statistical characteristic for high-throughput screening (HTS) assay. It has been used widely in several assay measures for quality assessment, optimization, comparison of instrumentation quality, and validation [19-21]. High-quality assays compatible with HTS should have an "excellent" Z' factor value (between 0.5 and 1.0). Z' factor takes into consideration most factors necessary for HTS assay characterization and is preferred to other APMs like signal-to-background ratio [22,23] that lack this feature. Consequently, Z' factor has become an accepted parameter for assessment of performance of assays and has been used widely in several assay measures [19-21]. IC₅₀ comparison, even though not an APM, has been used severally in assay performance comparison [12].

The purpose of this study is to evaluate CRA as a possible tool in respiratory mucosal cell irritation and toxicity screening. Since a previous study reported that FRA and MTT produce similar results, our approach was to observe whether values obtained using a respiratory mucosal cell line for Z' factor, signal-to-background ratio, and IC₅₀ for CRA and MTT were similar. There are justifications for this study. Absorbance measuring equipment is commonly available in most laboratories unlike fluorescence measuring devices. To overcome the limitation imposed by lack of fluorescence measuring equipment, there is need to develop CRA for respiratory mucosal irritation and toxicity screening. Furthermore, CRA can easily replace MTT with the added benefit that cells can be re-used (a feature MTT does not have). In addition, this study will quantitatively provide data on the performance of CRA in a mammalian cell.

Materials and Methods

Chemicals

Triton X-100 (TX) and benzalkonium chloride (BKC) were supplied by Sigma (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS) USP was from Fisher Scientific (Ottawa, ON, CA). Resazurin was obtained as resazurin sodium salt powder (Acros Organic NV). Tissue culture materials including Dulbecco's modified eagle's medium-Ham's F-12 nutrient (D-MEM/F-12), fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin/streptomycin, Glutamax, and phosphate-buffered saline-trypsin were purchased from Invitrogen (Burlington, ON Canada). Human bronchial/sub-bronchial gland cell line (Calu-3) was purchased from American

Type Culture Collection (Manassas, VA, USA).

Cell culture

The Calu-3 cells were grown according to a standard protocol. Cells were cultured in 96-well plates (Fisher Scientific, ON, Canada) in 1:1 D-MEM/F-12 supplemented with 10% FBS, 1% Glutamax, 100U/ml penicillin, and 100 mg/ml streptomycin. They were fed every other day with a DMEM-F12 containing 10% FBS, 1% Glutamax, 1% 10,000 units/mL penicillin and 1%, 100 mg/ml streptomycin and maintained at 95% O₂ and 5% CO₂.

Cell viability studies

Compounds known to be toxic or irritating to respiratory cells were selected. The test compounds (benzalkonium chloride, sodium dodecyl sulphate, triton X-100) were applied as solutions in DMEM/F-12 at 8 different concentrations in wells (n=4) incubated for 30 min and 2 h. The concentrations of the test solutions used were determined through a range finding procedure. Blank DMEM/F-12 was used as control. Cells used for the experiments were within 8-15 passages and at 70- 80 percent confluency. Before the experiments, cells were washed with DMEM/F-12, pH 7.4, followed by 30 min equilibration in the buffer. Subsequently, the supernatant was completely discarded from each well followed by addition of 200 µl of test compound or control and incubated. One batch of cells was used for each test compound to eliminate the effect of batch variation on the result. The test compounds and control were discarded after incubation and 100 µl of DMEM/F-12, pH 7.4 used to wash each well and subsequently discarded before cell viability assessment. The viability of the cells post-incubation was determined using separate wells for MTT and resazurin assay methods. The Z' factor, signal-to-background ratio and IC₅₀ were determined for each time frame. We calculated the IC₅₀ of the test compounds after the cell viability assay. think that comparable assay methods should have comparable inhibitory curves with similar IC₅₀ values. Both assays were done on submerged cells.

Resazurin reduction assay (CRA)

Resazurin stock solution 0.01% was prepared by dissolving resazurin powder (Sigma, St. Louis, MO, USA) in distilled water, filtered with a sterile 0.20 µm-pore filter (Corning Inc., MA, USA) and stored in the dark at 4°C for up to 1 week. An aliquot of the stock solution is diluted (1:10) before use. After treatment of cells, 200 µl of 0.001% resazurin solution was added to each well and incubated in 5% CO₂ at 37°C for 2 h. Subsequently, 100 µl of 3.0% SDS was added directly to each well to stop the resazurin reaction. The absorbance of the reduced dye was read at 570 nm [24-26] using 96-well plate reader (ABI systems).

3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay

After treatment of cells, 100 µl of MTT solution (5.0 mg/ml) prepared in distilled water was added to each well and incubated for 3 h in 5% CO₂ at 37°C. Subsequently, the supernatant was

discarded and replaced with 100 µl isopropyl alcohol per well. The plate was then sealed in an aluminium foil and incubated for 2 h to extract formazan produced by cellular mitochondrial dehydrogenase [27]. The absorbance of the solution was read within an hour after extraction on an ELISA plate reader (ABI systems) at 570 nm. One batch of cells was used on the plate for each test compound with control to eliminate the effect of batch variation on the result.

Data analysis

The Z'-factor, signal-to-background ratio (S/B) and IC₅₀ values were determined [12,22]. The optical density values was used to determine the viability per well. The mean absorbance of the control (blank DMEM/F-12) was considered equivalent to 100 % cell viability. Mean absorbance of treated samples in each assay was expressed as a percentage of the mean absorbance of control. The mean cell viability was used to calculate the Z'-factor [22] using the formula

$$Z' = 1 - \frac{3SD \text{ of sample} + 3SD \text{ of negative control}}{|\text{mean of sample} - \text{mean of negative control}|} \dots\dots\dots (1)$$

The signal-to-background ratio was calculated using the equation

$$\frac{S}{B} = \frac{\text{meansignal}}{\text{mean background}} \dots\dots\dots (2)$$

Where Z' means Z' factor value and SD is standard deviation.

Mean signal is the mean cell viability obtained from the negative control corresponding to 100 % cell viability or mean of the top (maximum) signal. Mean background is the mean cell viability obtained from the test sample corresponding to the least cell viability obtained or mean of the bottom (minimum) signal. The concentration of the test solutions causing 50% reduction in cell viability was calculated using values spread between maximum and minimum cell viability. The data was fitted into GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) and used to generate a sigmoidal dose-response curve. In comparing both values, we computed the ratio (IC₅₀ CRA/ IC₅₀ MTT). Comparable IC₅₀ values should be close to 1.0.

Results

The evaluation of CRA and MTT was based on the determination of the Z'-factor, S/B ratio and IC₅₀ using the percent viability of sample compounds. The result of values for Z'-factor is presented in (Table 1). All the Z' values for MTT were "excellent" (0.5 < Z' < 1.0) while most values for CRA were outside the acceptable range. On comparison, the Z' values for MTT from 2 h incubations have higher values than their corresponding values in 30 min incubations. All the Z' values obtained for MTT (30 min; 2 h) was positive while all the values for CRA at 2 h exposure was negative. Nevertheless, the values for CRA for SDS (0.72) and TX (0.68), both at 30 min exposure were excellent. The Z' values obtained for

MTT were within a narrow range (0.51 – 0.59) unlike CRA. MTT demonstrated a large S/B ratio both at 30 min and at 2 h for all the compounds as seen in (Table 2). The range of values obtained for the S/B ratio is 12-57 for MTT and 1-3 for CRA. A common trend for MTT at both incubation periods shows the order of decreasing S/B ratios as BKC < SDS < TX.

Table 1: Shows the tables of Z' values obtained for 1-3 reduction and MTT assays using cells treated with benzalkonium chloride (BKC), sodium dodecyl sulphate (SDS) and triton X-100 (TX) for 2 h and 30 min, All the Z' values for MTT were excellent (0.5 < Z' < 1.0) while most of the values for CRA were outside the acceptable range (Z' < 0, Z' > 1).

Compound	30 min incubation		2 h incubation	
	CRA*	MTT*	CRA*	MTT*
BKC	-	0.51	-11.52	0.58
SDS	0.72	0.55	-7.77	0.59
TX	0.68	0.56	-0.97	0.59

Table 2: Shows the Signal-to-background (S/B) ratio obtained using benzalkonium chloride (BKC), sodium dodecyl sulphate (SDS) and triton X-100 (TX). Two time frames (30 min and 2 h) were used for the compounds. The S/B ratios for resazurin reduction assay at both time frames were low. S/B ratios obtained for MTT assay at both time frames are high. Large S/B ratio is preferred in assay quality measurement.

Compound	30 min incubation		2 h incubation	
	CRA	MTT	CRA	MTT
BKC	1	13	1	12
SDS	3	49	1	38
TX	3	57	1	47

Table 3: Shows the IC₅₀ estimates obtained after treating cells with test compounds {benzalkonium chloride (BKC), sodium dodecyl sulphate (SDS) and triton X-100 (TX)} for 2 hand 30 min respectively. The results obtained from the resazurin assay were inadequate for estimation of IC₅₀ for some of the compounds. The result shows that the IC₅₀ values obtained from both assays are not comparable.

Compound	30 min incubation		2 h incubation	
	CRA	MTT	CRA	MTT
BKC	--	0.0081	--	0.0033
SDS	0.1065	0.016	--	0.0063
TX	0.3424	0.0209	0.0012	0.0095

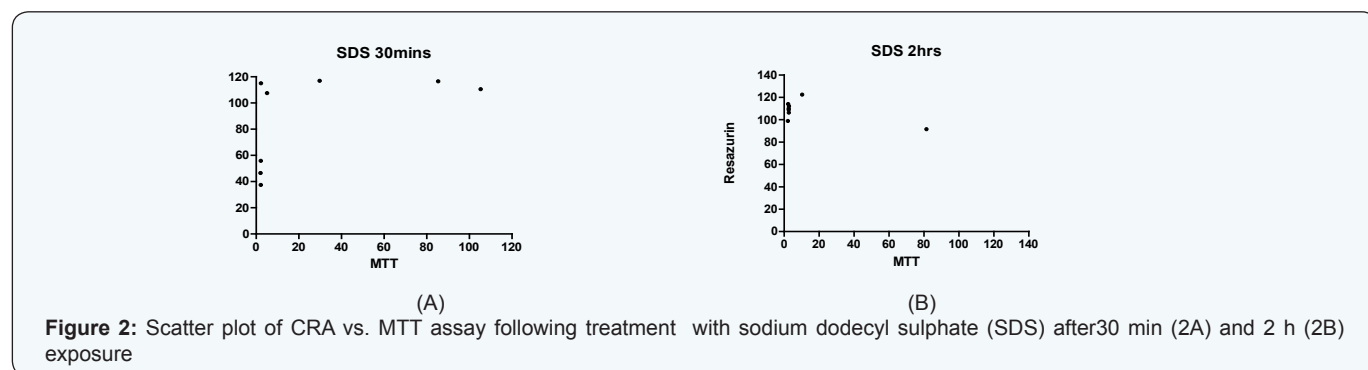
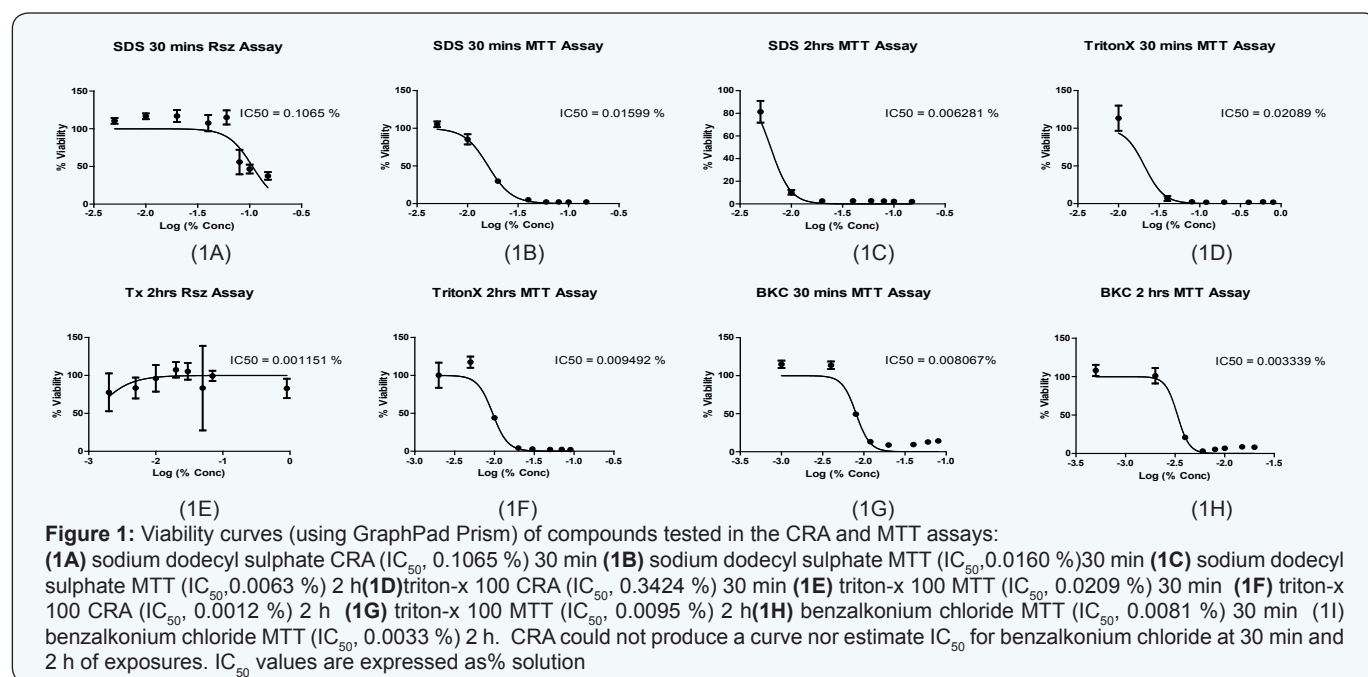
Table 4: Compares the IC₅₀ estimates from both assays by determining the ratio IC₅₀CRA / IC₅₀MTT. The value should be close to 1.0 if both assays are comparable. The result from CRA was not sufficient to generate an IC₅₀ value. Hence, the ratios for BKC, SDS and TX could not be determined. The values obtained are not close to 1.0. This implies that both assay methods are not comparable.

Ratio of IC ₅₀ CRA / IC ₅₀ MTT		
Compound	2 h	30 min
BKC	----	----
SDS	----	6.66
TX	0.13	16.38

The estimation of IC₅₀ from viability curves after treatment with SDS, triton X-100 and benzalkonium chloride is presented in

Table 3 and Figure 1. The order of decreasing value of IC_{50} both at 30 min and at 2h treatment for MTT is BKC < SDS < TX. In addition, the values for 30 min incubation are consistently higher than its corresponding value in 2 h incubation. The scatter plots for SDS, triton X-100 and benzalkonium chloride are presented in Figures 2-4 respectively. Figure 3A shows that only 1 point has value more than 80 % for both assay methods while the other points contrast sharply. Few of the values of the points for resazurin are similar to the values of negative control. All of the values of the points for resazurin are above 35 % while all of the points for MTT (except one) are less than 20 %. Figure 3B shows that only 2 points has value more than 80 % for both assay methods while the other points contrast sharply. Majority of the values of the points for resazurin are similar to the values of negative control while majority of the values for MTT are less than 50 %. The result demonstrates that except for a few cases, the percent viability reported by each assay is not comparable. In Figure 4, both plots show that only 2 points demonstrates cell viabilities more than

80 % for both assay methods while the other points contrast sharply. The values of all the points in Figure 4A for resazurin are similar to the values of negative control while majority of the values for MTT are less than 60%. Figure 4B shows that majority of the values of the points for resazurin are similar to the values of negative control while majority of the values for MTT are less than 30%. The results from Figure 4A and Figure 4B demonstrate that except for a few cases, the percent viability reported by each assay is not comparable. Scatter plots for BKC at 30 min and 2 h are similar. All the points of CRA for TX (30 min) show cell viabilities > 35% while most of the points for MTT were < 20%. Majority of points for CRATX (2 h) were similar to values of negative control while majority of points for MTT had viabilities < 50%. CRA failed to estimate IC_{50} for some compounds (BKC, 2 h; SDS, 2 h; BKC, 30 min) (Table 3&Figures 2- 4). The ratio (IC_{50} CRA/ IC_{50} MTT) showed a wide disparity between the IC_{50} estimates obtained for both assays (Table 4).



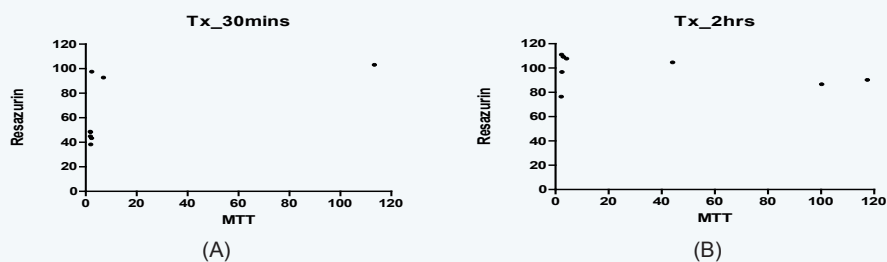


Figure 3: Scatter plot of percent viability of CRA vs. MTT assay following treatment with triton X-100 (TX) after 30 min (4A) and 2 h (4B) exposure.

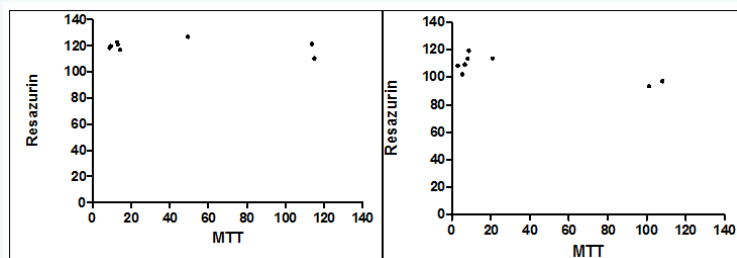


Figure 4: Scatter plot of CRA vs. MTT assay after treatment with benzalkonium chloride (BKC) after 30 min (4A) and 2 h (4B) exposure.

Discussion

There is no specific HTS assay for irritation or toxicity of respiratory mucosal cells. RA-based assay has been used for decades on different cell types and for various purposes [28]. As previously stated, CRA is less commonly used and data supporting this practice is scarce. The popularity enjoyed by FRA in cell viability assessment over CRA is due to its superior sensitivity which has been reported in literature [2,3]. Recently, an assay that uses Belgian slugs was developed for mucosal irritation [29].

variation of sample and control. Negative Z' value or values close to zero may result if the assay conditions are not optimized. The consequence of band overlap is that it is essentially impossible to use this assay for HTS screening. Since large S/B ratio is preferred in assay quality measurement, the excellent Z' values for MTT is corroborated by the large S/B ratio of MTT [30]. Both assay methods are not comparable since the inhibitory curves and IC_{50} values are at variance. This opinion is further strengthened as we could not obtain IC_{50} estimate of CRA for some of the compounds. The values of the ratio (IC_{50} CRA/ IC_{50} MTT) as seen in (Table 4) shows a difference in performance between both assays. This difference is further confirmed by the scatter plots (Figures 5,6). The Z' factor, signal-to-background (S/B) ratio and IC_{50} demonstrates that both CRA and MTT are not comparable.

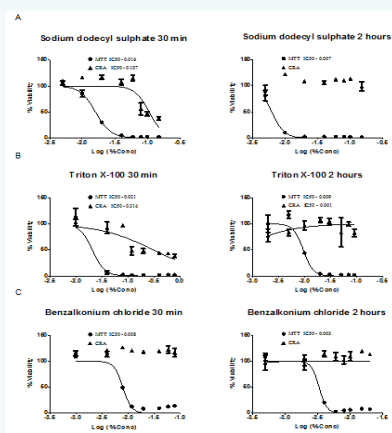


Figure 5: Scatter plot of CRA vs. MTT assay.

Our result show that the excellent Z' values for MTT indicates a quality HTS assay for respiratory mucosal cell irritation and toxicity. In contrast, Z' values from CRA for BKC (30 min, 2 h) and SDS (2 h) are null as they had values >1.0 and Z' values cannot be >1.0 [22]. Of all the 6 values for CRA, only one (SDS 30 min) is within the acceptable range. Negative Z' value of CRA for Triton-X 100 means that there is no separation band between signal

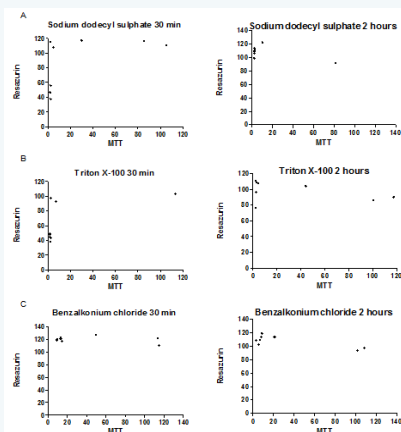


Figure 6: Scatter plot of CRA vs. MTT assay.

This finding agrees with previous reports that CRA is less sensitive than FRA. The Draizeeye test, which is a whole animal

test has been the standard for mucosal irritation and toxicity assay. Perrot et al. [26] previously used isolated pig cornea to demonstrate that FRA can be a substitute for Draize test for some class of compounds. Another study compared FRA and MTT and demonstrated that even though FRA was slightly more sensitive than MTT assay, the Z'-factor and EC₅₀ values were comparable in both assays [12].

It appears that CRA performs well in cell viability assays involving bacteria, and poorly in human cells. A multicenter study of MTT assay and CRA for testing drug susceptibility to anti-tuberculosis drugs revealed both assays as comparable, rapid, low-cost methods [31]. A similar study which compared nitrate reduction assay, MTT assay and Colorimetric Resazurin Microtitre Assay (REMA) showed that area under the curve (AUC) values obtained for the test drugs for MTT and REMA were similar [32]. That study further revealed that sensitivity and specificity for MTT and CRA were good and comparable [32]. These results may explain why CRA thrives in *Mycobacterial* culture studies [4,5]. To further show its usefulness in bacterial studies, CRA has been found reliable in HTS of bacteria for radiation sensitivity [28]. From our work and other previously published literature, it does appear that CRA has adequate sensitivity for bacterial but not for human cells. The dissimilarity in cellular constituents might be responsible for this variance. This study further agrees with previous reports that cell type may affect RA results [33].

Conclusion

This study evaluated CRA as a possible tool inhuman respiratory mucosal cell irritation and toxicity screening and found it less sensitive than MTT. The dissimilarity between the cellular constituents of human and bacterial cells may be affecting the sensitivity of CRA. Furthermore, we conclude that CRA is deficient as a tool in human respiratory mucosal cell studies.

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