Chemosensitivity of epirubicin and cyclophosphamide

Abstract

Epirubicin (PHARMORUBICIN®), anthracycline antibiotic, inhibits topoisomerase II which is essential for DNA replication, chromosome condensation and chromosome segregation. Inhibitors of topoisomerase II are important drugs used in the therapy of many neoplasms including breast cancer, lung cancer, testicular cancer, lymphomas and sarcomas. Similarly, cyclophosphamide (CYTOXAN®) is also a cytotoxic drug by cross-linking of strands of DNA and RNA with their alkyl group. So, it is known as an alkylating agent. It is also used in breast cancer, leukemia, lymphoma, Ewing's sarcoma, lung cancer etc. But, since cyclophosphamide is a prodrug which requires hepatic enzymes activation to form active metabolites, it does not work their cytotoxic action in vitro. In our paper, we identified the cytotoxic actions of both drugs in vitro by using the chemosensitivity assay. In this assay, we used MCF-7 breast cancer cell line. As a result, we found that the amounts of epirubicin are significance correlated with the percentage of cancer cell survivals. On the other hand, cyclophosphamide does not show proper effect on the breast cancer cell lines. In daily practice, these two drugs are widely used in many cancer therapies. Thus, we conclude that cyclophosphamide needs in vivo activation to express their oncolytic action otherwise epirubin does not need.

Introduction

Response of the various tumour cell lines (i. e. breast, colon, lung, prostate etc.) to the chemotherapeutic agents are evaluated by chemosensitivity assays. A chemosensitivity assay is a vitro laboratory test that can identify new compound or drug with anti-tumour properties. This type of study

demonstrates that tumour resistance was predicted with greater accuracy than sensitivity. Chemosensitivity assay may help in choosing the best drug or compound for the cancer being treated. Nowadays, there are many different types of chemo sensitivity assays to access the sensitivity have been developed. However, procedure of this assays are basically more or less the same. First of all, cancer cell line is extracted from the culture media and incubation of these cancer cells with tested drugs or compounds. Then, assessment of the cell survival and interpretation of the observation have been done. Therefore, generally for the chemo sensitivity to be done, the specific cell line to be assessed and cell cultures are required. There are a lot of cell lines. Firstly, primary cells which are explanted directly from a cancer patient cannot grow continuously in vitro and die eventually whereas the Secondary cells also from a donor. But their physical characteristic may change after they die. Another one is immortalized cells also known as transformed cells which can grow and divide in vitro when the appropriate conditions are maintained. Besides, eukaryotic cell lines are more difficult to culture compared to the prokaryotic cells.

In our study, the aim is to identify compounds (Epirubicin and cyclophosphamide) which have activity against a specific type of cancer (breast) have a novel mechanism of action. We use breast cancer cell line MCF-7 (Michigan cancer foundation-7). MCF-7 cells are oestrogen receptor positive control cell line and basic cell line of breast cancer. Moreover, we use MTT assay which is colorimetric assay, sensitive in vitro for measuring cell proliferation or apoptosis. MTT is added into the culture plate and incubated. This compound is reduced by mitochondrial reductase which is

present in active living cells to purple colour formazan crystals. Therefore, this reduction reaction is taken place only when reductase enzymes are active.

MTT Formazan

Acidified ethanol solution dissolves these crystals into colored solution. This solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. So, the reduction rate of tetrazolium is directly proportional to the rate of cell proliferation.

Epirubicin is an anthracycline antibiotic which is commonly used anti-neoplastic agent. Epirubicin is produced from Streptomyces Peucetius. It inhibits topoisomerase II which is essential for DNA replication, chromosome condensation and segregation. Epirubicin intercalate into DNA and forms a complex with DNA resulting in inhibition of DNA and RNA synthesis. It also generates reactive metabolites. So, these cytotoxic free radicals interact with many intracellular molecule and cell membranes. Thus, the biologic effects of the epirubicin may not be based solely on topoisomerase II activity.

Structure of Epirubicin

Epirubicin have some adverse effects. Among them, dose related myelosupression is the most common and even fatal. Also, it has cardio-toxic effect. Thus, it is contraindicated in severe cardiovascular diseases. But, side effects of epirubicin are relatively lesser than other anthracyclines. Besides, it has potential mutagenic and teratogenic effects.

Cyclophosphamide (prodrug) is an alkylating agent of the nitrogen mustard type. It is an inactive cyclic phosphamide ester of mechlorethamine. Inactive form transforms to an alkylating metabolites (Aldophosphamide). A small proportion of aldophosphamide is converted into phosphoramide mustard (active form) and acrolein by hepatic microsomal oxidation system (Cytochrome P450 enzymes) and some peripheral activation. Phosphoramide mustard alkylates or binds to DNA/RNA and then cross-linking of strands of DNA and RNA. Its action do not appear to be cell-cycle specific. Cyclophosphamide can prevent cell division.

It has some adverse effects especially haemorrhagic cystitis (up to 40%) which may be due to acrolein direct injury to the urothelium. Other significance side effects are myelosupression and secondary malignancies. Thus, cyclophosphamide is contraindicated in severe leucopoenia, thrombocytopenia, hepatic or renal dysfunction. It also has potential risk of congenital malformations when using in pregnancy.

Materials and Method

Materials

MCF – 7 (Michigan cancer foundation -7)

96 Well culture microtitre plates

RPMI 1640 medium (Roswell Park Memorial Institute)

Epirubicin

Cyclophosphamide

MTT assay proliferation kit

DMSO (Dimethyl Sulfoxide)

Universal Microplate Reader (Spectrophotometer)

Filtered Fume Enclosure

Method

MCF-7 breast cancer cell line is provided. First of all, the cap of MCF-7 cultured flask is opened and discarded the medium. 5ml of PBS (phosphate buffered saline) is added to wash this flask and allowed to mix then removed the PBS. 1-2ml of trypsin is added and keep the flasks on 1-2 minutes permitting to detach the cells. If required, tap periodically the flask from the bottom. After that, the whole solution is transferred to the 15ml falcon tube and then, centrifuged with 1200 rpm for 1-2 minutes. The supernatant is removed leaving behind the cell pellet. 180Î¼l of cancer cells (MCF-7) are put into the 96 wells microtitre plates from lane 2 to 12. After this, the microtitre plates are incubated at 37’C with 5% CO2, 95% air and humidity for 24 hours before any addition of the experimental drugs. 200Î¼l of RPMI 1640 containing 5% foetal bovine serum and 2mM L-glutamine is also added into the lane 1 and 20Î¼l to each well of lane 2 creating negative control and positive control respectively. 1µM of 20 µl of epirubicin is added to the lane 3 (row A to H) and 20µl (0. 5 µM) of epirubicin is also added to the lane 4. Similarly, from lane 5 to 12, decreasing concentration of the drug with same amount is placed (0. 25µM, 0. 125µM, 0. 0625µM, 0. 0312Î¼M, 0. 0156Î¼M, 0. 0078Î¼M, 0. 0039Î¼M and 0. 0019Î¼M respectively). After finishing, the plate is labelled and incubated at 37’C for 4 days. Repeating the same

procedure is done for cyclophosphamide. Overall procedure should be done under filtered Fume Enclosure to prevent contamination. Moreover, these two microtitre plates are stored wrapping with aluminium paper to avoid light because both drugs are photosensitive. After 4 days later, 20 µl of MTT (5mg/ml) is added to each well of the microtitre plates and cultured for 4 hours at 37’C for cleaving MTT. After 4 hours, all the solution (medium or drugs and MTT) within the plates is discarded carefully. Then, each well is filled with 150µl of DMSO to dissolve the crystals of formazan and mix the solution properly with stir bars. Finally, optical density (OD) of each well is read by spectrophotometer (Universal microplate reader) at 490nm wavelength.

Discussion

The respective tables and graphs show how to response the two drugs, Epirubicin and cyclophosphamide, on the MCF-7 breast cancer cell line. In graph 1, epirubicin killed the significant amount of the cancer cells at the concentration of 1 µM. In this concentration, only 12. 58% of the cancer cells are survived. Then, percentage of cell survival rates are gradually increased from lane 4 onwards while drug concentrations are also two fold diluted. Therefore, percentage of cell survival is inversely proportional to the epirubicin concentration. There is obviously seen that epirubicin has direct cytotxic effect in vitro. From graph 1, we assumed the IC50 (half maximum inhibitory concentration) which is about 0. 125Î¼M. IC50 is a measurement of effectiveness of compound or drug in inhibiting a biological or biochemical function. This quantitative measure indicates amount of a particular drug or other substance is needed to inhibit a given biological process by half.

Epirubicin is one of the compounds of anthracycline antibiotic and it can inhibit DNA topoisomerase II which is required for DNA break in replication. So, it effect on ‘ S’ phase of cell cycle. Moreover, anthracyclines can undergo one and two-electron reduction, since they are members of the quinone family, producing reactive compounds that damage macromolecules and lipid membranes. Epirubicin is mainly metabolized in the liver and excreted predominantly in the bile and then faeces. It can cross placenta and can be found in breast milk. Conclusively, we can assume that the cytotoxicity effect of epirubicin is still active in vitro chemosensitivity assay.

Next, according to the table 2, percentage of cell survival is not relevant with the concentration of the drug, cyclophosphamide. Apart from minor differences, there is no significant change in cell survivals. Similarly, in the graph of cyclophosphamide, the trend of cancer cell survivals is also swinging across the drug concentration but not correlated with cyclophosphamide concentration. Cyclophosphamide is the alkylating agent but it is prodrug. It is inactive in vitro. So, it needs to be activated with the action of hepatic and intracellular enzymes to form active one (4-hydroxycyclophosphamide, aldophosphamide, acrolein and phosphoramide mustard). Only active form can prevent the cell division by crossing-linking DNA (especially guanine base) and RNA strands with their alkyl group. So, it disrupts the DNA replication and subsequent steps. Cyclophosphomide is cell cycle non-specific drug. It is metabolized by liver enzymes (cytochrome P450) and excreted in bile. Therefore, main cytotoxic action of cytophosphamide is solely depended on the action of cytochrome P450 (CYP

450). Because of this reason, we can assume that cyclophosphamide has little or no cytotoxicity in vitro.

Chemo-sensitivity assays are intended to predict the sensitivity of in vitro cancer cell line to chemotherapeutic agents and identify more effective treatment protocols. So, the chemo-sensitivity assay may help in choosing the best drug or drugs for the cancer being treated. Despite there are many ways to analyse the effects of drugs on cell metabolism and cell morphology, chemosensitive assays can be utilized routinely in the clinical experiments. In our assay, MTT is used. It can measure cell proliferation, apoptosis and cell survivals. MTT compound is added into the MCF-7 cells cultured plates which is treated with anti cancer drugs (epirubicin or cyclophosphamide). These MTT is reduced by metabolically active cancer cells to insoluble purple formazan crystals. The rate of reduction reaction is proportional to cell survivals. So, we can calculate the optical density of formazan with spectrophotometer. Besides, for effective assays, proper handling and certain precautions are strictly essential. Otherwise, there will be errors in the assays and difficult to read the absorbance reading and the data interpretation will be inappropriate. Furthermore, proper experiment has to be done under aseptic condition as much as possible. Microtiter plates containing drugs and cell line need to store in the dark because most of the anti cancer drugs are photosensitive. Similarly, MTT should also be store at dark. It is also important to check the colour of the reagent before we use. However, there might be some problems and errors that likely to occur in the assays. If the MTT is in blue or green colour, it is likely to be contamination with the cellular or bacterial culture. If the absorbance readings are too low,

this might probably, the cells are not proliferations because of improper culture conditions, inadequate cell recovery time after plating or errors in the procedure.

In these days, there are many methods for anti cancer drug screening. The national cancer institute (NCI) use the COMPARE method in vitro. In this method, a probe or seed compound can be specified by using the NCI compound’s accession number (NSC number). And then compare algorithm is carried out to evaluate the entire database in the order of the similarity of the responses of the cell lines to the seed compound. By applying this algorithm, it is possible to assign mechanism of action to test or determine a compound or to determine that the response pattern is unique and not similar to that of any of the standard prototype compounds included in the NCI database.

DTP anti-cancer Screening Paradigm

- Test request
- Selection
- Cmp Submission
- Cellular Assays
- Data review
- (Drugability, novelty, reproducibility, potency, selectivity, mechanism)
- Acute Toxicity (Max. Tolerated Dose)
- Hollow Fiber Assay
- Data Review
- Xenograft Evaluation

Adopted from http://dtp. nci. nih.

gov/docs/misc/commonfiles/ACSPFlowChart. pdf

The screening is a two-stage process, starting with the evaluation of all compounds (single dose of 10Î¼M) against 60 different human tumour cell lines representing leukaemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate, and kidney. The outcome is then reported to analyze by the COMPARE program. Moreover, in vitro identification of potential anticancer agent is required to enhance with demonstration of in vivo animal models for screening the preclinical development. Then, the hollow fibre assay can be used for in vivo animal models. It is a current initial in vivo screening activity for the potential anticancer drugs with cytotoxicity in vitro screening. On the other hand, when potential anticancer drug screened in vitro needs to demonstrate for in vivo efficacy, xenograft models are used to test the efficacy. NCI currently research with developmental Therapeutics Program (DTP) Human Tumour Xenograft Models. The compounds are examined for anti tumour activity in human tumour xenograft model in nude mice or rodent tumour models after screening with NCI 60 cell lines and hollow fibre assay.

Conclusion

In conclusion, the anticancer drugs, epirubicin and cyclophosphamide sensitivity are assessed in vitro with MCF-7 breast cancer cell line by using MTT chemo sensitivity assay. Cytotoxic effect of these two drugs is different in vitro assay, although these two drugs play an important role in anticancer regime. As a result, epirubicin has significant cytotoxic effect on MCF-7 cell line in vitro whereas cyclophosphamide does not. Cyclophosphamide is

prodrug which needs to be activated by cytochrome P450 (hepatic enzymes) in vivo. So, the whole study suggests that their cytotoxic effects differ significantly in vitro. Anyway, the chemo-sensitivity assay is important and useful in vitro method for research to develop anti-cancer drugs, their efficacy and therapeutic effects.