Anticancer drugs doxorubicin and cyclophosphamide biology essay

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The available anticancer drugs doxorubicin and cyclophosphamide are used in several types of cancer treatment. In this practical, we will use the MCF-7 breast cancer cell lines to access the sensitivity of doxorubicin and cyclophosphamide in vitro with the method of MTT assay. MCF-7 breast cancer cells, ductal epithelial cell lines are characterized by receptor functions (estrogen receptor, progesterone receptor , Her2/neu-) and these cells are non-adherent cells and grow well in nutrient media with the formation of dome. Chemosensitivity of the drug effects are measured by MTT assay. It is a cell proliferation assay offering a quantitative, convenient method for evaluating a cell population’s response to external factors, whether it be an increase in cell growth, no effect, or a decrease in growth due to necrosis or apoptosis. Both drugs are widely used in clinical practice but we evaluate the effect of sensitivity of both drugs in vitro with MTT assay. The practical and statistical results show that doxorubicin have the significant anti-tumor effect on MCF-7 cells although cyclophosphamide didn’t work well in vitro on breast cancer cells. Hence, with the help of the

MTT assay, the drug efficacy can be determined for further improvement of drug development.

Introduction

Cancer cells are proliferating continuously by cell division and replication. Chemotherapeutic agents are the drugs that inhibit the cell proliferation at specific phase of cell cycle and destroy the malignant cells as well as normal cells. With the advancements in our understanding of the effects of chemotherapeutic drugs on cancer cells have more beneficial effects by treated with the combinational chemotherapy. In our practical, we will evaluate the efficacy of cyclophosphamide and doxorubicin on MCF-7 breast cancer cell in vitro with MTT assay. MCF-7 (Michigan Cancer Foundation-7) is a breast cancer cell line isolated in 1970 from a 69-year-old Caucasian woman, and they have been studied extensively as a model for breast cancer cell growth. The MCF-7 cells are characterized by autocrine receptor such as estrogen, progesterone and epidermal growth factor of Her2/neu-. MCF-7 cells are isolated from mammalian cell lines of breast cancer cells and they are maintained to growth in cell culture. There are several types of cell cultures: primary cell culture in which cells are directly from a subject, secondary cell culture, continuous cell culture, immortalized cell culture and hybridoma cell culture. In our practical, MCF-7 are supplied from ATCC (biological resource centre) and then subcultured by outgrowth explants for the ability of proliferation. Then, store the cells by cryopreservation method of freezing the cells in isopropanol which prevent stuck lid and frozen finger at 1’C/min until reaching the -80’C and transfers the cells into liquid nitrogen

vessel at -196’C without substantial loss of cell viability. In this way, MCF-7 breast cancer cells are maintained for further laboratory research.

Cyclophosphamide is a cell cycle dependent DNA and nitrogen mustard alkylating agent that has a broad spectrum of activity against a variety of neoplasms. An alkylating agent adds an alkyl group to DNA and attaches the alkyl group to the guanine base of inter and intra DNA strands, at the number 7 nitrogen atom of the imidazole ring and it leads to cell death. It is a prodrug and the cytotoxic effects of cyclophosphamide depend on its metabolism by hepatic microsomes. Cyclophosphamide is converted to metabolite 4-hydroxycyclophosphamide (isomer of aldophosphamide) by oxidase enzymes in the liver . Phosphoramide mustard is most likely alkylating metabolites which alkylates under physiological condition which is directly cytotoxic to cells. Cyclophosphamide is also converted to acrolein which is toxic to bladder epithelium and can lead to hemorrhagic cystitis.

Structure of cyclophosphamide

(Figure is taken from http://en. wikipedia. org/wiki/File: Mttscheme. png)

Doxorubicin is the member of anthracycline family and it is a hydroxylated version of daunorubicin. It intercalates between the base pairs in DNA double helix, thereby preventing DNA replication and ultimately inhibiting protein synthesis. Furthermore, it also inhibits the activity of an enzyme, topoisomerase II which results in an increased and stabilized cleavable enzyme-DNA linked complex during DNA replication and subsequently prevents the ligation of the nucleotide strand after double strand cleavage, thereby stopping DNA replication. Doxorubicin is widely used in several types

of cancer in hematopoietic, lymphoma, breast and lung cancer, but has the undesirable cardiotoxic affect which damage to the heart in some individual.

structure of Doxorubicin

(Figure is taken from http://en. wikipedia. org/wiki/File: Mttscheme. png)

For the detection of drug sensitivity to tumor cells, there are many chemosensitivity assays used in clinical research. They are 3-(4-5-dimethylthiazole-2-yl)-2-5-diphenyltetrazolium bromide(MTT)assay, 2, 3-bis(2-methoxy-4-nitro-5-[(phenlamio)carbonyl)]-2H-tetrazolium hydroxide (XTT)assay, sulforhodamine B(SRB)protein stain assay, colony forming assay, estrogen receptor assay, standard soft-agar assay and hollow fibre (HF)assay. These tests are also necessary for identification of new anticancer drugs. MTT assay has been used in the chemotherapeutic agents screening program of the National Cancer Institute (NCI) since 1991 and was reported to show the accuracy in more than 90% of patients (Kim et al. 2003) In our practical, we will apply the MTT assay to fulfill our objective of evaluation of two drugs. It is a laboratory test and standard colorimetric assay was introduced for use in immunology in 1983. It is also amenable for use in growth inhibition assays. The yellow tetrazolum salt (MTT) is reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of detergent. It gives a linear relationship between cell number and absorbance is established, enabling accurate, straightforward quantification of changes in proliferation. Utility of MTT method provide the accurate measurements by detecting the changes in cell metabolism with spectrophotometric procedure. Moreover, it is non

radioactive substances so safer reagents than others. It is easy to use and rapid processing and convenient storage for a prolonged period.

(Figure is taken from http://en. wikipedia. org/wiki/File: Mttscheme. png)

Material and Methods

Materials and Equipments for cell culture

MCF-7 breast cancer cell lines are supported from ATCC or NCI , NCC

cell culture medium, appropriate for cell line

Tissue culture flasks of appropriate sizes

Tissue culture plates-96 well

Sterile pipettes, assorted sizes

Multichannel pipette and sterile tips

Pasteur pipets, sterile unplugged

70% alcohol

Sterile Petri dishes

Laminar Flow Hood

Inverted Microscope

Vacuum pump and flask

Methods

I. Protocol for cell culture

Prepare the hood, wipe all surfaces with 70% ethanol for aseptic condition and brought the reagents and materials to the hood to begin the procedure.

The cryovials which has been kept in liquid nitrogen or -196’C freezer and thaw vial immediately in 37’C water bath. Thaw content with slight shake until only small ice is left in vial. It usually takes 1 min. Spray vial with 70% ethanol all over and wipe its surface with clean tissue in the hood

Open the vial and transfer the content to a 15ml Falcon tube already containing 5ml of fresh medium.

Spin down at 1000 rpm or 200g for 35 min at 4’C. Aspirate supernatant.

Resuspend cells in fresh medium and transfer to culture dish.

Cells are cultured in incubator and medium need to be changed for every 3 days.

Check the cell under microscope

For passaging the cells, when subculture is appropriate confluence, subculture can be conducted.

Remove media from the dish.

Briefly rinse the cell layer with 0. 25 %(w/v)Trypsin-0. 53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.

Add 2 to 3 ml of Trypsi-EDTA solution to flask and observe cells under an inverted microscope until to detach. Cells that are difficult to detach may be placed at 37’C to facilitate dispersal.

Add 6 to 8 ml of complete growth medium and aspirate cells by gently pipetting.

Transfer the cell suspension to the centrifuge tube with the medium and cells, and centrifuge at approximately 12000rpm for 2 min. Discard the supernatant.

Resuspend the cell pellet in fresh growth medium . Add appropriate aliquots of the cell suspension to new.

Keep the cell into 37’C 5% CO2 incubator.

II. Setting up of the Experiment lane

Under aseptic condition of Laminar Flow Hood, we are provided with 96 wells containing 2 culture plates and 2 different drugs (doxorubicin and cyclophosphamide) with different concentration.

First of all, we labeled each plate according to the drug we will access. 200ul of RPMI 1640 medium was added into lane 1 from row A to H as a negative control. 180 ul of MCF-7 cells suspension were already filled by lab technician.

Then, 20ul of RPMI 1640 medium was added into lane 2 from row A to H as a positive control. For a test, we put 20 ul of Doxorubicin with decreasing the

doubling concentration (1, 0. 5, 0. 25, 0. 0625, 0. 03, 0. 016, 0. 008, 0. 004, and 0. 002) from lane 3 to lane 12 in the row from A to H respectively.

Then the plate was incubated at 37’C for 4 days for the appropriate reaction.

This procedure was repeated the same for cyclophosphamide.

III. Accessing Cellular Response to Cytotoxic drug

After 4 days, 20 ul of MTT (5 mg/ml) was added.

The plates were then cultured at 37’C for 4 hours in which 37’C is the temperature for cleavage of MTT and optimal time may vary according to the assay, but 4 hours is suitable for most purposes.(this step was done by lab technician).

Then, 200 ul of medium plus MTT from each well was carefully removed and discarded without touching the bottom of the well to avoid the disturbance of the cells. 150ul of DMSO was added to each well to dissolve the formazon crystals homogenously. The amount of DMSO depends on the formazon crystals and more DMSO give higher color.

The solution was mixed thoroughly and the absorbance reading was read at 490nm on the spectrophotometer.

Results

The following formula is used to get the final concentration of the drug.

M1V1 = M2V2

In our table , the percent of cell survival of different concentration of both drugs are calculated using the following formula:

Percentage of cell survival of specific lane= Absorbance of drug treated in specific lane f Mean absorbance of positive control

Table 1: Absorbance reading at 490nm, percent survival, and Standard deviation values for Cyclophosphamide

Lane (Drug conc.)(uM)

1(-ve control)

2(+ve control)

3 (1)

4(0. 5)

5(0. 25)

6(0. 125)

7(0. 0625)

8(0. 03)

9(0. 016)

10(0. 008)

11(0. 004)

12(0. 002)

A

0. 144

0. 676

0. 529

0. 637

0. 665

0. 653

0. 607

0. 671

0. 615

0. 578

0. 623

0. 701

B

0. 091

0. 612

0. 491

0. 468

0. 511

0. 503

0. 487

0. 51

0. 523

0. 55

0. 486

0. 603

C

0. 088

0. 516

0. 456

0. 409

0. 484

0. 467

0. 482

0. 479

0. 446

0. 51

0. 428

0. 484

D

0. 078

0. 576

0. 477

0. 471

0. 49

0. 497

0. 465

0. 482

0. 45

0. 497

0. 445

0. 559

E

0. 115

0. 525

0. 508

0. 454

0. 516

0. 528

0. 528

0. 488

0. 454

0. 531

0. 448

0. 519

F

0. 113

0. 577

0. 509

0. 438

0. 509

0. 597

0. 57

0. 507

0. 53

0. 51

0. 499

0. 512

G

0. 11

0. 627

0. 6

0. 513

0. 579

0. 609

0. 56

0. 592

0. 597

0. 559

0. 55

0. 615

H

0. 089

0. 69

0. 495

0. 078

0. 623

0. 309

0. 704

0. 665

0. 68

0. 656

0. 666

0. 652

Average

0. 1035

0. 599875

0. 508125

0. 4335

0. 547125

0. 520375

0. 550375

0. 54925

0. 536875

0. 548875

0. 518125

0. 580625

% cell survival

84. 705

72. 265

91. 207

86. 747

91. 748

91. 56

89. 498

91. 499

86. 372

96. 79

Standard Deviation

0. 021253571

0. 063846775

0. 043142

0. 159351

0. 067234

0. 106737

0. 079071

0. 081577

0. 08711

0. 051354

0. 08756

0. 075193

Table 1 shows that the average absorbance readings of viable MCF-7 cells from lane 1 to lane 12 ranging from 0. 599875 to 0. 580625, and also the percentage of cell survival. There is no significant different in concentration and percent of cell survival among the lanes between different concentrations of cyclophosphamide. From our calculation, the largest standard deviation is (+/-0. 08756) shows the least consistency between replicates

Table 2: Absorbance reading at 490nm, percent survival, and Standard deviation values for Doxorubicin

Lane (Drug conc.)(uM)

1(-ve control)

2(+ ve control)

3(1)

4(0. 5)

5(0. 25)

6(0. 125)

7(0. 0625)

8(0. 03)

9(0. 016)

10(0. 008)

11(0. 004)

12(0. 002)

A

0. 072

0. 693

0. 072

0. 088

0. 172

0. 314

0. 518

0. 553

0. 634

0. 661

0. 61

0. 742

B

0. 072

0. 733

0. 073

0. 084

0. 155

0. 294

0. 409

0. 434

0. 52

0. 517

0. 551

0. 598

C

0. 067

0. 554

0. 07

0. 096

0. 19

0. 265

0. 423

0. 449

0. 45

0. 504

0. 504

0. 629

D

0. 068

0. 572

0. 074

0. 09

0. 169

0. 293

0. 422

0. 475

0. 596

0. 659

0. 549

0. 544

E

0. 078

0. 531

0. 085

0. 106

0. 16

0. 297

0. 392

0. 445

0. 486

0. 444

0. 487

0. 53

F

0. 083

0. 532

0. 088

0. 093

0. 157

0. 292

0. 377

0. 531

0. 591

0. 509

0. 503

0. 576

G

0. 072

0. 56

0. 072

0. 091

0. 157

0. 291

0. 433

0. 486

0. 554

0. 604

0. 551

0. 656

H

0. 073

0. 647

0. 068

0. 078

0. 157

0. 309

0. 482

0. 657

0. 578

0. 584

0. 886

0. 56

Average

0. 073125

0. 60275

0. 07525

0. 09075

0. 164625

0. 294375

0. 432

0. 50375

0. 551125

0. 56025

0. 580125

0. 604375

% cell survival

12. 484

15. 056

27. 312

43. 839

71. 67

83. 575

91. 435

92. 949

96. 246

100. 27

Standard deviation

0. 005194434

0. 077801487

0. 007226

0. 008294

0. 011987

0. 014579

0. 046648

0. 074747

0. 061689

0. 078881

0. 129578

0. 069902

Table 2 shows the average absorbance reading and percent cell survival of MCF-7 viable cells from lane 1 to lane 12 increases as the concentration of drug decreases(from 0. 07525 in 1uM to 0. 604375 in 0. 002uM) and (from

12. 484% in lane 1 to 100. 27% in lane 12). The drug works well on MCF-7 cells in vitro. The largest standard deviation is (+/-0. 129578) in lane 11.

Graph 1 for Cyclophosphamide

From graph 1, cyclophosphamide doesn’t work although the concentration of the drug is increasing gradually because cyclophosphamide needs the metabolic activity by oxidase enzyme which is present in the liver. Therefore, cyclophosphamide does not affect the tumor cells in vitro.

Graph 2 for Doxorubicin

From graph 2, percentage of cell survival is directly proportional to the drug concentration (doxorubicin). It doesn’t need the metabolic activity as in cyclophosphamide. The concentration value of Doxorubicin for IC50 is round about 0. 1uM. Therefore, doxorubicin is effective in vitro.

Table 3: IC50 (inhibitory concentration) values for cyclophosphamide and doxorubicin

Drug	IC50
Cyclophosphamide	
Doxorubicin	round about 0. 1uM

From Table 3, there is no inhibitory concentration effects of cyclophosphamide to MCF-7 in vitro, but there is an inhibition of the cell growth to MCF-7 at the concentration of 0. 1uM in doxorubicin.

Discussion

In our practical, we measured IC50(inhibitory concentration) of both drugs to determine the efficacy of drugs in inhibiting a biological or biochemical function of tumor cells. IC50 is the quantitative measure of the particular drug that is needed to inhibit the biological process. It is commonly used as a measure of antagonist drug potency in pharmacological research. According to FDA, IC50 represents the concentration of a drug that is required for 50% inhibition in vitro cell line. IC50 of the drug can be obtained by dose-response curve with different concentration of the drugs against the percent cell survival.

In this practical, we use Doxorubicin and Cyclophosphamide to access the efficacy of these drugs on the MCF-7 breast cancer cell lines. It is significantly seen that although cyclophosphamide metabolites block proliferation at several stages of cell cycle in treatment of many cancer (Cancer Biology, third edition, Roger J. B. King), it doesn’t affect on MCF-7 cell lines in vitro showing no difference in percent cell survival which have been treated with different concentration of cyclophosphamide. Cyclophosphamide acts as a prodrug and converted to aldophosphamide which has cytotoxic effect to tumor cells catalysed by liver oxidase enzyme; therefore, cyclophosphamide does not work in vitro.

However, Doxorubicin (anthracycline family) works well in vitro according to our practical results. This drug prevents DNA replication of the cancer cells by intercalating into DNA. Hence, the percent cell survival increases in each lane as the concentration of the drug decreases showing concentration of the drug is inversely proportional to the percent cell survival of MCF-7 breast cancer cell lines. Also from Graph2, round about 0. 1uM of the drug is required for 50% inhibition of growth of the cell. Therefore, the chemosensitivity assay is a valuable test which may help to choose the drug in treatment of cancer.

The behavior of particular cell line is well displayed graphically from these calculated mean value , but is not a particularly useful depiction of the pattern of response for multiple cell lines to antineoplastic agents like doxorubicin and another topoisomerase-directed agent which had similar , although not identical, patterns of activity. Series of previously uncharacterized structures was found to have a pattern of action resembling antimicrotubule agents(Bar et al., 1991; Paull et al., 1992). Thus, although the particular growth inhibitory response of a single cell line was relatively uninformative, the pattern of response of the cell lines as a group could be used to rank compounds according to likelihood of sharing a commonality of mechanism. To overcome this problem, Developmental Therapeutic Program’s (DTP) anticancer screening program generates the COMPARE algorithm in which vast quantities of data are captured in computerized database created by Dr. Kenneth Paull and is initiated to use in April 1990.

Chemosensitivity assays are reasonably important for the future development of effective drugs. MTT assay seems to be an easy process for

the prediction of chemosensitivity of isolated malignant cells in vitro; however each method represents a sample tool, which can provide false results if incorrectly performed. Numerous limitations significantly reduce the successful evaluation (e. g. metabolic interference).

Both Doxorubicin and Cyclophosphamide are widely used in the treatment of cancer and their sensitivity in vitro is very different. It is important for treatment in antiproliferative activity in human. The National Cancer Institute (NCI) of USA has been at the forefront of cancer drug discovery since 1955. The ultimate goal of the NCI’s drug development program is to expedite the best molecules for cancer treatment from discovery into clinical trials, utilizing any or all of the resources available.

The new NCI screen is appealing in its simplicity with semi-automated, the broad spectrum of human tumors it encompasses, and its low cost, relative to the cost of conventional in vivo screen. The assessments of in vitro cytotoxicity were focused on the use of tetrazolium salt-based assays such as the 3-(4, 5-dimethylthiazol-3-yl)-2-5-diphenyltetrazolium bromide(MTT)assay, XTT assay and more recently emerged the sulforhodamine B protein assay. The ultimate value of these screening program based on the response of cell lines in vitro will depend on the demonstration of a strong correlation between in vitro and in vivo response to cytotoxic drugs. For the analysis of the activity of the compound, the NCI’s DTP has utilized various experimental screening models tested in pre-clinical in vivo and vitro assays since its inception 1955. The choice of screening model was based primarily on response of the models to agents already identified as clinically active(Gellhorn and Hirschberg, 1955, Zubrod et al.

1966). The screening model first included only rodent tumor but was later enhanced to include human tumor xenograft with the intent of their serving as potentially better predictors of clinical activity against human tumor. In the late 1980, the US NCI60 human tumor cell line anticancer was involved from the narrow concept of an in vitro disease oriented screening model intended to be used in anticancer drug screening. Panels of cell lines were assembled that ultimately represented nine distinct tumor type: leukemia, colon, lung, CNS, renal, melanoma, ovarian, breast and prostate. In early 1990, the p388 pre-screen was replaced by an in vitro human tumor cell line with 60 different cell types. The nude mouse xenograft models are studied in small no of sensitive human tumor cells for further clinical development.

Conclusion

To bind up, the results calculated from the sensitivity of Doxorubicin and Cyclophosphamide to MCF-7 in vitro point out that the efficacy of doxorubicin has good correlation with different concentration of Doxorubicin, but the cyclophosphamide does not work will in vitro. Although the limitations imposed on invitro screening program, chemosensitivity testing in vitro are likely to be a major challenge for chemotherapy in clinical practice.