Review of Evaluation Methods of Tumor Pharmacodynamics

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Abstract: Treating cancer has become one of the hottest issues in the world today. Accurately assessing the efficacy of the body in scientific research and clinical treatment is the key to treatment. The use of pharmacodynamic testing to judge is an efficient evaluation method which pharmacodynamics is a study of the biochemical and physiological effects of drugs. This article reviews the different antitumor activity tests in vitro and in vivo.

1. Introduction

The National Cancer Institute (NCI) defines cancer as a group of diseases which have the abnormal cells split and diffuse to nearby tissues. According to relevant data, one of every six people in the world die because of cancer, making it the second primary cause of death (rank only second to cardiovascular disease). In 2017, an estimated 9.6 million people died because of different cancers. Therefore, how to cure cancer has become one of the hottest problems in the present world. Nowadays, the treatment means include surgical resection, chemotherapy, and radiation therapy, all of which can’t do without the necessary key medications. Therefore, how to accurately evaluate the efficacy of the body in scientific research and clinical treatment is essential.

Pharmacodynamics (PD) is to study the biochemical and physiological effects of the medicine. These effects may include those demonstrated by animals (including humans), microorganisms, or organism combinations (such as infections). In addition, we have to know that pharmacodynamics studies how drugs affect organisms, and pharmacodynamics specifically emphasizes dose-response relationships and concentration-effect relationships of the drugs.

The important pharmacodynamic experimental models (including in vitro antitumor tests, in vivo antitumor tests) must be able to demonstrate the intended indications of the treatment and should be related with the therapeutic effect of human diseases. Specifically, in vitro tests study the direct effects of drugs on enzymes, receptors, cells, tissues, pathogens and so on, and provide a basis for designing in vivo test schemes. Our research emphasis—in vivo test(namely, the entire animal models) models are generally divided into two categories: rare spontaneous animal models and common induced or experimental animal models. The common in vivo pharmacodynamic test usually requires two animals, two or more methods, two approaches (at least one is the clinical administration approach), three agents, a blank control, a positive control, and a model control. In the end, in evaluating the anticancer activity of the drug, the correct conclusion is made with in vivo test results as the primary and in vitro test results as the reference. This paper briefly reviews the evaluation methods of in vivo pharmacodynamic, with an expectation of making a certain contribution to the research of anticancer drugs.
2. In Vitro Antitumor Activity Experiment

In vitro tests mainly study the direct effects of drugs on enzymes, receptors, cells, tissues, pathogens and so on, and provide a basis for designing in vivo test schemes, so as to screen the in vitro antitumor activity drugs.

2.1 Dye Exclusion Method

The test principle of the dye exclusion method is that living cells have the ability to repel certain dyes such as eosin trypan blue and nigrosine, but dead cells can be colored because the membrane integrity has been damaged[1]. Therefore, these dyes are added to cultured tumor cells, and the proportion of the killed cells can be calculated by counting the colored and uncolored cells after a certain period. Some of the key test steps we have to know are to add a certain amount of certain dye liquor to the cell suspension to be tested in a certain volume. 0.4% trypan blue solution is the most frequently used. It shall be mixed and dyed for 5-15 min in the room temperature. Afterwards, the dyed cell suspension would be dropwise added onto the blood cell counting plate, and then the number of living cells and cells will be counted under a light microscope. The observation index of the test results should be used to calculate the proportion of the living cells by (the number of uncolored cells /the number of total cells) X100%, and the proportion of the living cells in the control group should be above 90%. The 50% lethal dose (IC50) of the test sample shall be calculated based on the proportion of the living cells, which shall be used as an indicator of the antitumor activity of the drug.

2.2 Microculture Tetrozolium Test(MTT)

The test principle of MTT is MTT, 3-(4,5-dimethylbibiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, which is a kind of dye that can accept the hydrogen atom[2]. NADP-related dehydrogenases in living cell mitochondria can convert yellow MTT into insoluble blue-purple formazan in vivo, while dead cells have no such function. After dissolving formazan with DMSO, the optical density will be determined with the microplate reader under a certain wavelength, so as to quantitatively determine the living rate of cells. The essentials of the test are: we add the MTT solution in 5mg/ml for 20 ml/hole after the cell treatment of the test sample, continue to cultivate for four hours, and the add the trigeminy liquor and cultivate overnight. In the end, we will test the OD value on the microplate reader at the place of 570nm wavelength. The observation indicators of this method are as follows: directly measured OD value of each measured well in a 96-well plate, and the calculated corresponding cell growth inhibition rate according to the formula (OD value of the control group-OD value of the treatment value) / OD value of the control group × 100%, and the IC50 value of the 50% growth inhibition concentration, which is further calculated by the Logit method as practical condition.

2.3 Alamalan Method

The test principle of the Alamalan method is that the non-fluorescent blue substrate Alamalan can be reduced to a strongly fluorescent pink substrate by NADPH-related dehydrogenases in mitochondria in living cells and then a microplate reader is used to read the corresponding fluorescence intensity values at the excitation and emission wavelengths of 530nm and 590nm, respectively. The fluorescence intensity values at these different wavelengths have a good linear relationship with the number of living cells, thus helping us judge and evaluate the antitumor activity of the cells. The essential point of this method is that 10-20ul Alamalan dye solution is added after the cells are treated by the test samples, the solution shall be continued to be cultured for a period, and then a microplate reader is used to read the corresponding fluorescence intensity values at the excitation and emission wavelengths of 530nm and 590nm, respectively. In the end, the observation index we need is the cell growth inhibition rate calculated based on the fluorescence intensity. In addition, we can further calculate the IC50 value when appropriate.
2.4 Sulforhodamine Staining Method

The test for the sulforhodamine staining method is based on SRB [3], a protein-binding dye, pink and soluble in water. SRB can bind to basic amino acids in biological macromolecules. Its OD reading at 515nm wavelength has a good linear relationship with the number of cells, so it can be used to quantify the number of cells. One disadvantage of the MTT method is that the OD value can be changed with the storage time, while the SRB method does not have this phenomenon, thus being more suitable for large-scale tests. The essential steps of the method: we add 10% cold trichloroacetic acid into the 4℃ fixed cells which have been treated by the samples for 1 h, wash and dry, add 4mg/ml SRB liquid 100ml/hole to dye for 15min, wash with 1% glacial acetic acid and the dry, add Tris solution of 150ml/hole, and test the OD value at 515nm wavelength on th microplate reader. The observation indexes we need to know are the same as those of the MTT method. In addition, NCI currently uses the following indicators to evaluate the in vitro antitumor activity of compounds: the measured OD values include the OD values of the control group, the drug addition group, and the cells at the time of drug addition C, T, and T0, and then the dose-effect relationship curve of the sample concentration is compiled according to the growth inhibition rate and cell death rate, so as to calculate the following three data: GI50 (the sample concentration at a growth inhibition rate of 50%), TGI (the sample concentration at a growth inhibition rate of 100%); LC50 (the sample concentration at a cell death rate of 50%).

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\text{Growth inhibition rate (\%) = } \frac{(T-T_0)}{(C-T_0)} \times 100\%
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\text{Cell death rate (\%) = } \frac{(T-T_0)}{T_0} \times 100\%
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2.5 Colony Formation Method

The test principle of the colony formation method is based on cloning the continuous proliferation ability of the original cells. When a single cell divides for 6 or more generations, the population (colony) of its progeny contains more than 50 cells [4]. The cloned original cells can be conducted with the quantitative analysis through the colony counting. It reflects the proliferation potential of a single cell, so it can more sensitively determine the activity of anticancer drugs. As a result, it has been considered as an ideal detection method recently. The method steps that need to be known are that we need to seed 2 ml of the logarithmic growth phase cell suspension with a concentration of 500 cells / ml to a culture dish of 35 ml, add the appropriate amount of test sample, culture for 7 days or longer, carry out Giemsa stain and count colonies containing more than 50 cells under a dissecting microscope. The observation index is to calculate the colony formation rate based on the number of colonies, the colony formation rate of the control group should not be less than 40%, and then calculate the colony formation inhibition rate of the drug group, and can further adopt the Logit method to calculate the IC50 value of the tested sample according to the practical conditions.

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\text{Colony formation rate=colony number/number of cell inoculation X100\%}
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\text{Colony formation inhibition rate= (1-colony formation rate of the drug group) / colony formation rate of the control group X100\%}
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The in vitro screening method has both advantages and disadvantages. The advantages of in vitro screening methods lie in simple operation methods, few drug usage amounts and direct application of the materials on body tumors, and shorter required time in achieving results with these methods. The disadvantages of in vitro screening methods are mainly because cytotoxicity tends to be positive, and toxic substances are often positive, so the false positive rate is high, and drugs that do not directly affect tumor cells also show false negatives. In the end, in vitro cells cannot completely represent the tumorous malignant cells in the body, showing certain limitations.

3. In Vivo Antitumor Activity Test

The in vivo antitumor activity tests mainly include spontaneous tumors and transplanted tumors. The next paragraph will focus on the in vitro tumor activity tests mainly used in contemporary medical tests. Furthermore, we need some corresponding efficient monitoring technologies to explore the anti-tumor activity, such as the living body bioluminescence imaging technology and living animal fluorescence imaging technology.
3.1 Concrete Model of Transplanted Tumors

Transplanted tumors mainly include the following three models: subcutaneous tumor model, ascitic tumor model and in situ inoculation model [5]. The specific steps of the subcutaneous tumor model are that the experimenter selects tumor-bearing mice with strong tumor growth and no ulceration, kills it by dislocating the cervical vertebra, disinfecting the skin with iodine, ethyl alcohol or bromogeramine in the aseptic condition (extremely purified platform or inoculation cover), cut apart the skin, and peel off the tumor, cut the tumor organization into 1.5mm3, inoculate subcutaneously on one or both sides of the animal's armpit with a trocar; or make a cell suspension, and then add sterile physiological saline at a certain ratio. Generally, the number of tumor cells per mouse is (1-5) × 10^6. The ascitic tumor model requires the experimenter to sterilize the animal skin under aseptic conditions, absorb the ascites of the well-growing animals, dilute with physiological saline at a certain ratio and then inoculate the animal's abdominal cavity. The number of inoculated cells is generally (1-5) × 10^6. The last in situ inoculation model is to inoculate a tumor derived from an organ in an organ of an animal, such as inoculating human liver cancer into the liver of a nude mouse. In situ inoculation is not a routine method, but it has its advantages and is an encouraged method. There are mainly lung, liver, stomach, intestine, breast and intracranial inoculation methods [6-8].

When making transplanted animal models, malignant cells or tissues are directly implanted into the host animal, so they are not suitable for studying early events, such as tumorigenesis and formation [9]. When establishing and using the above three models, experimenters should pay attention to: transplanted tumors are generally established through transplanting the corresponding cell strains, so it is required to comprehend the chemosensitivity of cell lines and transplanted tumors; the transplantation tumors can be used for in vivo antitumor tests 2-3 generations after the tumor recovery; it is necessary to fully understand the growth of the model, especially the fast-growing model; the transplanted tumor should be handed for over 15-20 generations after recovery to maintain the biological characteristics and hereditary characteristics of the transplanted tumors.

3.2 Living Body Bioluminescence Imaging Technology

In the field of tumor research, the scientific research team of AntiCancer, Inc. took the lead to create and apply a visual tumor model system, use a live molecular fluorescence imaging system to observe the proliferation, movement, invasion, metastasis, mutual effect between host and tumor cells, occurrence of tumor vessels, as well as the dormancy, proliferation and dying of the tumor cells and a series of other biological behaviors, so as to conduct visual, qualitative and quantitative research of the biological process in the molecule or cell levels, thus providing more authentic data for the research of tumor biology and pharmacology. It mainly uses two methods to detect: living bioluminescence imaging technology and living animal fluorescence imaging technology.

The marking principle of in vivo bioluminescence imaging technology is the luminescence of mammalian organisms [10]. Generally, the Firefly luciferase gene (consisting of 554 amino acids, about 50KD), namely, the luciferase gene, is integrated into the chromosomal DNA of the cell to be observed to express luciferase, and culture the cell strains which can express the luciferase in a stable manner. During the cell division, metastasis, and differentiation processes, luciferase will also be continuously and stably expressed. All genes, cells and living animals can be marked with the luciferase gene. After inoculating the marked cells into experimental animals, if the outer source (peritoneal or intravenous injection) provides luciferin to the substrate, the luminescence can occur within minutes. In the presence of ATP and oxygen, this enzyme can catalyze the oxidation reaction of fluorescein to illuminate, so the phenomenon of luminescence occurs only in living cells, and the intensity of the luminous light is linearly related to the number of marked cells.

In addition to Firefly Luciferase, Renilla Luciferase can sometimes be used. The substrates of the two are different. The former has the substrate of D-luciferin while the latter has the substrate of coelentarizine. The two have different illumination wavelengths. The former emits light at a wavelength of 540 to 600 nm, and the latter emits light at a wavelength of about 460 to 540 nm. The light emitted by the former is easier to pass through tissues, and the latter is metabolized faster in the body than the
former, and its specificity is not as good as the former. Therefore, most in vivo experiments use Firefly Luciferase as the reporter gene. If double marks are required, the latter can also be used as an alternative scheme. The marking means that can be used here is that we can insert the gene of luciferase into the chromosome of the cell that is expected to be observed through molecular biology cloning technology, and screen with the monoclonal cell technology to cultivate the cell strains which can express the renilla stably. After injecting the marked cells into the mice, the substrate of renilla-luciferin will be injected before observation, which are micromolecules of about 280 Daltons. A single injection of fluorescein can keep the luminase-marked cells in mice glow for 30-45 minutes. In the end, we should use a highly sensitive refrigerated CCD camera and a specially designed imaging camera and imaging software to achieve long-term and accurate observation. Therefore, by establishing a spontaneous tumor model, long-term observation can be achieved, which is more convenient and accurate, and the mechanism of tumor occurrence can be observed.

3.3 Living Animal Fluorescence Imaging Technology

The optical principle of living animal fluorescence imaging technology is to excite the fluorophores to a high energy state through excitation light, and then emit light [11]. Similar to the penetration of bioluminescence in animals, the penetrability of red light is much better than that of blue-green light in small animals. As the illuminance signal increases in the depth in body, the light penetration ability is stronger as the wavelength approaches closer to 900nm. In the meanwhile, it can eliminate the noise interference, near infrared fluorescence is the best choice of observing the physical indexes. If the experimental condition allows, we usually try to choose the fluorescent protein or dye with longer emission wavelength. There are three main methods of marking in vivo fluorescence imaging. The first is fluorescent protein marking: fluorescent protein is suitable for marking cells, viruses, genes and so on, usually using GFP, EGFP, RFP (DsRed) or the like; the second is fluorescent dye marking: fluorescent dye marking is the same as the in vitro marking method, with Cy3, Cy5, Cy5.5 and Cy7 being frequently used, which can be used to mark small molecule drugs, antibodies, peptides and so on; the third type is quantumdote marking: QuantumDot is a new type of semiconductor nanocrystal that can emit fluorescence. It is an atom cluster consisting of hundreds to tens of thousands of atoms, with the size of below 10nm and appearance extremely similar to extremely small dots. Compared with traditional organic fluorescent reagents, the emission light of the quantumdot fluorescence is 20 times stronger and the stability is 100 times than that of the organic fluorochrome. It has many advantages such as narrow fluorescence emission spectrum, high quantum yield, difficult bleaching, wide excitation spectrum, adjustable color, high photochemical stability, and difficult decomposition. It is mainly used for real-time dynamic fluorescence observation and imaging of living cells, which can perform cell differentiation and lineage observation in several days, as well as in-situ real-time dynamic tracing of various interactions between cells, in cells and between organelles. Moreover, quantum dots can also be marked on other substances to be researched, such as drugs, specific biomolecules and so on, tracking their activities and functions.

4. Conclusion

Through these in vitro and in vivo tumor detection methods that we have discussed above, we can gradually evaluate the disease of tumor more accurately. This is also the key issue of efficacy in the present field of scientific research and clinical treatment. If the in vivo and in vitro detection methods are combined to build a comprehensive detection model, this model system will help researchers to more comprehensively and quickly judge the in vivo drug response and its possible mechanism of action, and it is expected to become the drug screening technology of the new generation.

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