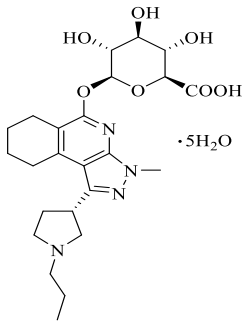
PHARMACEUTICAL COMBINATION COMPRISING TSL-1502M AND USE THEREOF

Technical Field

The invention belongs to the technical field of drug preparation, and specifically, relates to a pharmaceutical combination comprising an intermediate TSL-1502M for a PARP inhibitor TSL-1502 and the use thereof.

Background Art

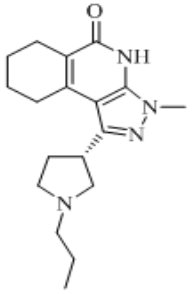
PARP inhibitor TSL-1502, a compound with a chemical name of (2S,3S,4S,5R,6S)-3,4,5-trihydroxy-6-(3-methyl-1-((S)-1-propylpyrrolidin-3-yl)-6,7,8,9-tetrahydro-3H-pyrazolo[3,4-c]isoquinolin-5-oxy)tetrahydro-2H-pyran-2-carboxylic acid (designated as TSL-1502), was first described as the compound in the paragraph 0177 in ZL201180002886.8 (Publication No. CN102510863A, Announcement No. CN102510863B, hereinafter the 2011 Patent), with the structural formula as Formula I.



Formula I

The above patent discloses in claim 16 the specific tumor types such as head carcinoma, thyroid carcinoma, neck cancer, eye cancer, skin cancer, oral cancer, throat cancer, esophagus cancer, breast cancer, bone cancer, leukemia, myeloma, lung cancer, colon cancer, carcinoma of sigmoid, rectal cancer, gastric cancer, prostate cancer, breast cancer, ovarian cancer, kidney cancer, liver cancer, pancreatic cancer, brain cancer, intestinal cancer, heart cancer, adrenal carcinoma, subcutaneous tissue cancer, lymph node cancer, malinant melanoma, malignant glioma and the like. However, in fact, only the treatment effects in melanoma and human breast cancer cell line were proven in the Example 19, while other tumors were not mentioned.

TSL-1502M is an intermediate for preparation of TSL-1502, as described in the patent application CN201910465780.X, with the title of invention as "AN INTERMEDIATE TSL-1502M OF A PARP INHIBITOR AND THE PREPARATION METHOD THEREOF", (application date of May 31, 2019, Publication No. CN112010850A), with the structural formula as Formula II,



Formula II.

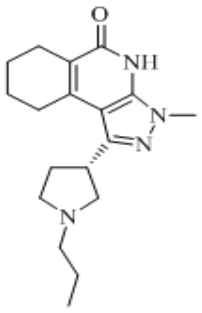
Formula II compound is designated as TSL-1502M, and it can be used as an intermediate to prepare TSL-1502, and can be used for the detection of TSL-1502, and is useful as a reference compound. However, the document did not disclose the medical use information for TSL-1502M and the information related to the combination with other anti-cancer drugs.

During the research on TSL-1502M, the inventors unexpectedly found that its inhibitory activity on PARP enzyme was higher than that of TSL-1502, suggesting its pharmaceutical use as a PARP inhibitor.

The combination application of TSL-1502M and other drug is not reported. The invention studied the combination application of TSL-1502M and other drugs and it is surprisingly found that the combination administration of TSL-1502M and temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan and topotecan has synergistic effect.

Summary of the invention

The invention provides a combinatory pharmaceutical combination comprising TSL-1502M and another anti-tumor drug, wherein the structure of TSL-1502M is as follows,



Formula II.

wherein the another anti-tumor drug is selected from a group consisting of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, and topotecan.

The invention encompasses a pharmaceutical preparation composition comprising the two active ingredients, TSL-1502M and another anti-tumor drug, or a combination of pharmaceutical preparations individually comprising TSL-1502M and the another anti-tumor drug, wherein the two preparations are packaged together.

The preparation method of the pharmaceutical combination according to the invention comprises a step of mixing TSL-1502M and the another anti-tumor drug.

Alternatively, the preparation method comprises formulating TSL-1502M and the another anti-tumor drug into individual pharmaceutical preparations, and packaging and combining the two preparation together, such that the two drugs can be administrated in combination when being use. In such combinatory packages, TSL-1502M can formulated into injectable preparation, and the another anti-tumor drug can be formulated into injectable preparation, and two unit doses of injectable preparation can be packaged into one packaging box, and can be injected separately or together when being used.

In the pharmaceutical combination comprising TSL-1502M and another anti-tumor drug according to the invention, the dose of each drug is in a pharmaceutically effective amount, wherein the "effective amount" refers to an amount in which each drug, alone or in combination, is effective to achieve clinical prevention or treatment of diseases. For example, the injection preparation is formulated in unit dose form, such as packaging into sterile vials of different colors, different specifications and sizes, each vial containing 0.1-2000mg of pharmaceutically active ingredient, packaging the vials,

and placing into a packaging box; and it can be further placed into a packaging box capable of containing 2 to 100 vials for convenient storage and transportation.

The pharmaceutical combination according to the invention comprises TSL-1502M and another anti-tumor drug, wherein the weight ratio of the two can be determined according to each effective amount, for example, 1:1000 to 1000:1.

For breast cancer, the weight ratio of TSL-1502M and temozolomide is 3-10nM: 10-30μM, preferably 3nM: 10-30μM.

For colorectal cancer or colon cancer, the weight ratio of TSL-1502M and 7-ethyl-10-hydroxycamptothecin or irinotecan is 3-10μM: 0.1-1nM, preferably 3-10μM: 0.1nM.

The preparation method of the pharmaceutical combination according to the invention comprises the following steps of: preparing the required amount of TSL-1502M and another anti-tumor drug together or respectively as pharmaceutical active ingredients into a pharmaceutical preparation combination which can be taken according to the conventional technology of pharmacy, wherein the dosage form comprises injectable preparation, preferably a dry powder injection preparation, and particularly preferably a lyophilized injectable preparation.

The injectable preparation of the invention can be prepared without adding excipients or adding one or more pharmacetical excipients such as glucose, lactose, mannitol, sodium chloride, hydroxypropyl-B-cyclodextrin and the like, and formulated into an injectable preparation by using a proper method.

The use method of the invention comprises the following steps of: preparing the required amounts of TSL-1502M and another anti-tumor drug together as two pharmaceutical active ingredients into an injectable preparation for injection. Alternatively, the required amounts of TSL-1502M and another antitumor drug can be separately prepared into injectable preparations for injection.

The invention further provides a pharmaceutical preparation combinationcomprising TSL-1502M, wherein the pharmaceutical preparation combination can be in any ingestible pharmaceutical form, such as tablet, sugar-coated tablet, film-coated tablet, enteric-coated tablet, capsule, hard capsule, soft capsule, oral liquid, buccal tablet, granule, electuary granule, pill, pulvis, ointment, pellet, suspension, powder, solution,

injectable preparation, suppository, soft ointment, hard ointment, cream, spray, drop, patch.

The pharmaceutical preparation combination of the invention is preferably in the form of a unit dose of the pharmaceutical preparation, and for example, when formulating into a pharmaceutical preparation, the unit dose of the pharmaceutical preparation can comprise 0.1-1000mg of TSL-1502M and another anti-tumor drug according to the invention, with the remainder being pharmaceutically acceptable excipients. The pharmaceutically acceptable excipient can be 0.01-99.99% by weight of the total weight of the preparation.

The pharmaceutical preparation combinationof the invention can determine the dosage regimen according to the condition of the patient when administered, such as 1-3 times daily, 1-20 tablets per time.

Preferably, the pharmaceutical preparation combination according to the invention can be oral preparation or injectable preparation. Among others, the oral preparation is one selected from a group consisting of capsule, tablet, dripping pill, granule, concentrated pill, and oral liquid. Among others, the injectable preparation is one selected from a group consisting of liquid, semi-solid, solid, and powder form, preferably injectable solution, and injectable powder.

Among the pharmaceutical preparation combination of the invention, the orally administered preparation can comprise excipients, such as binder, filler, diluent, tableting agent, lubricant, disintegrant, colorant, flavoring agent and wetting agent, and if necessary the tablet can be coated.

Suitable fillers include cellulose, mannitol, lactose and other similar fillers. Suitable disintegrants include starch, polyvinylpyrrolidinone and starch derivatives, for example, sodium starch glycolate. Suitable lubricants include, for example, magnesium stearate. Suitable pharmaceutically acceptable wetting agents include sodium lauryl sulfate.

The pharmaceutical preparation combinationof the invention can be formulated into solid oral composition by conventional methods such as mixing, filling and tableting. By repeating the mixing, the active ingredient can be distributed into those combination using a large amount of fillers.

The form of the oral liquid preparation can be for example aqueous or oily suspension, solution, emulsion, syrup or elixir, or can be a dry product that can be reformulated with water or other suitable carriers before use. Such liquid preparations can comprise conventional additives, such as suspending agent, for example, sorbitol, syrup, methylcellulose, gelatin, hydroxyethylcellulose, carboxymethylcellulose, aluminium stearate gel or edible hydrogenated oil, emulsifying agent, for example, lecithin, sorbitan monooleate or arabic gum; non-aqueous carrier (it can include edible oil), for example, almond oil, fractionated coconut oil, such as oily ester of the glycerol ester, propylene glycol or ethanol; preservative agent, for example, methylparaben or propylparaben or sorbic acid, and if necessary, can comprise conventional flavoring agent or colorant.

For an injectable preparation, a prepared liquid unit dosage form comprises the active ingredient of the invention and a sterile carrier. Depending on the carrier and the concentration, the compound can be suspended or dissolved. The preparation of the solution generally includes dissolving the active ingredient into a carrier, filtering and sterilizing before loading into a suitable vial or ampoule, and then sealing. The excipients, such as local anesthetic, preservative agent and buffer can also be dissolved into such a carrier. In order to improve its stability, such composition can be frozen after loading in a vial, and be subjected to the water removal in vaccum.

The present invention further provides the use of the combination of the present invention in the manufacturer of an anti-tumor drug in which the dosage of TSL-1502M and another anti-tumor drug may be from 0.01 mg to 2000 mg per day.

The application of the invention obtains unexpected technical effects, and application examples are shown in experimental examples of the invention.

The anti-tumor drug combination provided by the invention has the following characteristics:

1. The inventor has found in the test that TSL-1502M obviously enhances the proliferation inhibition effect of temozolomide on MDA-MB-436 cells (breast cancer cell line). The combined use of temozolomide induces a significant increase in the

phosphorylation of γ-H2AX in MDA-MB-436 cells and promotes the apoptotic process of tumor cells.

2. The combination of TSL-1502M and temozolomide inhibits tumor cells better than that of TSL-1502M or temozolomide alone. As shown in Table 3-1, the inhibition rate of TSL-1502M alone on MDA-MB-436 cells (breast cancer) at a concentration of 30nM is 67.5±1.5%, and that of TMZ alone at 30μM is 8.5±3.5%. The combination of the two could further increase the proliferation inhibition rate of MDA-MB-436 cells to 80.2±3.5%, and this synergistic effect was more significant at lower drug concentrations.

3. TSL-1502M significantly increased the inhibitory effect of irinotecan active metabolite SN38 on the proliferation of SW620 cells (colon cancer cell line).

4. The combination of TSL-1502M and irinotecan active metabolite SN38 shows better inhibition effect on tumor cells than single use of TSL-1502M or temozolomide. As shown in Table 3-2, TSL-1502M alone inhibited the proliferation of SW620 cells (colorectal cancer, and specifically colon cancer) by 37.2±17.9% at a concentration of 10μM, and SN38 (the active metabolite of irinotecan) alone by 62.3±18.1% at 1nM. The combination of the two further improved the proliferation inhibition rate of MDA-MB-436 cells to 98.3±1.3%, and this synergistic effect was more significant at lower drug concentrations.

Brief Description of The Drawings

Figure 1: The effect of TSL-1502 and TSL-1502M on cultured cell proliferation *in vitro*.

Figure 2: The effect of the combination therapy of TSL-1502, TSL-1502M and cytotoxic drugs on the proliferation inhibition in MDA-MB-436, SW620 cells.

Figure 3: The effect of TSL-1502M on the γ-H2AX phosphorylation in MDA-MB-436 cells.

Detailed Description of The Invention

Example 1

The invention provides a combinatory drug lyophilized injectable preparation, consisting of TSL-1502M and another anti-tumor drug temozolomide, 7-ethyl-10-

hydroxycamptothecin, irinotecan, topotecan.

Preparation Example:

TSL-1502M 20g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 10g, were respectively dissolved into a 1000ml aqueous solution containing 100g mannitol, and respectively filled into a 2g vial for 500 vials. Before capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, each one vial was taken to package into combination, such that the product was obtained.

Example 2

TSL-1502M 5g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 10g were dissolved together into a 1000ml aqueous solution containing 100g mannitol, mixed, and filled into a 2g vial for 500 vials. Before capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, they were packaged, such that the product was obtained.

or

TSL-1502M 5g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 20g were dissolved together into a 1000ml aqueous solution containing 100g mannitol, mixed, and filled into a 2g vial for 500 vials. Before capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, they were packaged, such that the product was obtained.

or

TSL-1502M 5g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 50g were dissolved together into a 1000ml aqueous solution containing 100g mannitol, mixed, and filled into a 2g vial for 500 vials. Before capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, they were packaged, such that the product was obtained.

or

TSL-1502M 5g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 100g were dissolved together into a 1000ml aqueous solution containing 100g mannitol, mixed, and filled into a 2g vial for 500 vials. Before capping,

they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, they were packaged, such that the product was obtained.

or

TSL-1502M 10g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 5g were dissolved together into a 1000ml aqueous solution containing 100g mannitol, mixed, and filled into a 2g vial for 500 vials. Before capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, they were packaged, such that the product was obtained.

or

TSL-1502M 20g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 5g were dissolved together into a 1000ml aqueous solution containing 100g mannitol, mixed, and filled into a 2g vial for 500 vials. Before capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, they were packaged, such that the product was obtained.

or

TSL-1502M 50g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 5g were dissolved together into a 1000ml aqueous solution containing 100g mannitol, mixed, and filled into a 2g vial for 500 vials. Before capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, they were packaged, such that the product was obtained.

or

TSL-1502M 100g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 5g were dissolved together into a 1000ml aqueous solution containing 100g mannitol, mixed, and filled into a 2g vial for 500 vials. Before capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, they were packaged, such that the product was obtained.

Example 3

TSL-1502M 2g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 1g, were respectively dissolved into a 1000ml aqueous solution containing 100g mannitol, and respectively filled into a 2g vial for 500 vials. Before

capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, each one vial was taken to package into combination, such that the product was obtained.

or

TSL-1502M 10g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 5g, were respectively dissolved into a 1000ml aqueous solution containing 100g mannitol, and respectively filled into a 2g vial for 500 vials. Before capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, each one vial was taken to package into combination, such that the product was obtained.

Example 4

TSL-1502M 5g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 2.5g, were respectively dissolved into a 1000ml aqueous solution containing 100g mannitol, 罐装到 2g 的 vial 中 for 500 vials. Before capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, each one vial was taken to package into combination, such that the product was obtained.

Example 5

TSL-1502M 5g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 5g, were respectively dissolved into a 1000ml aqueous solution containing 100g mannitol, and respectively filled into a 2g vial for 500 vials. Before capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, each one vial was taken to package into combination, such that the product was obtained.

Example 6

TSL-1502M 50g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 5g, were respectively dissolved into a 1000ml aqueous solution containing 100g mannitol, and respectively filled into a 2g vial for 500 vials. Before capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, each one vial was taken to package into combination, such that the product was obtained.

Example 7

TSL-1502M 5g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 50g, were respectively dissolved into a 1000ml aqueous solution containing 100g mannitol, and respectively filled into a 2g vial for 500 vials. Before capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, each one vial was taken to package into combination, such that the product was obtained.

Example 8

TSL-1502M 5g, and any one drug of temozolomide, 7-ethyl-10-hydroxycamptothecin, irinotecan, topotecan 10g, were respectively dissolved into a 1000ml aqueous solution containing 100g mannitol, and respectively filled into a 2g vial for 500 vials. Before capping, they were placed into a lyophilizer, and dried in vaccum for 24 hours. After capping, each one vial was taken to package into combination, such that the product was obtained.

Example 9: The preparation of TSL-1502M

The Example 1 in the application CN201910465780.X, with the title of invention as "AN INTERMEDIATE TSL-1502M OF A PARP INHIBITOR AND THE PREPARATION METHOD THEREOF" is referred.

Reaction: In the reaction vessel, the main reagent (*S*)-3-methyl-1-(pyrrolidin-3-yl)-3,4,6,7,8,9-hexahydro-5*H*-pyrazolo[3,4-*c*]isoquinolin-5-one (2*S*,3*S*)-2,3-di(benzoyloxy)succinate (630.0g, 1mol) and ethanol (3800mL) were added, stirred, and cooled to 10ºC. Sodium cyanoborohydride (125.7g, 2mol) was added. Then propanal (116.2g, 2mol) was added dropwise, wherein during the dropwise addition, the temperature was less than 20ºC. After completing the dropwise addition, the reaction was continuously stirred at 10ºC~25ºC for 1h~2h.

Quenching: After the reaction was completed, hydrochloric acid having a concentration by mass of 36 to 38% (73g, 2mol) was slowly added dropwise to the reaction mixture. The pH was adjusted between 4 and 5. The addition speed was controlled. The large amount of generated gas was absorbed by 20% sodium hydroxide in order to prevent overflowing, and the temperature was controlled at 20ºC to 30ºC.

Post-treatment: S1, After quenching, the reaction solution was concentrated under reduced pressure to evaporate ethanol, at a temperature less than 70ºC.

S2, To the residue of S1, water and dichloromethane (the weight ratio of water and dichloromethane was 1: 4) were added, with an amount of 7 times of the weight of the compound of Formula I. At a temperature of 5ºC~15ºC, the pH of the mixture was adjusted to 9 to 10 with 40% sodium hydroxide aqueous solution. The solution was stirred, stood, and seperated, and the organic phase was kept.

S3, The aqueous phase obtained in S2 was continuously extracted once with dichloromethane. The obtained organic phase and the organic phase obtained in S2 were combined, and the aqueous phase was kept.

S4, The combined organic phase obtained in S3 was extracted with 40% sodium hydroxide aqueous solution. The obtained aqueous phase and the aqueous phase obtained in S3 were combined, and the organic phase was kept.

S5, The combined aquesou phase obtained in S4 was adjusted with 36~38% hydrochloric acid to pH 4 to 5. Then dichloromethane was added, with an amount of dichloromethane of 5 times of the weight of the compound of Formula I. The solution was stirred, stood, and seperated, and the organic phase was kept.

S6, The aqueous phase obtained in S5 was continuously extracted twice with dichloromethane. The obtained organic phase and the organic phase obtained in S4 and S5 were combined.

S7, The combined organic phase obtained in S6 was washed twice with 3% sodium bicarbonate aqueous solution, and then dried with anhydrous sodium sulfate. The sodium sulfate solid was removed by filtering. The filtrate was concentrated under reduced pressure, and then dried under vacuum to yield the intermediate of a PARP inhibitor TSL-1502M 226g.

Experimental Example 1

I, Experimental Material

1, Tested drug

TSL-1502: white powder, Lot. No. 120301, purity 99.56%, water content 16.65%, sealed, preserved at 2-8ºC, prepared by the Applicant;

TSL-1502M: white powder, Lot. No. 20150801, purity 98.6%, sealed, preserved at 2-8ºC, prepared by the Applicant;

ABT-888 (Veliparib): white powder, Lot. No. HM-069-8-20101112, purity 98.12%, sealed, preserved at 2-8ºC, purchased from Shanghai Haoyuan. ABT-888is a novel potent PARP-1 and PARP-2 inhibitor, mainly used in breast cancer.

AZD2281 (Olaparib): white powder, Lot. No. 20131105, purity 99.15%, sealed, preserved at 2-8ºC, purchased from Shanghai Mode. AZD2281 is a PARP inhibitor, with approved indication including ovarian cancer, breast cancer, pancreatic cancer, prostate cancer;

Temozolomide(abbreviated as TMZ): white powder, Lot. No. 20150418, sealed, preserved at 2-8ºC. Temozolomide is an imidazole-tetrazine derivative, mainly used in glioblastoma multiforme.

SN-38 (active metabolite of irinotecan, 7-ethyl-10-hydroxycamptothecin. cas No. 86639-52-3): ligh yellow powder, purity 99.1%, Lot. No. E060206, sealed, preserved at 2-8ºC, purchased from Shanghai Junjie Biotechnology. Irinotecan is a semi-synthesized derivative of camptothecin, mainly used in colorectal cancer.

2, Formulation method of the drug

TSL-1502, TSL-1502M, AZD2281 and ABT-888 were formulated into 10 mM stock solution with DMSO, packaged and stored at -70ºC. TMZ was formulated into 200mM stock solution with DMSO, , packaged and stored at -70ºC.

3, Cell line selection criteria and cell line source

By referring to the pharmacological study on the FDA approved PARP inhibitors Olaparib (LYNPARZA), Rucaparib (RUBRACA) and Niraparib (ZEJULA), the BRCA1/2 mutated-type and wild-type cells were selected to evaluate the in vitro anti-tuomr activity of TSL-1502 and its metabolites. Cell source and culture condition are shown in Table 1.

Table 1: Cell source and Type

Cell Line	Source	Tissue Source and Type	BRCA Genotype	Culture Condition
V-C8	Delivered abroad	Chinese Hamster Ovarian cell	BRCA2 defective	10% FBS containing F-10

		derived line		
V-C8 #13-5	Delivered abroad	Chinese Hamster Ovarian cell derived line	Normal	10% FBS containing F-10
MDA-MB-436	cell library	human breast cancer cell	BRCA1 mutated	10%FBS and 10 μg/ml insulin containing L-15
Capan-1	ATCC	humanpancreatic cancer cell	BRCA2 mutated	20%FBS containing IMDM
HCC1937	ATCC	human breast cancer cell	BRCA1 mutated	10% FBS containing RPMI1640
MDA-MB-468	Cell library	human breast cancer cell	Normal	10% FBS containing L-15
UWB1.289	ATCC	human ovarian cancer cell	BRCA1-null	3% FBS containing RPMI1640/IMDM
UWB1.289 BRCA1	ATCC	human ovarian cancer cell	BRCA1-expressed	3% FBS containing RPMI1640/IMDM
SW620	ATCC	human colon cancer cell	Normal	10% FBS containing L-15

4, Reagents and Instruments

RPMI1640, L-15, F-10, DMEM and IMDM were purchased from Gibco BRL. FBS was purchased from Gibco BRL. SRB was purchased from Sigma. Anti-Rad51 primary antibody was purchased from Santa Cruz. Anti-P-γ-H2AX and PARP primary antibody were purchased from Cell Signaling Technology. Anti-β-Tubulin primary antibody was purchased from Sigma. horserdish peroxidase-labeled goat anti-rabbit and goat anti-mouse secondary antibody were purchased from Calbiochem. Alexa Fluor® 488 goat anti-rabbit fluoresence secondary antibody was purchased from Molecular Probes. Immobilon Western HRP Substrate luminal reagent was purchased from Millipore. Other conventional chemical reagents were domestic analytical pure (AR) reagents.

Multifunctional microplate reader Synergy H4 was purchased from BioTek. carbon dioxide incubator (Model 3111) was purchased from Thermo. Inverted microscope XDS-1B was purchased from Chongqing Optoelectronic Instrument Co., Ltd. Flow cytometer (FACS Calibur flow cytometer) was purchased from Beckman Dickson. Laser confocal microscope was purchased from Olympus. Western blot imager was purchased from GlinxScience Instruments.

II, Experimental Method

1, Sulfonyl Rhodamine B protein staining method(SRB method)

A certain number of cells in logarithmic phase were inoculated in 96-well plates. After 24 hours of adherent growth, drugs with different concentrations (1, 3, 10, 30, 100, 300, 1000, 3000, 10000 nM) were added. After 10 days of drug action, the cells were fixed with trichloroacetic acid, and then stained with SRB solution. Finally, Tris solution was added to dissolve SRB, and the OD value was measured by a microplate reader at the wavelength of 510nm. The cell growth inhibition rate was calculated according to the following formula.

Inhibition Rate = (OD Value$_{\text{Control Well}}$ − OD Value$_{\text{Drug Well}}$) / OD Value$_{\text{Control Well}}$×100%

The IC_{50} of the half inhibitory concentration was calculated based on the inhibition rate at each concentration.

2, Western Blot Method

Detection of γ-H2AX phosphorylation

MDA-MB-436 was inoculated into a 6-well plate (2×10^5/ml) and treated with different concentrations of TSL-1502M or AZD2281(100, 1000, and 10000 nM) for 1.5 h, followed by 1mM TMZ for 0.5 h. Then, 1×SDS gel loading buffer (50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 10% glycerol, and 0.1% bromophenol blue) were added to lyse the cells. The cell lysate was heated and denatured in a boiling water bath, and was subjected to SDS-PAGE electrophoresis. After the electrophoresis was completed, the protein was transferred to PVDF membrane by a wet transfer system. The PVDF membrane was placed in a blocking solution (5% defatted milk powder diluted in TBS/T) for blocking at room temperature, and was subjected to I and II anti-reaction. After the membrane was washed, the color was developed using the Immunologic Western HRP Substrate Luminal Reagent and photographed using the Western blot Imager.

III, Experimental Results

1, TSL-1502, TSL-1502M inhibit the proliferation of various in vitro cultured tumor cells

TSL-1502, AZD2281, and ABT-888 were tested for cell proliferation inhibition after 240 hours of exposure (replacement of fresh drug every 120 hours). The results are

shown in Table 2 and Figure 1.

In BRCA-deficient or mutated cells (V-C8, MDA-MB-436, Capan-1 and UWB1.289), the proliferation inhibition effect of TSL-1502M was significant, and the IC_{50} were, respectively, 0.2±0.0nM, 0.9±0.2nM, 100.6±73.8 nM, 1.9±0.4nM). The inhibition effect was significantly stronger than its prodrug TSL-1502 (IC_{50} were, respectively, 132.1±4.7nM, 79.6±12.5nM, ≈10000 nM, 935.7±526.6nM), and the reference drugs AZD2281 (IC_{50} were, respectively, 18.6±1.3 nM, 4.0±0.7nM, 586.1±89.4 nM, ≈1nM (The inhibition rate was slightly weaker than TSL-1502M)) and ABT-888 (IC_{50} were, respectively, 314.2±24.4 nM, 114.7±60.8nM, NA, 2102.5±1470.1nM) ;

However, in BRCA1 mutated HCC1937 cells, the proliferation inhibitions of TSL-1502M, TSL-1502, AZD2281 and ABT-888 were all weak.

In BRCA-normal cells (V-C8#13-5 and UWB1.289 BRCA1), the proliferation inhibition effects of TSL-1502M, TSL-1502, AZD2281 and ABT-888 were all weak. However, in MDA-MB-468 cells with normal BRCA function, TSL-1502M and AZD2281 showed proliferation inhibition effect to some extent (IC_{50} were, respectively, 73.6±19.5 nM and 541.1±93.8 nM) .

In summary, TSL-1502M is more sensitive to the cells with BRCA1/2 function defects, and shows selectivity on the proliferation inhibition effect in the cells. The function features on selectivity are similar to AZD2281 and ABT-888, and the inhibition effect is stronger than TSL-1502, AZD2281 and ABT-888.

Table 2: The effect on the proliferation of in vitro cultrated cell (n=2)

Cell Line	IC50 (nM, Mean ± SD)			
	TSL-1502	TSL-1502M	AZD2281	ABT-888
V-C8	132.1±4.7	0.2±0.0	18.6±1.3	314.2±24.4
V-C8#13-5	>10000	>10000	≈10000	>10000
Capan-1	≈10000	100.6±73.8	586.1±89.4	NA
HCC1937	>10000	>10000	≈10000	>10000
MDA-MB-468	>10000	73.6±19.5	541.1±93.8	NA
UWB1.289	935.7±526.6	1.9±0.4	≈1	2102.5±1470.1
UWB1.289 BRCA1	>10000	2110.0±865.5	2917.5±79.9	>10000
MAD-MB-436	79.6±12.5	0.9±0.2	4.0±0.7	114.7±60.8

2, TSL-1502, TSL-1502M enhances cytotoxic drug anti-tumor activity

The anti-tumor effect of the combination of TSL-1502 and TSL-1502M and cytotoxic

drug were detected. The results are shown in Tables 3-1, 3-2, 3-3, 3-4 and Figure 2.

2.1 The proliferation inhibition effect of TSL-1502 and TSL-1502M on MDA-MB-436 cells: see Table 3-1

Table 3-1: The proliferation inhibition effect on MDA-MB-436 cells

Inhibition Rate (%, Mean±SD)

		AZD2281			
		0 nM	10 nM	30 nM	100 nM
TMZ	0 μM	0	25.0±3.6	36.4±4.1	47.0±5.2
	10μM	2.7±2.5	42.0±0.4	56.8±1.6	61.5±2.2
	30μM	9.1±4.8	59.8±2.9	69.6±3.5	71.7±1.4
		ABT888			
		0 nM	30 nM	100 nM	300 nM
TMZ	0 μM	0	4.9±0.8	18.9±6.6	23±3.2
	10μM	0.9±2.2	18.1±1.4	30.1±0.7	40±0.5
	30μM	6.2±3.5	38.2±1.5	50.5±0.3	60±2.6
		TSL-1502			
		0 nM	30 nM	100 nM	300 nM
TMZ	0 μM	0	1.3±2.1	15.6±3.3	30.2±1
	10μM	3.8±0.8	7±4.2	24.6±2.7	48.1±2.8
	30μM	11.2±2.4	22.7±2.1	42.3±3	63.4±3
		TSL-1502M			
		0 nM	3 nM	10 nM	30 nM
TMZ	0 μM	0	28.6±0.1	61.9±1.3	67.5±1.5
	10μM	-0.9±0.8	45.2±1.3	69.3±0.6	74.1±1.7
	30μM	8.5±3.5	63.8±3.3	75.6±1.1	80.2±3.5

Table 3-2 Calculation of Q-value of TSL-1502M and TMZ combined action by King's formula

C_{1502M}(nM)	C_{TMZ}(μM)	Q	Synergistic Effect
3	10	1.62	Yes
10	10	1.13	No
30	10	1.10	No
3	30	1.84	Yes
10	30	1.16	Yes
30	30	1.14	No

In BRCA1 mutated MDA-MB-436 cells, TSL-1502, TSL-1502M and the reference compound AZD2281, ABT-888 could all significantly enhance the proliferation inhibition effect of TMZ on MDA-MB-436 cells. TMZ (≤30μM) alone had almost no inhibition effect on the proliferation of MDA-MB-436 cells, while the combination of

10μM TMZ and 3 nM TSL-1502M showed a Q Value of 1.62, having synergistic effect; and the combination of 30μM TMZ and 3nM or 10nM TSL-1502M showed Q Values of 1.84 and 1.16 respectively, having synergistic effect. That is, in the tumor cells or tumor tissues, the drug concentrations of TSL-1502M and TMZ respectively in the ranges of 3-10nM and 10-30μM showed synergistic effect on the tumor inhibition, with a preferred combination of TSL-1502M 3nM and TMZ 10-30μM, and more preferred combination of TSL-1502M 3nM and TMZ 30μM.

2.2: The proliferation inhibition effect of TSL-1502 and TSL-1502M on SW620 cells. See Table 3-3

Table 3-3: The proliferation inhibition effect on SW620 cells

Inhibition Rate (%, Mean±SD)

		AZD2281		
		0μM	3μM	10μM
SN38	0 nM	0	15.4±23.0	70.2±18.2
	0.1nM	1.0±11.2	32.2±27.9	94.5±5.0
	1nM	73.5±8.0	92.0±9.3	97.1±1.4
		TSL-1502M		
		0μM	3μM	10μM
SN38	0 nM	0	18.8±13.4	37.2±17.9
	0.1nM	0.0±4	37.7±16.6	56.3±12.7
	1nM	62.3±18.1	98.0±0.7	98.3±1.3

Table 3-4 Calculation of Q-value of TSL-1502M and SN38 combined action by King's formula

C_{1502M}(μM)	C_{SN38}(nM)	Q	Synergistic effect
3	1	1.41	Yes
10	1	1.29	Yes
3	0.1	2.01	Yes
10	0.1	1.51	Yes

In BRCA-normal SW620 cells, TSL-1502M and the reference compound AZD2281 can all significantly enhance the proliferation inhibition effect of TMZ on SW620 cells. SN38 (≤1nM) or TSL-1502M (≤10μM) alone showed inhibition rates on SW620 cell up to 62.3±18.1, 37.2±17.9, respectively. When the two are combined, the inhibition rates could be enhanced to 98.3±1.3%. The combination of TSL-1502M (3μM~10μM) and SN38 (0.1nM~1nM) showed Q Values of more than 1.15, having synergistic effect. These results indicate a synergistic effect of TSL-1502M on cytotoxic drugs in both

BRCA mutated and normal tumor cells.

3, The combination of TSL-1502M and TMZ significantly enhances the phosphorylation of MDA-MB-436 cell γ-H2AX

Phosphorylated histone H2AX(γ-H2AX) is a marker of DNA damage. We detected the effect of TSL-1502M combined with TMZ on DNA damage repair in BRCA1 mutant MDA-MB-436 cells. The effects of TMZ combined with TSL-1502M and the reference control compound AZD2281 on γ-H2AX are presented in Figure 3, wherein the combination is shown in the circles, and indicates the follows.

After the treatment of the combination of TSL-1502M or reference compound AZD2281 and TMZ, the phosphorylation of γ-H2AX was significantly enhanced.

The above results indicates that, TSL-1502M and AZD2281 can inhibit DNA damage repair, and the activity of TSL-1502M is stronger than AZD2281.

Experimental Conclusion:

TSL-1502M significantly enhanced the proliferation inhibition effect of temozolomide on MDA-MB-436 cells (BRCA1 mutation) and irinotecan active metabolite SN38 on SW620 cells (BRCA1/2 normal). The combination with temozolomide induced a significant increase in the phosphorylation of γ-H2AX in MDA-MB-436 cells, suggesting that the synergistic effect was achieved by promoting cell apoptosis of tumor cells. The action characteristic of TSL-1502M is similar to that of the reference compound AZD2281, and the activity of TSL-1502M is stronger than that of AZD2281.