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Protocol for screening cytotoxic activity of natural products against KB cells

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Abstract. In our studies on the anti-tumour bioassay on the human laryngeal carcinoma KB cell line, modifications were made to the protocol used by Institut de Chimie des Substances Naturelles (ICSN), Centre National de Ia Recherche Scientifique (CNRS) of Paris, France, to compensate for differences with respect to environment, type, concentration of chemicals and growth factors. In the preparation of the culture medium, sodium hydroxide was omitted, penicillin/streptomycin levels were doubled, and kanamycin was used at 50 IU mL^{-1}. The tests were carried out in 4 mL culture media and each flask had 20,000 – 30,000 cells mL^{-1}. Adriblastine was used in the *in vitro* cytotoxicity test at 1 x 10^{-8} to 2.5 x 10^{-7} M; Neutral Red dye was used to detect cell damage. Our studies showed that the ED_{50} of adriblastine on KB cells was 0.1 x 10^{-7} to 0.3 x 10^{-7} M.

Abstrak. Dalam mengkaji sistem bioasei anti-tumour terhadap sel karsinoma larinks manusia, iaitu leluhur sel KB, pengubahsuaian telah dilaksanakan kepada protokol yang digunakan di Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, Paris, Perancis, untuk mempampas perbezaan dari aspek sekitaran, jenama dan kepekatan bahan kimia dan faktor pertumbuhan. Dalam penyediaan medium kultur, natrium hidroksida tidak dimasukkan, paras penicillin/streptomycin digandakan, dan kanamycin digunakan pada kepekatan 50 IU mL^{-1}. Kajian dijalankan di dalam kelalang yang mengandungi 4 mL media kultur dan kelalang mempunyai 20,000 – 30, 000 sel mL^{-1}. Adriblastine digunakan dalam kajian sitotoksisiti *in vitro* pada kepekatan 1.0 x 10^{-8} kepada 2.5 x 10^{-7} M; pewarna Neutral Red digunakan untuk mengesan kerosakan pada sel. Dalam kajian kami, ED_{50} adriblastine ke atas sel KB ialah 0.1x 10^{-7} hingga 0.3 X 10^{-7} M.

Introduction

In vitro cell cultures techniques are the common methods for determining the acute toxicity of chemicals. The present study utilises the KB cell culture for this purpose. The KB cells were first isolated from 54-year old white man who developed an ulcerated friable tumor on the floor of the mouth and the tongue [1]. The KB cells were cultivated directly onto a glass surface in a medium consisting of 13 amino acids, 7 vitamins, glucose, salts and 10% human serum, these components being essential to the growth of cells. Next, the KB cells were grown in Medium 199 with Earle's salt supplemented with 10% fetal calf serum.

The method for measuring the damage caused to the cell culture by adriblastine is conducted according to protocol [2]. The extent of the cell damage in different cultures is shown by the relative amounts of dye in the cell sheets; this is measured colorimetrically. This method has been used in virus infectivity and neutralization assays, virus growth studies and cytotoxicity tests [2]. The cytotoxicity test on KB cells was carried out to determine its ED_{50} value and to compare this value with the value obtained by the laboratory at ICSN.

Experimental

Chemicals. Adriblastine was purchased from Farmitalia Carlo Erba Ltd., Italy. It was

dissolved in dimethyl sulfoxide, DMSO (Sigma, USA) and was kept refrigerated at a stock concentration of 10^{-3} M.

Cell line. The KB cell line of the laryngeal carcinoma was provided by the ICSN, and the cells were grown in the Medium 199 with Earle's salt. Medium 199 (Flow Lab., Australia) was supplemented with 10% inactivated foetal calf serum (FCS), and was filter sterilized by using a 0.22 μm cellulose acetate filter membrane (Gelman Sc., Australia). It was stored at 4°C .

Culture medium. The supplemented maintenance Medium 199 culture medium was prepared with Medium 199 supplemented with 0.03g L^{-1} of L-glutamine, 10% of inactivated FCS, 100 μg mL^{-1} and 100 IU mL^{-1} of streptomycin and penicillin, 20 mM final Hepes. The reagents used were obtained from Flow Lab., Australia. The culture medium was changed once every two days and the cell population was divided once every four days.

Cryopreservation of cells. The preservation solution consisted of 60% FCS, 10% DMSO and 30% Medium 199. Cells that were growing at the exponential stage were washed twice and then treated with 4 mL of 0.25% trypsin (Amresco, USA) for 5 minutes. The cells were spun down at 1000 rpm for 5 minutes on a bench centrifuge. The cells were re-suspended in 2 mL fresh preservation solution and the solution aliquotted into sterile provials, in 1 mL aliquots. The provials were each kept in a 1-inch polystyrene box left to stand in ice. The box was finally kept in a freezer at -70°C overnight. Provials were then transferred into liquid nitrogen (-196°C).

Reviving of cells. The reviving medium was similar to the supplemented culture medium except that it contained 20% FCS. The provial was removed from the liquid nitrogen and transferred to a 37°C water bath for rapid thawing. The thawed cells were transferred into 5 mL of 20% supplemented medium in a 1 mL polypropylene tube and spun at 1000 rpm for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 8 mL of 20% supplemented medium in a 260 mL tissue-culture flask (Falcon, USA) and incubated at 37°C in a 5% CO_2 water-jacketed incubator (Jouan, France).

Cytotoxicity assay. The KB cells were maintained in Medium 199 with Earle's salt supplemented with 10% FCS. The medium was changed a day before testing. The confluent cells were washed twice with PBS and treated with 0.25% trypsin (Amresco, USA) for 5 minutes. The detached cells were then centrifuged at 1000 rpm for 5 minutes. The pellet was re-suspended in culture medium and adjusted to obtain a concentration of 20,000 – 30,000 cells mL^{-1}, and a 4 mL portion of the cells was transferred into each flask [3]. The *in vitro* cytotoxicity test was carried out with adriblastine at the concentrations of 2.5 x 10^{-7}, 1 x 10^{-7}, 0.5 x 10^{-7}, 0.25 x 10^{-7} and 1 x 10^{-8} M. The cells were incubated with adriblastine at 37°C in a humidified atmosphere of 5% CO_2 in air for 72 hours. At the end of the incubation, 1% Neutral Red dye was added and the cells and the cells were further incubated for another 2 to 3 hours. The cells were then rinsed twice with phosphate buffered saline (PBS) and 1% of sodium dodecyl sulphate was added. After 1 hour of contact, the optical density at 540nm was read on a spectrophotometer. The experiment was repeated four times. The ED_{50} of adriblastine on KB cells was determined from the mortality curve.

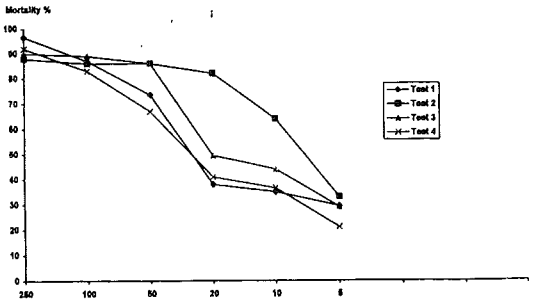

Figure 1. Mortality curve of KB cells after 72 hours incubation with adriblastine.

Results

The results of the set of four consecutive trials are shown in Figure 1. At a concentration of 2.5 x 10^{-7} M, adriblastine was toxic to the cells, as it caused over 95% mortality. From the mortality curve, the ED_{50} of adriblastine on KB cells was found to be within the range of 0.1 – 0.3 x 10^{-7} M. The present protocol differs from that used by ICSN in several ways. Sodium hydroxide was omitted since the Hepes used has kept the medium well balanced. The penicillin/streptomycin concentrations were increased two-fold and kanamycin was used in place of gentamycin. Medium 199, chemicals and growth factors used were purchased from different suppliers to ensure reproducibilities. The dye incubation period was shortened as little additional dye was taken up by the cells after an incubation exceeding 3 hours. The ED_{50} value for adriblastine obtained by ICSN was 0.5 x 10^{-7} M to 1.2 x 10^{-7} M (Tempete, *personal communication*). The ED_{50} value of adriblastine obtained in the present study is lower compared with that obtained by ICSN. This suggests that the KB cells maintained in our laboratory are more sensitive towards adriblastine but the reason for this behaviour is not known. The KB cells are fast growing and are relatively easy to maintain. The bioassay described here is fast and not cumbersome to perform. We highly recommend it for screening bioactive compounds.

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References

1 Finter, N.B. (1969). *J. Gen. Virol.* **5**: 419.
2 Geran, R.I., Greenberg, N.H., MacDonald, M.M., Schumacher, A.M. and Abbot, B.J. (1972). *Cancer Chemother. Rept.* **3**: 16.
3 Eagle, H (1955). *P.S.E.B.M.* **89**: 362.