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Bioassays for anticancer activities

Janice McCauley
University of Wollongong, jim479@uowmail.edu.au

Ana Zivanovic
University of Wollongong, az993@uow.edu.au

Danielle Skropeta
University of Wollongong, skropeta@uow.edu.au

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Abstract

The MTT/MTS in vitro cell proliferation assay is one of the most widely used assays for evaluating preliminary anticancer activity of both synthetic derivatives and natural products and natural product extracts. The highly reliable, colorimetric based assay is readily performed on a wide range of cell lines. This assay gives an indication of whole cell cytotoxicity; however, to determine the exact molecular target further assays need to be performed. Of these, kinase inhibition assays are also one of the most widespread enzyme inhibition screening assays performed. Kinases are enzymes that play a key role in a number of physiological processes and their inhibitors have been found to exhibit anticancer activity against various human cancer cell lines. Herein, we describe the methods for performing both in vitro MTT/MTS cytotoxicity and kinase enzyme inhibition assays. These are two of the most useful anticancer screening techniques available that are relatively economical and can be easily and routinely performed in the laboratory to characterize anticancer activity. Both assays are highly versatile and can be modified to test against targeted disease processes by using specific kinase enzymes or cell lines.

Keywords

MTS/MTT assays, Cytotoxicity, Anticancer activity, Human cancer cell lines, Enzyme inhibition, Kinases, CMMB

Disciplines

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Bioassays for Anticancer Activities

Janice McCauley,[1,2] Ana Zivanovic[1,3] D. Skropeta*[1,3]

[1] School of Chemistry, University of Wollongong, Wollongong, 2522, NSW Australia

[2] Shoalhaven Marine and Freshwater Centre, School of Biological Sciences University of Wollongong, Shoalhaven Campus, 2541, NSW, Australia.

[3] Centre for Medicinal Chemistry, University of Wollongong, Wollongong, 2522, NSW Australia

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Corresponding Author*

Danielle Skropeta

Tel: 02 4221 4360

Fax: 02 4221 4287

Email: skropeta@uow.edu.au

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Summary

The MTT/MTS *in vitro* cell proliferation assay is one of the most widely used assays for evaluating preliminary anticancer activity of both synthetic derivatives and natural products and natural product extracts. The highly reliable, colorimetric based assay is readily performed on a wide range of cell lines. This assay gives an indication of whole cell cytotoxicity, however to determine the exact molecular target further assays need to be performed. Of these, kinase inhibition assays are also one of the most widespread enzyme inhibition screening assays performed. Kinases are enzymes that play a key role in a number of physiological processes and their inhibitors have been found to exhibit anticancer activity against various human cancer cell lines. Herein, we describe the methods for performing both *in vitro* MTT/MTS cytotoxicity and kinase enzyme inhibition assays. These are two of the most useful anticancer screening techniques available that are relatively economical and can be easily and routinely performed in the laboratory to characterise anticancer activity. Both assays are highly versatile and can be modified to test against targeted disease processes by using specific kinase enzymes or cell lines.

Key words: MTS/MTT assays, cytotoxicity, anticancer activity, human cancer cell lines, enzyme inhibition, kinases

Introduction

A goal of many natural product chemistry and organic synthesis laboratories is ultimately drug discovery. An important aspect of the drug development process is testing both natural products and synthesised compounds for bioactivities that are

involved in targeted diseases processes. Cancer is a general term to define a number of diseases that are characterised by the uncontrolled proliferation of cells resulting from the disruption or dysfunction of regulatory signaling pathways that are normally under tight control *(1-2)*. Cancer can spread rapidly and invade other tissues and organs and different cancers are recognized to have unique characteristics or markers *(3)*.

To assess for preliminary anticancer activity in terms of cell viability, the MTT and MTS *in vitro* cytotoxicity assays are considered two of the most economic, reliable and convenient methods. This is based on their ease of use, accuracy and rapid indication of toxicity *(4)*, as well as their sensitivity and specificity *(5)*. Both assays are *in vitro* whole cell toxicity assays that employ colorimetric methods for determining the number of viable cells based on mitochondrial dehydrogenase activity measurement and differ only in the reagent employed. In the MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is bioreduced by dehydrogenase inside living cells to form a coloured formazan dye, while in the MTS assay, a similar bioconversion takes places utilising 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt and an electron coupling reagent (phenazine ethosulfate) PES (**Fig. 1**).

The MTT assay requires the addition of solubilising agents to dissolve the insoluble formazan product formed, while the MTS assay generates a water-soluble formazan product, thus simplifying the assay. The number of viable cells is measured through colorimetry and works on the principle that the mitochondrial dehydrogenase enzymes which produces NADH or NADHP, reduces the colourless tetrazolium salt into a coloured aqueous soluble formazan product by the mitochondrial activity of

viable cells at 37°C (**Fig. 1**). The quantity of the coloured product is directly proportional to the number of live cells in the culture since the MTT/MTS reagent can only be reduced to formazan by metabolically active cells.

[Insert Figure 1 near here]

This is a useful assay to characterise potential anticancer agents and can be performed routinely and easily in the laboratory without the need to forego intellectual property. The MTT and MTS assays assess for toxicity to the particular cell under investigation (not anticancer activity *per se*). Therefore, most researchers screen for cytotoxicity against either murine or human cancer cell lines, as well as against a normal cell line such as peripheral blood lymphocytes. A selectivity index of the compound for cancer cells over normal cells can then be determined. It is also advisable to confirm MTS/MTT results with qualitative observations under the microscope of the cell morphology both before and after the assay. This can often assist in identifying potential modes of actions and deciding which further assays should be performed such as caspase activation, assessing stage of cell cycle arrest and microtubule stabilisation or destabilisation *(6)*.

In addition to performing MTT or MTS assays against specific cell types, many researchers also submit their compounds to screening by the National Cancer Institute (NCI). The NCI offers a rapid *in vitro* primary anticancer drug screen to support cancer researchers worldwide *(7)*. The screen, which is performed at no cost to the researcher other than shipping of their sample, consists of a panel of 60

different human tumour cell lines from several cancer types including leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate and kidney. The NCI60 screen tests the degree of growth, inhibition or cytotoxicity of a compound against each cell line over a range of concentrations to generate a characteristic profile or fingerprint of cellular response *(8)*. A computer program (COMPARE) is used to assess the pattern of response across the cell lines, which can point towards a likely mechanism of action or identify compounds with unique modes of activity and/or selectivities for specific cell types. Operational details are provided by the NCI Developmental Therapeutics Program (DTP) and can be found at http://dtp.nci.nih.gov and for information regarding the submission of compounds for testing in the NCI screens see http://dtp.nci.nih.gov/docs/misc/common_files/submit_compounds.html *(9)*. Only pure compounds are screened in the assay and the supplier of the sample is required to provide the molecular structure of any compounds submitted, which are then reviewed and considered for testing.

The above assays are whole cell assays, however, often a researcher will want to determine activity against a specific molecular or cellular target in order to confirm the mechanism of action and to assess for selectivity towards different targets and any off-target effects. This can be achieved using *in vitro* inhibition assays against either purified enzymes or cell-free extracts enriched in the enzyme target of interest. One such anticancer screening method that can be easily and routinely performed in the laboratory in low-, medium- or high-throughput format is protein kinase inhibition *(10)*. After G protein-coupled receptors (GPCRs), protein kinases are the second most important anticancer drug target being pursued today.

Unlike MTS-type assays, which are cell based, these assays are based on detecting the degree to which the potential drug compound can inhibit an enzyme's activity. Most drugs used today demonstrate their bioactivity by acting as either receptor antagonists or as enzyme inhibitors. Enzymes are popular drug targets as they play a significant role in a number of disease processes and are susceptible to inhibition by small drug-like molecules *(11)*.

Protein kinases are an abundant group of enzymes in the human body with approximately 518 different protein kinases encoded in the human genome *(12)*. Kinases catalyse the chemical transfer of a phosphate group from a high energy molecule such as adenine triphosphate (ATP) to a hydroxyl-containing substrate such as serine, threonine and tyrosine, and are divided into different families based on their selectivity for these amino acids.

As one of the most abundant groups of enzymes in the human body, protein kinases are important in almost every major pathway in eukaryotic cells. They play a central role in the regulation of cellular activities and in signal transduction in signal transmission pathways. Moreover, kinases have important roles in metabolism, cell growth, apoptosis, immune response, gene expression, oncogenesis, cell differentiation and proliferation, metabolism, DNA damage repair and cell motility *(2)*. As a result, the deregulation of kinases has been identified to be the main cause in an increasing list of diseases *(13)*. An estimated one-third of pharma drug discovery programs now focus on targeting cancer-related kinases with the aim of developing potent and selective inhibitors with lower side effects than treatments that traditionally focus on DNA and chromosome regulation *(14-17)*. An example of a

kinase inhibitor that has successfully proceeded onto the pharmaceutical market is Imitinib (Gleevec, Novartis) which is a tyrosine kinase inhibitor that has dramatically improved the prognosis for sufferers of chronic myeloid leukaemia after being the first small-molecule kinase inhibitor to be approved for human use *(13)*.

c-AMP Dependent protein kinase A (protein kinase A, PKA) is an enzyme involved in the phosphorylation of a wide range of proteins, ion channels and transcription factors *(18)*. It has been demonstrated to regulate a number of physiological processes including cardiovascular function, steroid biosynthesis, reproductive function, myogenesis, adipocyte metabolism, exocytotic processes and immune function *(19-20)*. PKA was also found to play a key role in memory processes *(21)*. The cAMP-PKA pathway has been linked to the promotion of malignant phenotypes of head and neck squamous cell carcinoma *(22)* and demonstrated to be activated in a range of tumours *(23)*. Conversely, PKA inhibitors have been found to display both *in vitro* and *in vivo* anti-tumour activity against various human cancer cell lines and to enhance monocyte function in HIV-infected patients *(24)*. Thus, it is becoming increasingly apparent that the ability to selectively inhibit PKA provides a new way of potentially modulating cancer, immune function, and memory disorders such as Alzheimer's disease, Parkinson's disease and schizophrenia *(19,21,25)*.

Herein, we describe methods for performing both the MTT/MTS cell proliferation cytotoxicity assay and for screening against the enzyme PKA. Both methods are widely used in our laboratory *(6,26-27)*.

2. Materials

Carry out all procedures at room temperature unless otherwise specified.

2.1 Protein kinase assay

This protocol is for the analysis of 14 samples per plate – each sample is run twice at 100 μg/mL and in triplicate. This gives six data points per % inhibition reading. Alternatively, 14 samples can be run once at two different concentrations (100 μg/mL, 1 μg/mL) in triplicate. This will give three data points per % inhibition reading (adjust protocol accordingly). The volumes provided in this protocol are intended for a 96-well plate. The actual volumes used can be adjusted as needed.

1. ***Prepare sample solutions:*** Prepare a 5 mg/mL stock solution of sample in 100% dimethyl sulfoxide (DMSO).
2. ***Sample dilution:*** Make 1 mL of a 1 mg/mL secondary stock solution (in 20% DMSO) of the sample by taking 200 μL of the above 5 mg/mL stock and adding 800 μL of Milli Q water. During the assay, this will be diluted by 1:10 to give a final concentration of 100 μg/mL in 2% DMSO [*see* **Notes 1** & **2**].
3. ***Prepare kinase reaction buffer:*** Dissolve 48.5 mg of tris(hydroxymethyl)aminomethane (Tris), 19.0 mg of magnesium chloride ($MgCl_2$) and 1.0 mg of bovine serum albumin (BSA) in 2 mL of ultra-pure H_2O and then make up to 10 mL in a falcon tube. Adjust the pH to 7.5 using aqueous hydrochloric acid (HCl) if required. This will make 10 mL of reaction buffer that is sufficient for 1 x 96-well plate (i.e. 45 μL x 96 wells = 4.3 mL, plus 5 mL for adenosine triphosphate (ATP) stock) [*see* **Note 3**].

4. ***Enzyme preparation:*** The PKA enzyme (Promega) used herein is supplied in buffer as 2500 units at 114 units/μL. Dissolve whole contents of tube containing enzyme in a total of 2 mL of kinase reaction buffer (see step 3 above). Aliquot out 10 x 200 μL of this solution into labeled Eppendorf tubes and store at -70 °C, to give 250 units of activity per tube. The assay described herein utilises 2.50 units per well, i.e. 2.50 units x 96 wells = 240 units in total. Thus the supplied enzyme will enable testing of 10 x 96-well plates [*see* **Note 4**].
5. Kemptide (PKA) specific substrate (Promega, Australia) (10 mg/mL). The substrate is used as supplied and stored at -20 ºC [*see* **Note 5**].
6. ***Prepare ATP solution:*** Label 3 tubes: ATP stock 1, ATP stock 2 and ATP stock 3 (the last one is used in the assay). Weigh 5.51 mg of ATP and make up to 500 μL with kinase reaction buffer to give a 20 mM solution of ATP (stock 1). Take 100 μL of ATP stock 1 and dilute to 500 μL in kinase reaction buffer to give a 4 mM solution. Take 15 μL of the ATP stock 2 and dilute to 3000 μL in kinase reaction buffer to give a 20 μM solution. This last solution is used in the assay to give a final concentration of 10 μM [*see* **Note 6**].
7. 96-well opaque white (non-sterile) plates (Corning #3912).
8. Micropipettes.
9. Luminometer compatible for 96-well plates.
10. ***20% DMSO solution:*** Add 800 μL of distilled water to 200 μL of pure DMSO.
11. A known kinase inhibitor standard reference for positive control, e.g. staurosporine, H-9 or H-89 (Sigma-Aldrich) [*see* **Note 7**].

12. ***Kinase-Glo® Reagents:*** Kinase-Glo® Buffer and Kinase-Glo® Substrate (Promega Corporation, Australia). Store at -20°C.

2.2 MTS assay

Plates containing compound dilutions should be discarded in cytotoxic waste bins. All pipette tips that came into contact with the test compounds should also be disposed of in cytotoxic waste bins

1. ***Sample preparation:*** Prepare a 4 mg/mL stock solution of sample in DMSO.
2. ***MTS reagent:*** Should be stored at -20 ºC for long-term storage and protected from light. Reagent should be warmed up to room temperature before use in the assay.
3. RPMI tissue culture medium containing 5% fetal bovine serum (FBS) [*see* **Note 8**].
4. Stock cultures of cells should be maintained in RPMI medium containing 5% FBS (fetal bovine serum).
5. Clear, sterile 96-well microplates.
6. Trypan blue solution (0.4%, liquid, sterile-filtered, suitable for cell culture).
7. Dimethyl sulfoxide (DMSO).
8. Human, leukemic, monocyte-like, histolytic lymphoma (U937) and human, metastatic breast adenocarcinoma (MDA-MB-231) cancer cell lines, or other cell lines as required [*see* **Note 9**]
9. Haemocytometer.
10. Centrifuge.

11. Sterile incubator.
12. UV spectrophotometer compatible for 96-well plate.

3. Methods

The kinase assay described here utilises the Kinase-Glo® Luminsecent Kinase Assay Platform from Promega and is performed according to the manufacturer's instructions *(28)* with minor modifications as used in our laboratory. The method below is relevant to screening against PKA, however a wide range of kinases can be used in this assay including GSK-3β, PI3K, Src and MAPK *(29,28)*. In theory, potentially any kinase could be used, provided the appropriate substrate is also utilised.

3.1 Kinase Assay

1. To wells A/F1-12 of a 96-well plate, add 5 µL of the 1 mg/mL stock solution of the test compounds, which will give a final concentration of 100 µg/mL sample in 2% DMSO (**Table 1**). To the positive control wells (G1-6) and the negative control wells (H1-6), add 5 µL of the 20% DMSO solution.

[Insert Table 1 around here]

2. To wells G/H7-12, add 5 µL of the internal standard (e.g. staurosporine).
3. Thaw a single tube of the enzyme (250 units per tube) and make up to 2 mL with the kinase reaction buffer to give a concentration of 125 units/mL. This will give a final concentration of 50 units/mL (or 2.50 units/well).

4. To six of the positive control wells (G1-6), add 20 μL of the reaction mixture containing 2.5 X the optimal concentration of kinase in kinase reaction buffer. The positive control should provide 100% luminescence. There will be 1880 μL of this reaction mixture remaining.
5. Add 20 μL of the kemptide (PKA peptide substrate, 10 mg/mL) to the remaining 1880 μL of kinase mixture. This will provide 200 μg of the kinase substrate in 1900 mL to give a 140 μM substrate/enzyme solution in buffer. This amount is sufficient for 1 x 96-well plate, giving a final concentration of 56 μM in each well per 50 μL reaction.
6. To all remaining wells, add 20 μL of the above reaction mixture (step 5) containing 2.5 X the optimal concentration of kinase and kinase substrate in 1X kinase reaction buffer. (Negative controls, 0% luminescence).
7. To all wells, add 25 μL of the ATP solution (20 μM, stock 3 ATP solution). This will give a final concentration of 10 μM in each well per 50 μL reaction. See **Table 2** for a summary of the reagent volumes added to the 96-well plate.
8. Using a plate shaker, gently shake the plate and incubate at room temperature for the optimal amount of time [*see* **Note 10**].
9. ***Prepare Kinase-Glo® reagent:*** The kinase buffer should be stored in the freezer and thawed at room temperature. Add the Kinase-Glo® Substrate to the Kinase-Glo® Buffer and add 50 μL of this mixture to each well [*see* **Note 11**].
10. Mix the plate and incubate for 10 minutes at room temperature. Due to the long half-life of the Kinase-Glo® signal, the plates may be left longer before reading, if desired. Record luminescence, which will be directly proportional to percent inhibition of the controls [*see* **Notes 12-14**].

[Insert Table 2 around here]

3.2 MTS Assay

The MTS assay described here utilises Promega's MTS CellTiter 96® AQueous One Solution Cell Proliferation assay and is performed according to the manufacturer's instructions *(30-31)* .

1. ***Determination of cell number and viability:*** Place 20 µL cells + 20 µL Trypan blue on parafilm and place 20 µL of this mixture under a cover-slip on the haemocytometer. Count the number of cells using the equation provided [*see* **Note 15]** to determine the volume required to get a cell concentration of 111,000 cells/mL.
2. ***Day 1 - Setting Up the Plate:*** Pipette 610 µL of cells (as determined in Step 1 of the procedure) into a new Falcon tube and centrifuge at 1600 rpm for five min. (For four plates, pipette 2.44 mL of cells).
3. Drain off supernatant and resuspend the pellet into 2.5 mL of media. This will give you 2.5 mL of cell solution at 111,000 cells/mL. (For four plates, you would resuspend in 10 mL of media).

4. Pipette 100 µL of media into wells A1-A12, H1-H12, B1-G1, B11-G11, B12-G12, B5-B7, E5-E7, F5-F7 and G5-G7 as per **Fig. 2**. These wells are used as blanks to stop any interference that may occur when the spectrophotometer reads the absorbance values for the test wells.

[Insert Figure 2 near here]

5. Pipette 90 µL of media into wells B8-G8, B9-G9, B10-G10. These wells will form the sample background controls.
6. Pipette 90 µL of cell solution into wells B2-G2, B3-G3, B4-G4. These wells will form the sample wells.
7. Pipette 90 µL of cell solution into wells D5-D7. These wells will form the 2.5% DMSO controls.
8. Pipette 100 µL of cell solution into wells C5-C7. These wells will form the cell controls.
9. Incubate plates for 24 hours at 37 ºC with 5% carbon dioxide (CO_2).
10. ***Day Two - Diluting Test Compounds:*** Prepare a microtitre plate for serial dilutions of the test compounds as shown in **Fig. 3** for each compound to be tested. Wells A3-G3 should all contain a final volume of 100 µL.

[Insert Figure 3 near here]

11. ***Adding serial dilutions of samples to cells:*** Using the plate of cells prepared on Day 1, pipette 10 µL of each sample dilution of the test compound in 25% DMSO (i.e. A3-F3, step 11) in triplicate into the 18 wells containing 90 µL of cell solution (i.e. 10 µL of the sample at 500 ug/mL in triplicate into wells B2-B4, 250 ug/mL sample into C2-C4 etc, down to the 15.5 µg/mL sample into

G2-4). Repeat this step, by adding 10 µL of the serial dilutions of the sample in triplicate to the 18 wells containing 90 µL of media (e.g. wells B8-G8, B9-G9, B10-G10). The test compounds will now have a final concentration of 2.5% DMSO in all wells.

12. Pipette 10 µL of the 0 µg/mL in 25% DMSO (i.e. G3 from step 11) into the three remaining wells containing 90 µL cells (wells D5-D7). This will be the 2.5% DMSO control.
13. Incubate plate for 24 hours at 37 ºC and 5% CO_2 [*see* **Note 16**].
14. ***Day Three - Adding MTS Reagent and Plate Reading:*** Thaw MTS reagent before use (~1 mL MTS reagent required per plate).
15. Pipette 20 µL of the MTS reagent into all sample wells (e.g. B2-G2, B3-G3, B4-G4, B5-D5, B6-D6, B7-G7, B8-G8, B9-G9, B10-G10) [*see* **Note 17**].
16. Incubate for three hours at 37 ºC and 5% CO_2.
17. Wrap the plates in aluminium foil and take to the spectrophotometer. Read the absorbance of the whole plate at 490 nm [*see* **Note 18**].

4. Notes

1. A final concentration of 2% DMSO was used for the sample and was found not to interfere with results at this concentration. Standards and controls used in the assay were also dissolved in a final concentration of 2% DMSO. Other solvents such as 2% ethanol can be used, provided all standards/controls are also prepared in this solvent.
2. We found that a starting stock solution of 1 mg/mL of sample (diluted to 100 µg/mL in the assay) was appropriate to identify kinase inhibitory activity.

Samples can be tested at lower concentrations or over a range of different concentrations to determine IC_{50} values.

3. We found that it is best to prepare the buffer solution fresh each time and to store the buffer at room temperature.
4. For kinases other than PKA, the optimum amount of kinase enzyme will need to be determined and the quantity of other reagents adjusted accordingly *(28)*.
5. Kemptide (PKA Peptide Substrate) is a synthetic peptide substrate for PKA derived from the PKA phosphorylation site in liver pyruvate kinase.
6. This assay can be used to identify whether the kinase inhibitor is ATP competitive or non-competitive, by utilising various ratios of ATP (e.g. <10 μM ATP for ATP-competitive inhibitors and >100 μM for ATP non-competitive inhibitors). In general, we found it is best to prepare ATP solutions fresh, while the ATP reagent itself should be stored in the freezer.
7. We include an internal reference of a known kinase inhibitor such as staurosporine in our assays (IC_{50} = 7 nM *vs* PKA; IC_{50} = 0.7 nM *vs* PKC) by adding 5 μL of a 1 mg/mL solution of the inhibitor (in 20% DMSO) to six of the wells in place of a sample set. Staurosporine is a potent inhibitor of PKC and can induce apoptosis in Jurkat cells. Other PKA inhibitors include the isoquinoline sulfonamides H-9 (IC_{50} = 2 μM) and H-89 (IC_{50} = 48 nM).
8. RPMI-1640 was developed by Moore et al. *(32-33)* at Roswell Park Memorial Institute.
9. Human, leukemic, monocyte-like, histolytic lymphoma (U937) and human, metastatic breast adenocarcinoma (MDA-MB-231) cancer cells were obtained from American Type Culture Collection (ATCC, VA, USA) distributed by Cryosite, NSW, Australia. Cells were regularly cultured *in vitro* in culture

medium consisting of RPMI-1640 medium, along with 2 mM L-glutamine, 5.6% (2 g/L) $NaHCO_3$ and 5% foetal calf serum. The cells were maintained in a Huracell incubator (Kendro Laboratory Products, Langenselbold, Germany) at 37 °C with a humidified atmosphere containing 5% CO_2. However, a wide range of both normal and cancer cell lines can be used in this assay.

10. We found that thawing the Kinase-Glo® reagents (Kinase-Glo® substrate and Kinase-Glo® buffer) generally took one hour and we also allowed an hour for the incubation time. We strongly advise optimizing the kinase reaction conditions so that the reaction can be run at room temperature to avoid formation of temperature gradients. Information regarding optimization of kinase reaction conditions can be found in the manufacturer's Technical Bulletin *(28)*.
11. The choice of Kinase-Glo® reagent will depend on the desired ATP concentration to be used in the assay. This information can be found in the manufacturer's protocol, Promega Corporation, Australia *(28,34)*.
12. We find that it is best to read the plate within 15 mins after the addition of the Kinase-Glo® reaction mixture.
13. Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline. We found that the best plate reading is immediately after adding the Kinase-Glo® Buffer and when the incubation is performed at room temperature.
14. The most common assay detection methods are colorimetric or fluorescence-based. However, luminescent-based detection is particularly desirable for coloured products such as natural products, as coloured extracts and compounds can lead to false results when using absorbance as the end point.

Utilization of luminescence can be more beneficial for biological screening by identifying hits with a lower number of false positives *(35)*.

15. An example calculation of cell concentration of which the values obtained will be used in the method protocol described herein:

$$No.of\ Cells\ =\ \frac{(\#\ of\ cells\ in\ 4\ grids)}{4}\ \times\ 2\ \ \times\ 10000$$

For example if we count 91***** cells:

$$No.\ of\ Cells =\ \frac{91\ *}{4}\ \times\ 2\ \times\ 10000\ =\ 455000\ cells$$

Therefore, to get a cell concentration of *111,000 cells/mL*, you will need:

$$\frac{111000\ cells/mL}{455000 cells}\ =\ 0.244\ mL\ =\ 244\ \mu L$$

For the preparation of one plate the cells will be resuspended in 2.5 mL media therefore the volume of cells required would be:

$$244\ \mu L\ \times\ 2.5\ =\ \ 610\ \mu L$$

Adjust accordingly if more plates are required.

For example, the preparation of 4 plates will require:

$$244\ uL\ X\ 10\ mL\ (2.5\ ml\ per\ plate\ x\ 4\ plates)\ =\ 2.44\ mL$$

16. Longer incubation times of 48 – 72 hours are also routinely employed in this assay.
17. The MTS reagent is light sensitive so this step should be performed with the lights off in the cytotoxic cabinet.
18. As this is a colorimetric assay, it should be kept in mind that both coloured compounds and natural product extracts may interfere with the absorbance reading, and appropriate background controls should always be performed.

Acknowledgement

This work was supported by the School of Medicinal Chemistry and the School of Chemistry, University of Wollongong.

Tables

Table 1 Kinase assay plate preparation: wells A-F[1-12] are the test compounds; wells G1-6 are positive controls; wells H1-6 are negative controls; and H7-12 and G7-12 are internal reference standards (Std). The plate below shows the set-up for 12 test compounds run twice (samples a and b) in triplicate. This gives six data points per % inhibition reading.

	1	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***	***11***	***12***
A	1a	1b	2a	2b	3a	3b	4a	4b	5a	5b	6a	6b
B	1a	1b	2a	2b	3a	3b	4a	4b	5a	5b	6a	6b
C	1a	1b	2a	2b	3a	3b	4a	4b	5a	5b	6a	6b
D	7a	7b	8a	8b	9a	9b	10a	10b	11a	11b	12a	12b
E	7a	7b	8a	8b	9a	9b	10a	10b	11a	11b	12a	12b
F	7a	7b	8a	8b	9a	9b	10a	10b	11a	11b	12a	12b
G	Positive Control (100% Luminescence)						Std1a	Std1a	Std1a	Std2a	Std2a	Std2a
H	Negative Control (0% Luminescence)						Std1b	Std1b	Std1b	Std2b	Std2b	Std2b

Table 2 Kinase assay summary of reagent concentrations and volumes added to plate.

	Initial Stock Conc.	***Standard Wells (µL)***	***Positive Control (µL)***	***Negative Control (µL)***	***Final Conc. in 50uL***
Sample	1 mg/mL	-	-	-	100 ug/mL
DMSO solution	20%	-	5	5	2%
Enzyme/no substrate (2.5X)	125 units/mL	-	20	-	50 units/mL
Enzyme/substrate (2.5X)	140 µM	20	-	20	56 µM
ATP solution (2X)	20 µM	25	25	25	10 µM
Total reaction volume		50	50	50	
Kinase-Glo® reagent		50	50	50	

Figures

Figure 1: Janice_McCauley_Figure_1.tif at 600 DPI

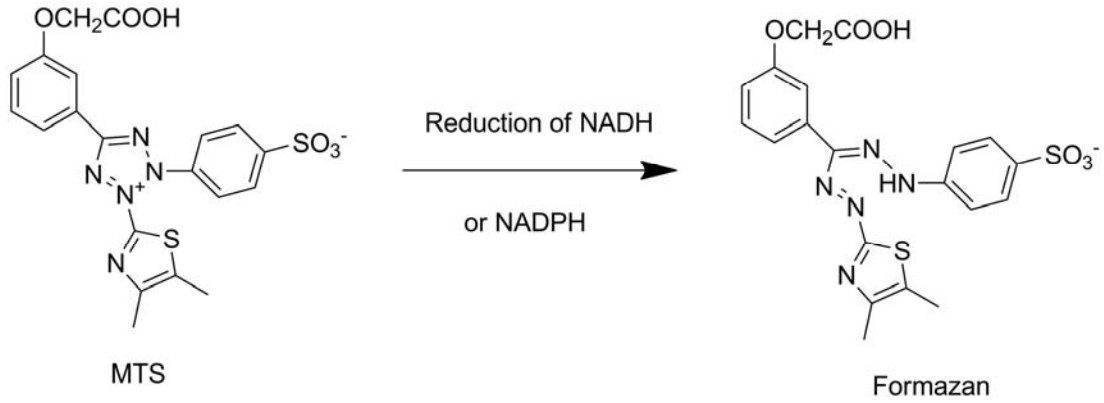

Fig. 1. The reduction of the MTS tetrazolium salt to the red formazan product by viable cells.

Figure 2: Janice_McCauley_Figure_2.tif at 600 DPI

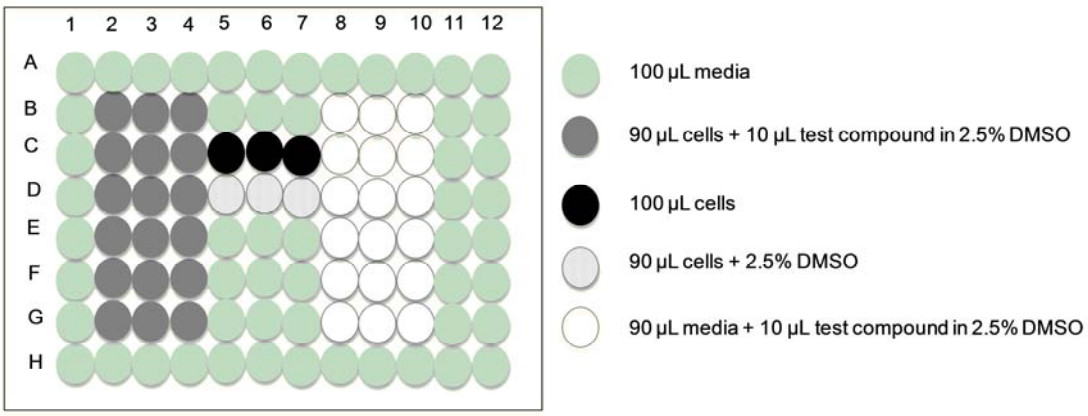

Fig. 2. 96-Well microplate set-up for the MTS assay.

Figure 3: Janice_McCauley_Figure_3.tif at 600 DPI

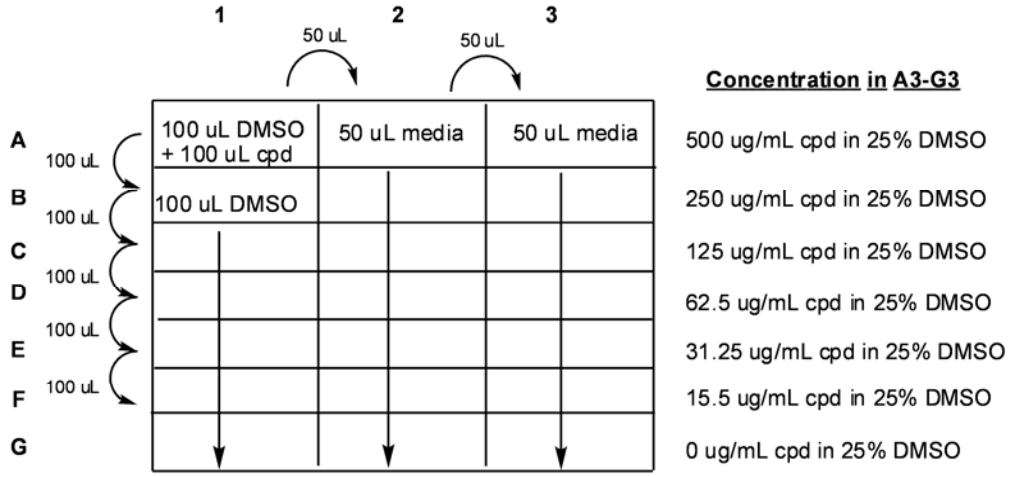

Fig. 3. Preparation of microtitre plate for 1:2 serial dilutions. Add 100 µL of DMSO and 100 µL of the test compound (4 mg/mL in DMSO) to well A1. Next, add 100 µL of DMSO to wells B1 to G1. Then, 100 µL is transferred from A1 to B1; B1 to C1 and so forth until F1, where 100 µL is removed and discarded. G1 should contain just 100 µL of 100% DMSO. Next 50 µL of media is added to wells A2-G2 and A3-G3 and 50 µL transferred from A1 to A2 and then from A2 to A3 to give 100 µL of the sample at a concentration of 500 µg/mL in 25% DMSO in media. This is repeated for wells B2-B3, C2-C3, etc to give a series of 1:2 dilutions of the sample.

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