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Potentiation of anti-cancer antibody efficacy by anti-neoplastic drugs: detection of antibody-drug synergism using the combination index equation.

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September 14, 2018

Alisha DSouza, Ph.D.
Senior Review Editor
JoVE

Manuscript # JoVE58291 "An effective method to investigate the synergism between anti-cancer antibodies and chemotherapeutic drugs in vitro and in mice."
Authors: Bahri et al.

Dear Dr. Alisha Dsouza,

Thank you for your letter dated 13 August 2018. We were please to know that that our manuscript was rated as potentially acceptable for publication in JoVE, subject to adequate revision and response to the comments raised by the reviewers, the editorial and the production managers.

We have revised the manuscript and the video based on the comments made by the reviewers. Revisions are indicated in red in the revised manuscript.

Based on the instructions provided in your letter, we uploaded the revised manuscript and the revised high-resolution Video on the journal's website.

Appended to this letter is our point-by-point response to the comments raised by the reviewers.

We also disclose the following potential conflict of interest with the subject matter discussed in the manuscript:

- SF, JF, and SB are named as inventor on patent applications describing ganglioside OAcGD2 as therapeutic target.

I hope that the revised manuscript is acceptable for publication in JoVE.

Sincerely yours,

Stéphane Birklé

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TITLE:

Potentiation of Anticancer Antibody Efficacy by Antineoplastic Drugs: Detection of Antibody-drug Synergism Using the Combination Index Equation

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KEYWORDS:

Cancer research; drug development; antibody-drug combination; antibody-drug interaction; MTT assay; antibody-drug synergy; combination index equation; *in vitro* cell line model; tumor xenograft model

SUMMARY:

This protocol describes how to assess synergism between an anticancer antibody and antineoplastic drugs in preclinical models by using the combination index equation of Chou and Talalay.

ABSTRACT:

Potentiation of hostile monoclonal antibodies (mAb) by chemotherapeutic agents constitutes a valuable strategy for designing effective and safer therapy against cancer. Here we provide a protocol to identify a rational combination at the preclinical step. First, we describe a cell-based assay to assess the synergism between anticancer mAb and cytotoxic drugs, that uses the

combination index equation of Chou and Talalay[1]. This includes the measurement of tumor cell drug- and antibody-sensitivity using an MTT assay, followed by an automated computer analysis to calculate the combination index (CI) values. CI values of <1 indicate synergism between tested mAbs and cytotoxic agents[1]. To corroborate the *in vitro* findings *in vivo*, we further describe a method to assess the combination regimen efficacy in a xenograft tumor model. In this model, the combined regimen significantly delays tumor growth, which results in a significant extended survival in comparison to single-agent controls. Importantly, the *in vivo* experimentation reveals that the combination regimen is well tolerated. This protocol allows the effective evaluation of anticancer drug combinations in preclinical models and the identification of rational combination to evaluate in clinical trials.

INTRODUCTION:

The conventional approach in the treatment of a large number of different types of cancer was based on monotherapy. Even if it is still used in many cases, this method met several obstacles leading to opting for combined therapies[2]. Particularly, cancer cells are more susceptible to develop resistance when treated with a single drug by inducing alternative survival mechanisms[3], resulting in therapeutic failure in patients[4]. Moreover, in monotherapy, drugs are usually administrated at a high dose. This situation often results in the occurrence of strong dose-dependent side effects that can be intolerable and force physicians to stop the treatment[2]. For these reasons, the association of anticancer molecules is now preferred to monotherapy.

Ideal drug combinations would be those that act in synergy against tumor cells, without increased toxicity against normal cells. Synergism refers to the interaction of two or more drugs that produces a therapeutic effect greater than the sum of each individual drug acting separately. Such interactions may result in enhanced clinical therapeutic efficacy[2]. It limits treatment resistance, increases efficacy, and can also reduce toxicity[2]. In fact, the dosage of each drug can be reduced to lower their side effects by targeting different pathways. In addition, one of the molecules can also serve as a sensitizing agent against cancer cells. The effect of the second drug may be enhanced on sensitized cells and fewer dosages can be used[5].

Combined therapy can include two or more chemotherapeutic drugs and/or biologics, such as monoclonal antibodies[6]. These mAbs specifically target cells expressing a cell surface antigen of interest and are able to kill tumor cells through immunological pathways including antibody-dependent cell-mediated cytotoxicity (ADCC), with the involvement of immune effector cells[7], and complement-dependent cytotoxicity (CDC)[6]. They can also act *via* a nonimmunological mechanism mediated by apoptosis[8-11]. In this case, the induction of the process of programmed cell death may sensitize cancer cells, weaken their function, and make the associated chemotherapeutic drug more effective at a lower dosage. As such, proapoptotic mAb are good candidates for designing combination regimens with antineoplastic drugs.

Different mathematical models have been described to assess drug synergism; one of them is based on the combination index method[1]. This method is based on the median-effect principle developed by Chou[1]. The median-effect equation correlates the drug dose and drug effect as follows.

$$D = Dm[Fa/(I - Fa)]^{1/m}$$

Here, *D* is the dose drug; *Dm* is the median-effect dose; *Fa* is the fraction affected by the dose; *m* is an exponent that signifies the shape of the dose-effect plot[1]. The median-effect dose is used to calculate the dose *Dx* of a drug that inhibits or kills "*x*" percent of cells. The CI value is then calculated to assess the additive effect of the drug combination, as follows[1].

$$CI = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2}$$

A CI value of 1 indicates an additive effect and a CI value of <1 indicates a synergistic effect, while a CI value of >1 indicates antagonism[1]. The application of this method is further facilitated by the availability of a computer program, CompuSyn, that determines synergism and antagonism at all doses or effect levels simulated automatically[12].

Our group has developed the mAb 8B6 specific for O-acetyl-GD2 ganglioside (OAcGD2) neuroblastoma antigen[13] and further demonstrated that this mAb is able to induce cell death with attributes of apoptosis[11]. To test whether mAb 8B6 can sensitize neuroblastoma cells to the antineoplastic agent topotecan, we adapted the above-mentioned method developed by Chou[1]. First, we determine the effective dose 50 (ED_{50}) values of mAb 8B6 and topotecan. Next, the neuroblastoma cells with equipotent ratios of the two compounds based on ED_{50} values are exposed to determine the CI values using the above-mentioned simulation software. This method allows us to demonstrate synergism between mAb 8B6 and topotecan *in vitro*. Next, we describe a protocol to further assess the potency and the safety of this combination regimen *in vivo*. This protocol can be easily applied to select potent and safe anticancer mAb and chemotherapeutic agent combinations in preclinical studies. A schematic representation of this study is provided in **Figure 1**.

PROTOCOL:

Animal housing and experimental procedure were approved by the French Government (agreements #C44-278 and #APAFIS 03479.01). Animal care and procedures were conducted under directive EU 2010/63/EU and French Law #2013-118 on the protection of animals used for scientific purposes.

1. Evaluation of the Drug Interaction Between mAb 8B6 and Topotecan *In Vitro*

1.1. 96-well sample preparation

CAUTION: Consult the institution's Health and Safety committee and follow local regulation rules related to laboratory safety. Review the Material and Safety Data Sheet information before working with any media, cell line, or reagents. Use proper sterile technique and work in a laminar flow hood. All solutions/equipment that are used to manipulate cells must be sterile.

NOTE: The following protocol was designed for use with adherent cells. Modifications are required to apply the method to nonadherent cells growing in suspension; this protocol uses quadruplicate for each experimental condition.

1.1.1. Grow IMR5 cells in a T75 flask.

1.1.2. On the first day (day 0), observe the cell culture under a microscope to check the cell confluency. Aspirate the cell medium from the flask, wash it with 5 mL of phosphate-buffered saline (PBS), and add 3 mL of 0.05% ethylenediaminetetraacetic acid (EDTA)/PBS solution. Return the flask to the incubator for 3 min (37 °C, 5% CO_2).

1.1.3. Examine the cell culture under a microscope for cell detachment.

NOTE: If necessary, return the flask to the incubator for an additional 3 to 5 min, depending on tumor cell type.

1.1.4. Add 10 mL of complete cell medium to the flask and transfer the cell suspension to a sterile 15 mL conical tube. Centrifuge the cells for 5 min at 300 x *g*. Count the cells using a hemocytometer.

1.1.5. Remove and discard the supernatant. Resuspend the cell pellet in complete growth medium. Adjust the medium volume to obtain a final concentration of 1 x 10^5 cells/mL.

1.1.6. Seed 84 wells of a 96-well culture plate with 10^4 cells each, which is 100 µL of cell suspension. Follow the experimental layout shown in **Figure 2**.

1.1.7. Incubate the cells for 18 h in the cell incubator (37 °C, 5% CO_2).

1.2. Drug solution preparation

NOTE: For drug/mAb sensitization studies, modify the timing, the length, and the concentration treatment to suit the particular drug/mAb in question. Note that the initial concentration is 3x the final concentration.

1.2.1. The next morning (day 1), prepare the following drug solutions using complete growth medium.

1.2.1.1. mAb solution preparation

1.2.1.1.1. Dilute mAb in 500 µL of complete growth medium to obtain an antibody working solution with an mAb concentration of 240 µg/mL.

1.2.1.1.2. Perform five two-fold serial dilutions as indicated in **Figure 2**.

1.2.1.2. Topotecan solution preparation

1.2.1.2.1. Dilute, as above, the drug in 500 µL of complete growth medium to obtain a drug working solution with a final concentration of 120 nM.

1.2.1.2.2. Perform five two-fold serial dilutions as indicated in **Figure 2**.

1.2.1.3. Antibody and drug solution preparation

1.2.1.3.1. Dilute the drug and mAb solutions in 500 µL of complete growth medium to obtain a solution at 120 nM drug and 240 µg/mL mAb (working solution).

1.2.1.3.2. Perform five two-fold serial dilutions as indicated in **Figure 2**.

1.2.2. To arrive at the final concentration, transfer 50 µL of each drug solution into the corresponding wells, as indicated in the experimental layout (**Figure 2**).

NOTE: Transfer 50 µL of complete growth medium into the untreated cell wells, as indicated in **Figure 2**.

1.2.3. Incubate the cells for 72 h in the incubator (37 °C, 5% CO_2).

1.3. MTT assay

1.3.1. Add 10 µL of MTT reagent solution into each well.

1.3.2. Incubate at 37 °C for 4 h.

1.3.3. Add 100 µL of lysis solution (10% SDS in 0.01 M HCl) into each well, using a multichannel pipette, and mix thoroughly by pipetting.

1.3.4. Incubate at 37 °C for 4 h in a humidified chamber (95% humidity).

1.3.5. Read the absorbance at 570 nm (A_{570}) and 620 nm (A_{620}) using a spectrophotometer.

NOTE: Mix each sample again by pipetting before reading the absorbance; absorbance at 620 nm allows the correction of nonspecific background values.

1.3.6. Calculate the corrected absorbance: corrected absorbance = $A_{570} - A_{620}$.

1.3.7. Calculate the cell viability as follows: cell viability = 100 x (sample mean corrected absorbance / control mean corrected absorbance).

1.3.8. Calculate the fraction-affected values (Fa) using the following equation: 1 - (sample mean corrected absorbance / control mean corrected absorbance).

1.4. Drug interaction analytical simulation software for single and drug combination studies

1.4.1. Run the simulation software to open the start window.

1.4.2. Click on the **New Experiment** button to open the **Main** window.

1.4.3. Type the name of the experiment in the **Name** window.

NOTE: A date can be added in the **Date** window.

1.4.4. Click on the **New Single Drug** button.

1.4.5. Type the name in the **Full Name** window.

1.4.6. Type the abbreviation in the **Abbrev** window.

1.4.7. Type the drug concentration unit in the **Units** window.

1.4.8. Enter **Data Point 1 Dose** and **Fa value**, press **Enter**.

1.4.9. Repeat this step until all Data Points are entered.

1.4.10. Click on the **Finished** button.

1.4.11. Follow the same steps to enter mAb Data Points.

NOTE: Use the same concentration unit as is used by Drug.

1.4.12. Click on the **New Drug Combo** button.

1.4.13. Select **Drug** and **mAb**.

1.4.14. Select **Constant Ratio** and click on **OK**.

1.4.15. Type the name in the **Full Name** window.

1.4.16. Type the abbreviation in the **Abbrev** window.

1.4.17. Type the drug/mAb ratio in the **Ratio of** window.

1.4.18. Enter **Data Point 1 Dose** and press **Enter**.

NOTE: The program will automatically calculate the doses of mAb and Combo.

1.4.19. Enter the **Data Point 1 Fa** value and press **Enter**.

1.4.20. Repeat this step until all Data Points are entered.

1.4.21. Click on the **Finished** button and, then, click on the **Generate Report** button.

1.4.22. Select drug and mAb and, then, click **OK**.

1.4.23. Select **Combo** and, then, click **OK**.

1.4.24. Select **Header**, **CI table**, and **Summary table**. Then, click **OK**.

1.4.25. Type the file name of the analysis file and click **SAVE** to generate the report.

NOTE: After clicking **OK**, the report will automatically open in the computer's default web browser.

1.4.26. To print the report, choose **Print** from the web browser's file menu. The report contains a Summary Table Section that includes title, date, file name, description note, parameters (m, Dm, and r), ED_{50} for either agent used in monotherapy or in combination, and the CI table for each combination at ED_{50}, ED_{75}, ED_{90}, and ED_{95}.

NOTE: A CI value of <1 indicates synergism, a CI value of =1 indicates additivity, and a CI value of >1 indicates antagonism.

2. Generation of Human Neuroblastoma Xenografts in Nonobese Diabetic NOD Scid Gamma Mice (NSG Mice)

NOTE: Exclude any contamination of the cell culture. Since the basement membrane matrix forms a gel above 5 °C, all cultureware or media coming in contact with the basement membrane matrix reagent should be prechilled/ice-cold. Keep the basement membrane matrix on ice during the entire process.

2.1. Preparation of the IMR5 cell suspension

2.1.1. Thaw the basement membrane matrix reagent overnight by submerging the vial in ice in a 4 °C refrigerator before use.

2.1.2. On day 0, harvest the cultured IMR5 cells as detailed above.

2.1.3. Transfer the cells to a 15 mL conical tube and centrifuge at 300 x *g* for 5 min.

2.1.4. Discard the supernatant. Wash the cells 2x with 15 mL of ice-cold PBS, and prepare a cell suspension of 5 x 10^7 cells/mL in ice-cold PBS.

NOTE: If necessary, transfer the cell suspension to a 1.5 mL microcentrifuge tube.

2.1.5. Swirl the basement membrane matrix vial.

NOTE: The basement membrane matrix reagent should be thawed and dispersed.

2.1.6. Add one volume of basement membrane matrix reagent and mix it by pipetting to obtain a cell suspension of 2.5 x 10^7 cells /mL.

2.1.7. Keep the cell suspension on ice.

2.2. Preparation of the mice

NOTE: The mice should be six to seven weeks old.

2.2.1. Maintain mice under a specific pathogen-free condition.

2.2.2. Allow a three- to five-day acclimatization period after the mice have arrived.

2.2.3. On the day of inoculation, shave the flank where the injection will be done (see step 2.3.6).

2.3. Preparation of the tumor cell injection

NOTE: Keep the ice-cold basement membrane matrix cell suspension aseptic throughout the procedure.

2.3.1. Mix the cells and carefully draw the cell suspension into a 1 mL syringe mounted with a 21 G needle.

2.3.2. Check to be sure that there are no air bubbles in the syringe.

2.3.3. Disinfect the inoculation area of the mouse with an antiseptic solution.

2.3.4. Gently squeeze the mouse's skin on the flank between fingers, at the injection site.

2.3.5. Insert the needle exactly into the skin fold. Do not place the needle deep into the tissue to ensure a subcutaneous injection.

2.3.6. Inject 100 µL of IMR5 cell suspension (*i.e.*, 2.5 x 10^6 cells) subcutaneously into the lower right flank of the mice.

2.3.7. Rotate the syringe to prevent leakage and withdraw the needle.

2.4. Monitoring of body weight changes and tumor growth

2.4.1. Measure the length (A) and the width (B) of the tumor with a caliper.

2.4.2. Calculate the tumor volume using the formula ($A \times B^2$) x 0.5.

2.4.3. Start therapy when the tumors have reached an average volume of ~50 - 60 mm^3.

3. Drug and Antibody Administration in Mice

3.1. Intravenous administration of mAb 8B6

3.1.1. Carefully fill a 1 mL syringe mounted with a 25 G needle with mAb solution.

3.1.2. Place the mouse under a heat lamp for 10 min to dilate the tail vein.

3.1.3. Restrain the mouse in a rodent restrainer.

3.1.4. Disinfect the inoculation area of the mouse with an antiseptic solution.

3.1.5. Insert the needle parallel to the tail vein, penetrating 2 - 4 mm into the lumen while keeping the bevel of the needle face upward (**Figure 3A**).

3.1.6. Inject 100 µL of antibody solution intravenously (i.v.).

3.1.7. When the injection is finished, gently pressure the injection site to prevent bleeding.

3.2. Intraperitoneal administration of topotecan

3.2.1. Draw the drug solution in a 1 mL syringe mounted with a 25 G needle.

3.2.2. Hold the mouse in a supine position, with its posterior end slightly elevated.

3.2.3. Disinfect the inoculation area of the mouse with an antiseptic solution.

3.2.4. Locate the mouse's abdomen midline and mentally divide the abdomen into quadrants. Locate the injection site in the right or left lower quadrant (**Figure 3B**).

3.2.5. Insert the needle into the abdomen (5 mm deep) at ~10° angle, in the right or left lower quadrant.

3.2.6. Inject 100 µL of drug solution intraperitoneally (i.p.).

3.2.7. Disinfect the inoculation site.

REPRESENTATIVE RESULTS:

The representative results and figures are adapted with permission from earlier published work[14].

Anti-OAcGD2 mAb 8B6 Synergistically Enhances the Inhibitory Effects of Topotecan on Neuroblastoma Cell Line Growth:

To establish the drug and the antibody concentrations to be used for assessing synergism between topotecan and mAb 8B6, the drug and the antibody sensitivities of human IMR5 neuroblastoma cells were measured first, using an MTT assay. Exposure to either mAb 8B6 or topotecan alone for 72 h resulted in a concentration-dependent inhibition of IMR5 cell viability (**Figure 4A**). Dose-response curves allowed the calculation of the ED_{50} values for each compound. To this end, Fa values were computed using the analysis simulation software. The calculated ED_{50} values were found to be 10 nM ± 1 for topotecan (**Figure 4B**) and 18 µg/mL ± 3 for mAb 8B6 (data not shown).

Based on these ED_{50} values, next, the potency of six combinational equipotent ratios of mAb 8B6 and topotecan were tested (**Figure 2**). The shift of the combination dose-response curve toward the sensitive side of the graph indicates that the combination regimen is more potent than each monotherapy (**Figure 4A**). To obtain the corresponding ED_{50} and CI values, Fa values were computed. The ED_{50} values of topotecan were significantly lower in the presence of mAb 8B6 ($p < 0.05$, **Figure 4B**), indicating that mAb 8B6 sensitizes the tumor cell to topotecan. Importantly, the CI values were significantly less than 1.0 ($p < 0.05$), demonstrating a synergistic interaction (**Figure 5**). Thus, mAb 8B6 has the potential as an adjuvant therapeutic agent for topotecan chemotherapy.

Anti-OAcGD2 mAb 8B6 Enhances Antitumor Activity of Topotecan *In Vivo*:

Because *in vitro* models neither take into account the drug half-life nor the drug metabolism, it is necessary to corroborate the *in vitro* findings *in vivo*. Moreover, *in vivo* models are very useful to assess the combination regimen safety. To confirm that the synergism between topotecan and mAb 8B6 was specific to cancer cells, the antineuroblastoma effects of the combination therapy in a tumor xenograft model were assessed. For this, the severe immunodeficient NSG mouse strain was selected. These mice lack both an innate and an adaptive immune system[15], and therefore, make it possible for researchers to exclude any immunomodulatory effects induced by topotecan therapy that can affect mAb potency. Treatment was started once the tumors displayed a mean volume of 50 ± 2.5 mm^3 (**Figure 6A**). The treatments consisted of either mAb 8B6 (150 µg i.v., day 7 and day 11), topotecan (0.36 mg/kg i.p., days 7 - 11), or a combination of mAb 8B6 and topotecan. The tumor volumes were monitored during the course of the experimentation. All therapies led to tumor growth retardation compared with control groups (**Figure 6A**). The combination regimen, however, induced the strongest effect (**Figure 6A**).

Tumor growth can be further analyzed to study the survival rate upon treatment. To this end, the event resulting in mouse euthanasia was defined as the tumor volume ≥ 1 cm^3 for ethical reasons. This allowed us to perform a Kaplan-Meyer survival analysis and to calculate the median event-free survival time for each experimental group. As shown in **Figure 6B**, both monotherapies improved the event-free survival (EFS) substantially when compared to control groups (the median EFS of the vehicle-treated group was 21 days; of the control antibody group, 22 days; of the topotecan group, 26 days; of the mAb 8B6 group, 29 days [**Figure 6B**]). Yet, the combined therapy had the strongest effect on the mice survival, with a median EFS extended to 39.5 days ($p < 0.05$, mAb 8B6 *vs.* combination; $p < 0.01$, topotecan *vs.* combination [**Figure 6B**]).

Because synergistic interactions can result in increased toxicity, the combination regimen safety needs to be assessed. As such, weight loss is commonly used as a sensitive marker for health monitoring in rodents[16]. Thus, weight loss—measured as a decline in percentage from the initial weight—was retained as an indicator of the systemic tolerability of each tested regimen. No loss of body weight was observed, suggesting that the treatment was well tolerated (**Figure 7**). These data suggest that the combination of topotecan plus mAb 8B6 represents a more potent antitumor efficacy *in vivo* than either agent alone, without detectable toxicity.

FIGURES AND TABLES LEGENDS:

Figure 1: Schematic representation of the study.

Figure 2: Layout of the combination experiment on a 96-well plate with ratios of topotecan-to-mAb 8B6, prepared as six solutions. The solutions were prepared as described in the protocol. Wells labeled 1 to 4 serve as mAb 8B6 solutions, wells labeled 5 to 8 serve as topotecan solutions, and wells labeled 9 to 12 serve as mAb 8B6 and topotecan (combination) solutions. Wells labeled A and H serve as controls; wells labeled B serve as 0.125 x ED_{50} concentration drug solutions; wells labeled C serve as 0.25 x ED_{50} concentration drug solutions; wells labeled D serve as 0.5 x ED_{50} concentration drug solutions; wells labeled E serve as ED_{50} concentration drug solutions; wells labeled F serve as 2 x ED_{50} concentration drug solutions; wells labeled G serve as 4 x ED_{50} concentration drug solutions, as indicated. Therapeutic agents with two different units (*i.e.*, µg/mL for mAb 8B6, and nM for topotecan) are analyzed in a fixed-ratio combination (0.075, topotecan/8B6).

Figure 3: Injections. (**A**) Intravenous injection into the tail vein of a restrained mouse, using an insulin syringe of 27 G x 1/2 in, 1 mL. (**B**) Intraperitoneal injection to the lower right quadrant of the mouse's abdomen, using a 1 mL syringe mounted with a 25 G needle.

Figure 4: Dose-effect relationship of topotecan, mAb 8B6, and their combination on growth viability inhibition of IMR5 neuroblastoma cells after 72 h of exposure. An MTT assay was performed as described in the protocol. (**A**) The dose-response curves shown are representative of three independent replicates, each run in quadruplicates. The data are presented as the mean ± SD; *** $p < 0.001$. (**B**) ED_{50} of topotecan used as a single agent or in combination with mAb 8B6. The data are presented as the mean ± SEM. This figure has been modified from Faraj *et al.*[14].

Figure 5: **Combination index values.** Percentage survival values were transformed into fraction-affected (Fa) values and used to calculate the combination index (measure of synergy, additivity, and antagonism) using computer software, as indicated in the text. In the combination index plots, data are presented as mean ± SD for three independent replicates. Results show that mAb 8B6 had a synergistic effect with topotecan (CI <1). This figure has been modified from Faraj *et al.*[14].

Figure 6: Combination treatment of IMR5 xenografts in NSG mice with mAb 8B6 plus topotecan. (**A**) Mice bearing human neuroblastoma IMR5 xenografts were treated with vehicle (PBS, i.p.), topotecan alone (0.36 mg/kg, i.p.), control IgG alone (150 µg, i.v.), mAb 8B6 alone (i.v.), or topotecan and mAb 8B6 combined, as indicated. The administration of mAb 8B6 or control antibody treatment started on day 7 after the IMR5 cell inoculation and was repeated once on day 11. The topotecan or PBS treatment was started on day 7 and given for five consecutive days. The tumor growth was monitored, and tumor volumes were calculated. The mean tumor volume ± SEM of each treatment group (PBS group, 9 mice; all other groups, 10 mice) are depicted (* $p < 0.05$ for mAb 8B6 against mAb 8B6 and topotecan together, ** $p < 0.01$ for topotecan against mAb 8B6 and topotecan together), as indicated. A significant reduction in xenograft volume was observed for the mAb 8B6/topotecan combination, compared to the drug-alone controls, as indicated ($p < 0.05$). (**B**) Event-free survival Kaplan-Meyer curves of different groups treated are shown. This figure has been modified from Faraj *et al.*[14].

Figure 7: Mean weight for each treatment group, as indicated. The mean weight of the mice on day 0 was defined as 100% weight. The weight in each group remained stable for the period of treatment. The data are presented as the mean ± SEM. This figure has been modified from Faraj *et al.*[14].

DISCUSSION:

To predict the effect of drug interactions, three methods can be used: the isobologram methodology[17], the nonlinear mixture model[18], and the combination index[1]. Combination index analysis is the most commonly used because its application is simplified by the availability of a user-friendly computer program. For this purpose, we first characterized the dose-effect response of each agent used alone or in combination, by performing an MTT assay[19]. This methodology relies on the ability of viable cells to reduce the tetrazolium salt in a purple-colored formazan product with an absorbance maximum of 570 nm[19]. At death, the cells lose the capacity to convert MTT into formazan. Thus, the quantity of the colored formazan product is proportional to the number of viable cells in culture[19]. Indeed, we chose this methodology as a nonradioactive alternative to the tritiated thymidine incorporation into DNA for measuring cell proliferation[19]. As such, MTT assays have been widely adopted and remain popular in academic labs as evidenced by thousands of published articles. While the quantity of formazan is measured by recording the absorbance at 570 nm using a plate-reading spectrophotometer, a reference wavelength of 630 nm is sometimes used but not necessary for most assay conditions. In consideration of the critical step described here, we found that the culture conditions used to grow cells can affect the results of MTT assays. We found that the viable cell number, the cell metabolic activity, the age of

cultures, and the number of passages and details of the growth medium can all be important factors. Thus, they must be taken into consideration when repeating the assay and analyzing data. Furthermore, cell culture conditions differ for each cell lines. It is, thus, complicated to provide any clear indications about the cell seeding density. As a rule of thumb, an average number of cells between 2,000 - 10,000 cells per well is recommended to achieve an optimum cell density within 72 h. Importantly, MTT reduction reflects viable cell metabolism and not, specifically, cell proliferation or cell death. Therefore, MTT assays should neither be described as measuring cell proliferation nor cell cytotoxicity[20,11]. The detection of cell death relies on specific cell-based assays. For example, in previous studies, we used western blot analysis to detect caspase 3 activation and/or flow cytometry analysis to detect phosphatidylserine in the outer plasma membrane leaflet, to evidence the proapoptotic activity of mAb 8B6[13].

When combination testing drugs, they can be delivered together or successively in time. Given the variety of the mechanism of action, it is difficult to provide precise guidelines for the relevant experimental setting. Yet, most investigators utilize direct testing of drug combinations. In addition, concentrations that produce a defined single-agent effect of 50% cell growth inhibition are commonly used. Drug concentrations reflecting plasma concentrations achievable in patients are commonly considered as relevant clinically. As such, the combination dose-effect analysis is performed for several doses, keeping the combination ratio constant with a serial dilution of the $(ED_{50})_{mAb}/(ED_{50})_{drug}$ ratio, although it is not an absolute requirement[1]. Of note, the CI values should be also calculated at various ED tested (*e.g.*, ED_{50}, ED_{75}, and ED_{90}) because they may change with the Fa in a nonlinear manner[1]. After performing the automated analysis of the CI values, the linear correlation coefficient *r*-value estimated by the algorithms should be >0.9, to reflect the goodness of the fit of the experimental data. CI values of <1 indicate synergy, CI values of =1 indicates additivity, and CI values of >1 denote antagonism[1]. Due to the variability in both the experimental methods and the biological system, the calculated CI should be further subjected to statistical consideration. As such, a simple method is to repeat the drug combination experiment several times followed by the calculation of the resulting CI values before determining the statistics of the mean ± SD[1]. It is also advised to test the combination in the largest possible panel of cell lines, to take into account the variability that exists between them.

Importantly, several parameters in the *in vitro* study are not taken into consideration for the *in vivo* extrapolation. For instance, the *in vitro* viability assays neither take into consideration the *in vivo* half-life of the drug, nor the *in vivo* drug metabolism that may result in drug inactivation. Thus, the extrapolation from *in vitro* to *in vivo* remains a separate question, and the beneficial drug interaction evidenced *in vitro* should be further confirmed *in vivo*. As such, a clear demonstration of synergy in mice bearing tumor xenografts, using the combination index method, has been published[21]. Yet, *in vivo* studies require a large number of animals to accurately define synergy and are more expensive and more time-consuming. The alternative effective model *F*-test still requires substantial resources[22]. As a different approach to limit the use of a large number of animals consists in demonstrating the drug synergy in cell culture models followed by the evidence of an increased antitumor response of the combination in a limited xenograft study[14], we used the human neuroblastoma IMR5 cells as the tumor target for the *in vivo* study. We retained IMR5 cells because they are tumorigenic in immunocompromised mice[23].

While human tumor xenograft models provide valuable models for testing drug combinations *in vivo*[24], it can be difficult to establish such models from a variety of cell lines[25]. To increase the incidence of tumor formation in mice, cells can be injected into mice with a membrane basement matrix as a vehicle[25]. Importantly, since membrane basement matrix polymerizes and solidifies at temperatures above 5 °C, all cultureware or media coming in contact with the membrane basement matrix should be prechilled/ice-cold, and the membrane basement solutions should be kept on ice during the entire process[25]. With the use of the membrane basement matrix, we observed a 100% take rate with IMR5 cells, while, in the absence of membrane basement matrix, this cell line demonstrated a <80% take rate (data not shown). In addition, to increase the incidence of tumor graft, membrane basement matrix can also increase the tumor growth rate[25]. We observed that all xenografts had appeared by day 11. However, during the first seven days following the tumor cell challenge, the presence of lumps could be observed, which may be caused by the presence of the initial inoculum (data not shown). Thus, we did not consider measurements of tumor size meaningful before this time. Using the membrane basement matrix made it possible to reduce the number of mice in the experimental setting and to perform the *in vivo* experiment within three months.

Drug/antibody dosages may vary depending on the cell line and mouse strain. Due to the limitation of the *in vitro* viability assay for extrapolating the *in vivo* drug/antibody dosage described above, clinically relevant dosages were retained for both topotecan and mAb 8B6 in the *in vivo* experimental setting, based on the data published by others[26,27]. To extrapolate the human dosage setting to mice, previously published guidelines[28] were followed. Per se, we injected 150 µg of antibody 8B6 i.v. at day 7, followed by a second injection five days later (total dose/mouse = 300 µg). Topotecan treatment started the same day as the first mAb 8B6 injection by injecting 0.36 mg/kg topotecan or PBS control i.p. for five consecutive days. In consideration of the critical step described here, we recommend starting antibody infusions when the tumor xenografts reach ~50 mm^3. Higher tumor xenograft volumes will result in a lower response rate because tumor burdens clearly correlate inversely with antibody concentration, antibody exposure, and antibody efficacy[29]. We also retained weight loss as an indicator of systemic tolerability of each tested regimen[16]. However, the interpretation of collected weights may not be helpful with a large tumor mass. In this case, body condition scoring, as described elsewhere[16], is advised.

Tumors can be collected at different time points for immunochemistry analysis, to provide further information about the mechanism of action triggered *in vivo*. In a previous work, the tumor apoptotic index was assessed after mAb 8B6 infusion[13]. To this end, tumor apoptotic cells were detected using a TUNEL assay[13]. In addition, the percentage of tumor cell nuclei was scored with Ki67 antigen-positive staining to analyze the tumor proliferation index[13].

Severe immunodeficient mice lack both innate and adaptive immunity with the loss of T cells, B cells, and natural (NK) cells with reduced macrophage and antigen-presenting cell functions and the absence of circulating complement[30]. Thus, this protocol does not allow researchers to draw any conclusions regarding putative effects of chemotherapy in potentiating mAb therapy by altering the tumor microenvironment *in vivo*[31]. However, both putative immunomodulatory

effects of the chemotherapeutic agent and the role of the fragment-crystallizable (Fc) region of the mAb in enhancing the drug antitumor potency merit consideration, but these questions require the availability of a syngeneic model with an intact immune system and a physiological tumor microenvironment.

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DISCLOSURES:

S.Fa., J.F., and S.B. are designated as inventors of pending patents covering the clinical application of anti-O-acetyl-GD2 therapeutic antibodies.

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Figure 1

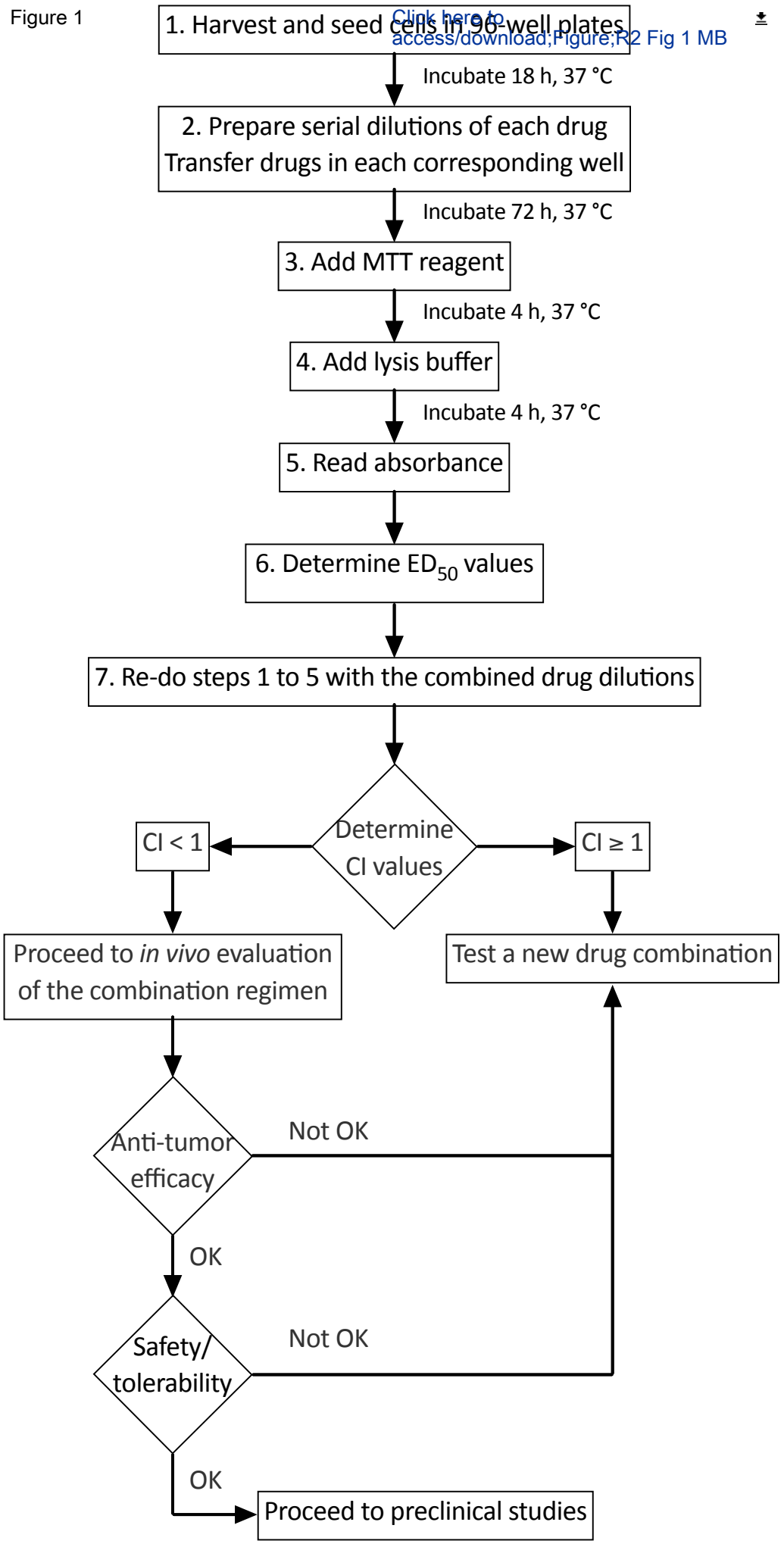

Figure 2

		Concentrations	
		8B6 (µg/mL)	Topotecan (nM)
	0	0	0
Point 1	0.125 x ED_{50}	2.5	1.25
Point 2	0.25 x ED_{50}	5	2.5
Point 3	0.5 x ED_{50}	10	5
Point 4	**ED_{50}**	**20**	**10**
Point 5	2 x ED_{50}	40	20
Point 6	4 x ED_{50}	80	40
	Control	Control	Control

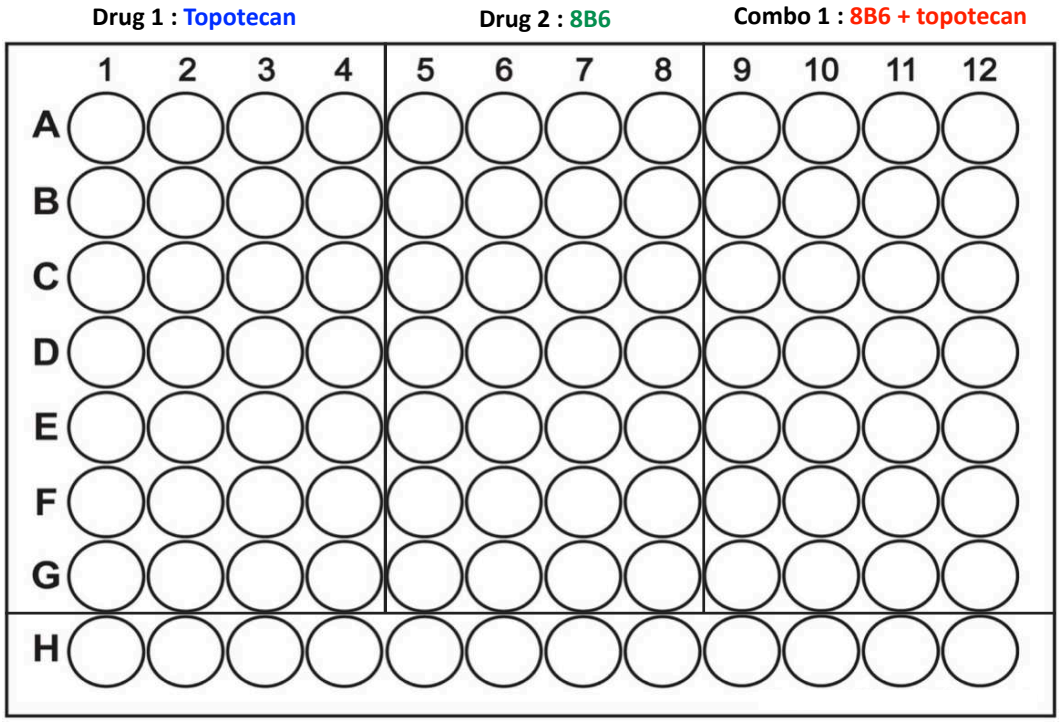

Figure 3

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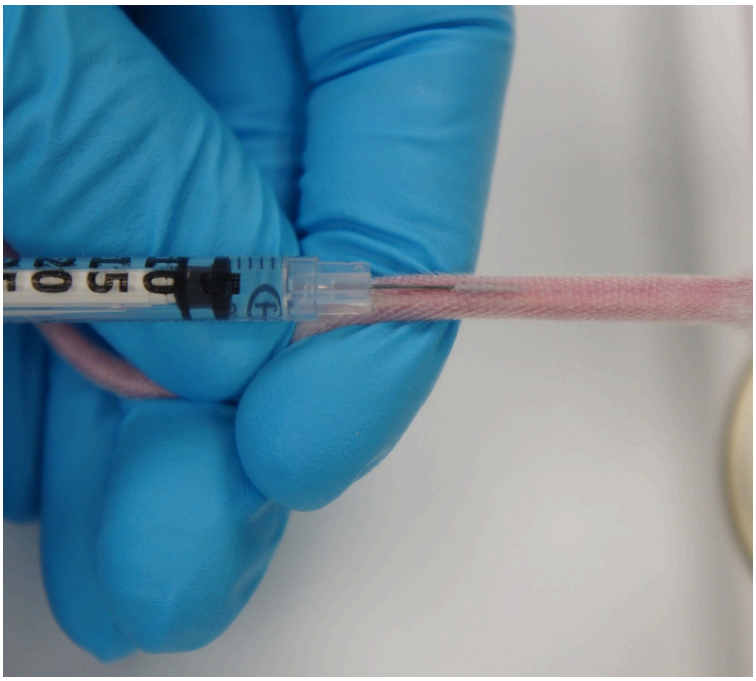
A



B

Figure 4

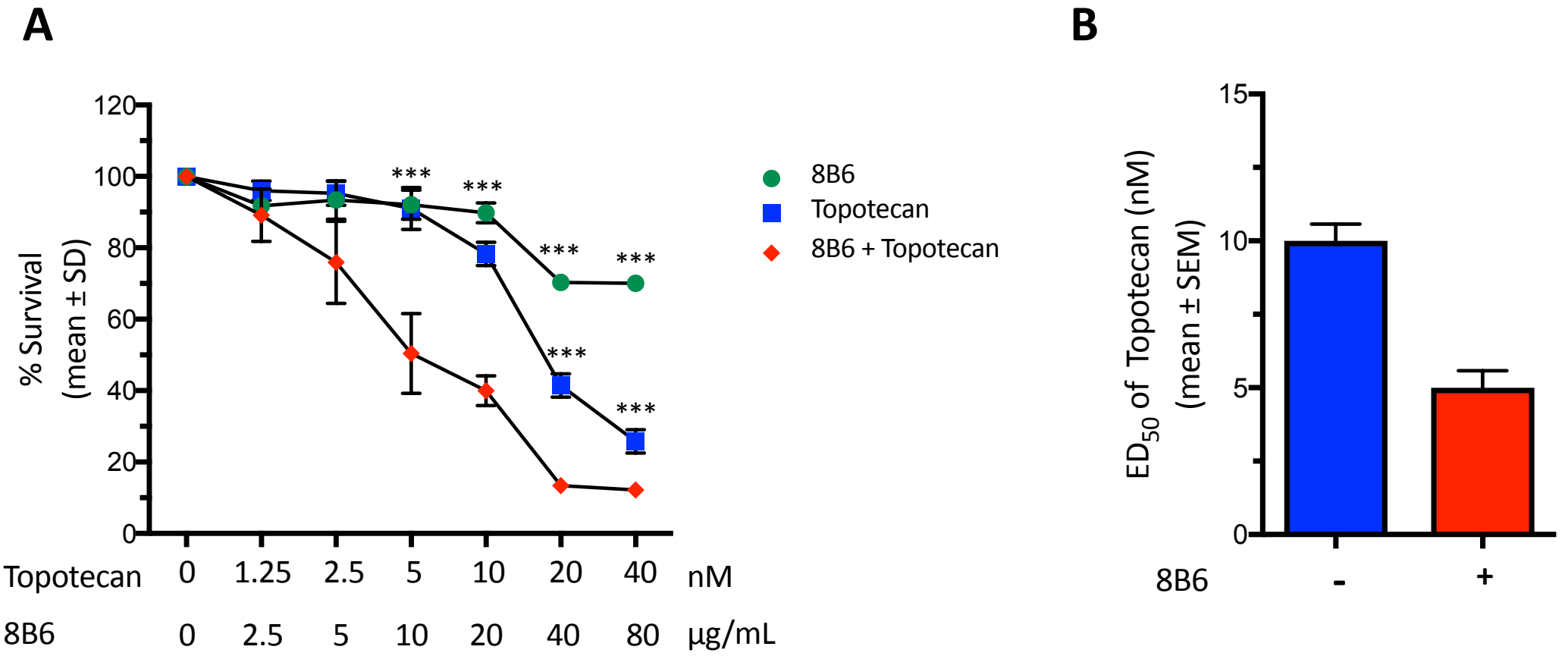
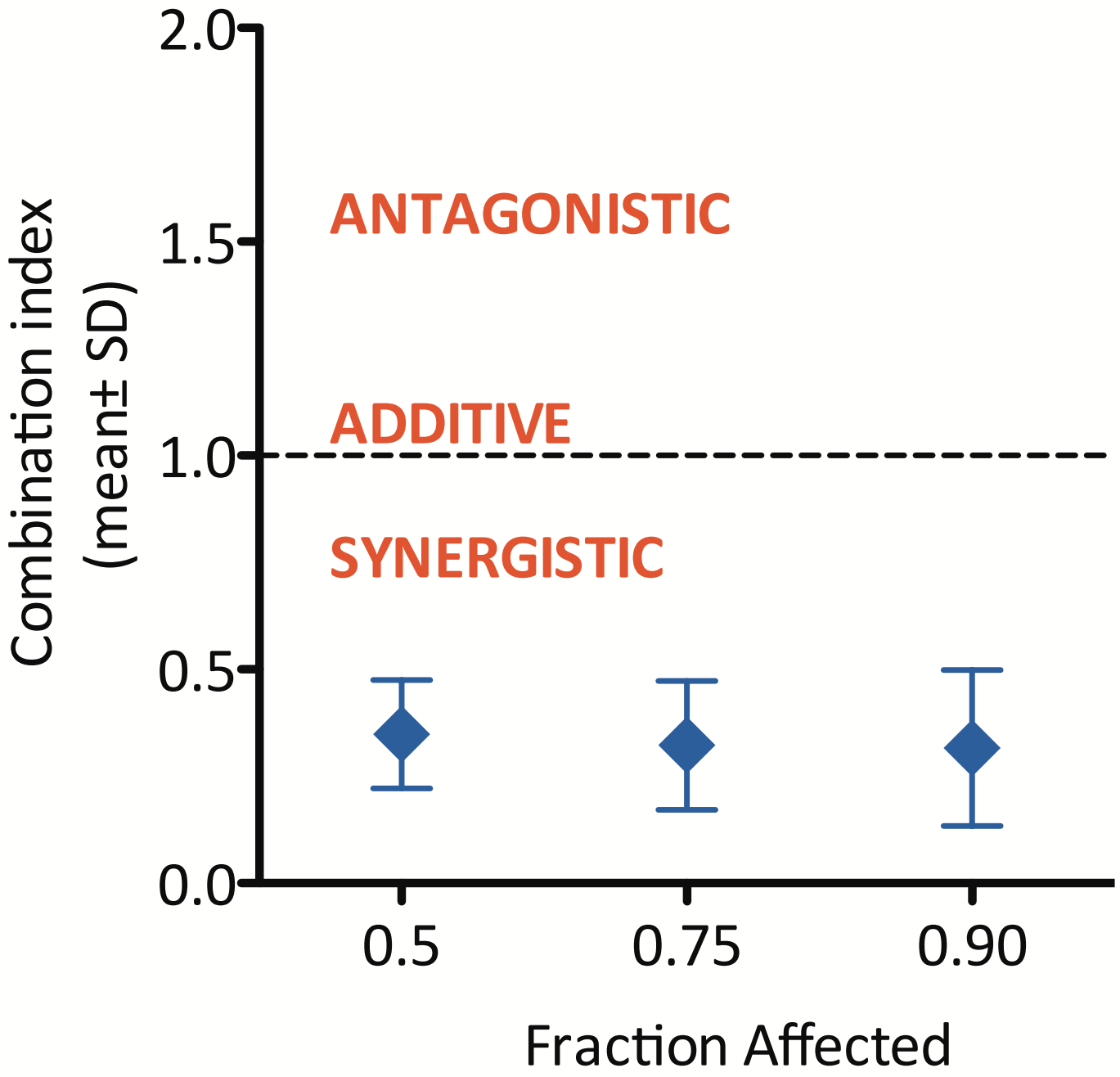


Figure 5



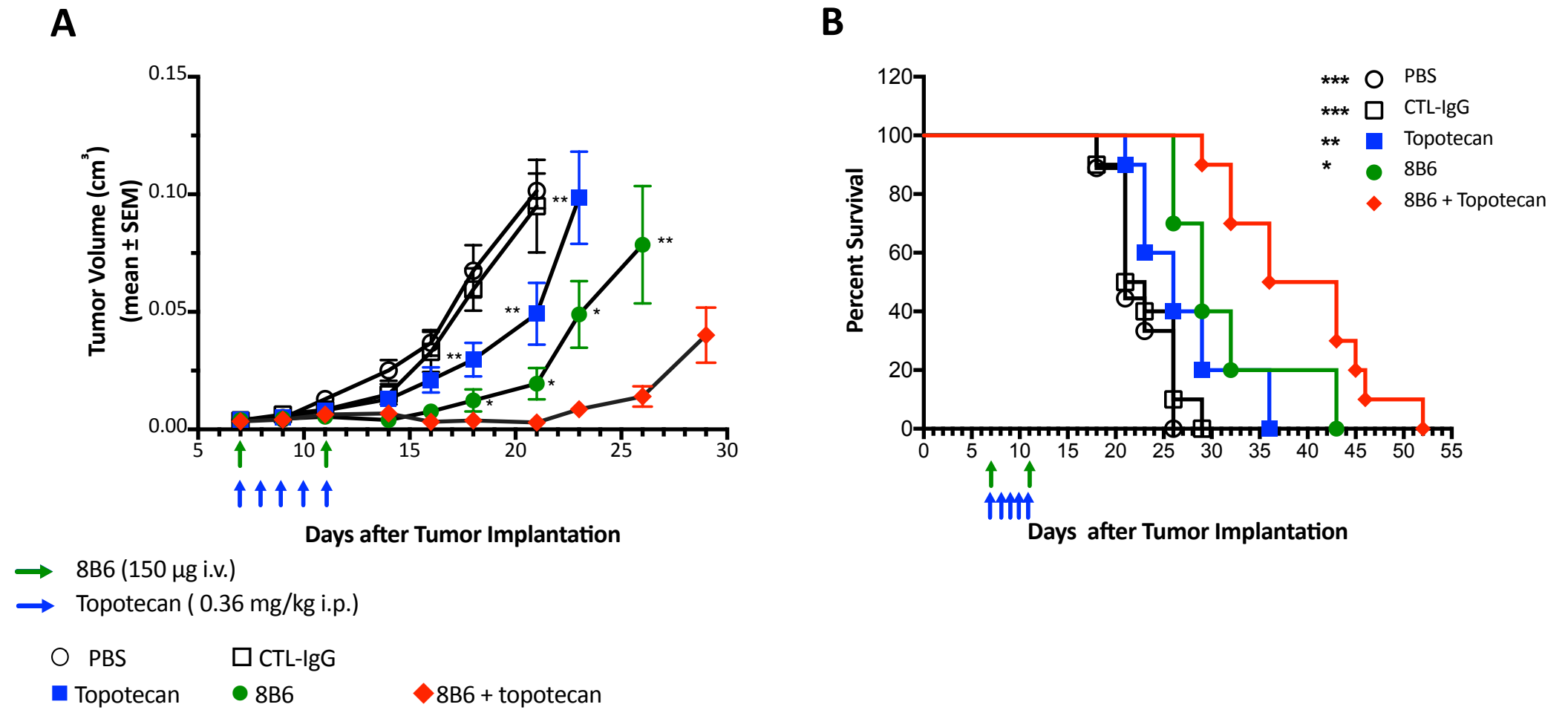


Figure 7

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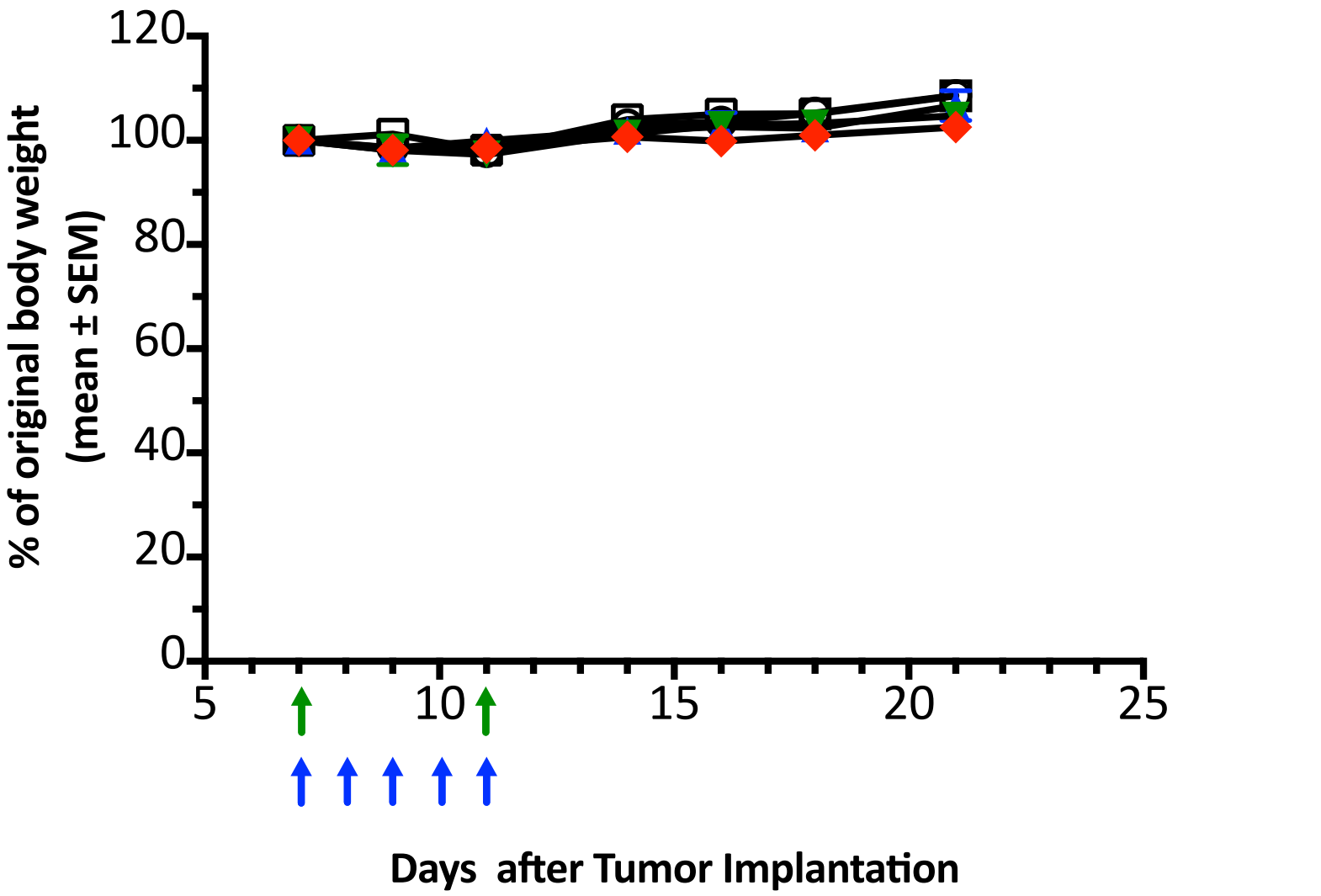

8B6 (150 µg i.v.)

Topotecan (0.36 mg/kg i.p.)

PBS

CTL-IgG

Topotecan

8B6

8B6 + topotecan

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Cell Proliferation kit (MTT)	Roche	11-465-007-001	
CompuSyn software	ComboSyn		Combosyn can be downloaded for free at http://www.combosyn.com
Electric shaver	Bioseb	BIO-1556	
Fetal calf serum	Eurobio	CVFSVFF00-01	10% heat-inactivated fetal calf serum in RPMI 1640
Firefox	Mozilla Corporation		Firefox can be downloaded for free at http://www.mozilla.org/en-US/firefox/
Heat lamp	Verre&Quartz	4003/1R	
Human neuroblastoma IMR-5 cell line	Accegen Biotechnology	ABC-TC0450	IMR-5 is a clone of the human neuroblastoma cell line IMR32 5459762. IMR-5 cells were generously provided by Dr. Santos Susin (U.872, Paris, France)
L-glutamine	Gibco	25030-024	2 mM in RPMI 1640
Lysis solution	Roche	11-465-007-001	
mAb 8B6	University of Nantes	N/A	
Matrigel	Corning	354248	
Multiskan FC	Thermofischer Scientific	N08625	
Needle 21G 1 ½	BD Microlance	304432	
Needle 25G 1	Terumo	NN-2525R	
NSG mice	Charles River Laboratories	5557	
Nunc MicroWell 96-well microplates	Thermofisher	167008	
PBS	VWR	L182-10	
PBS, 0,05% EDTA	Sigma-Aldrich	E9884	
PC that runs windows 7	Microsoft		Windows 7 can be purchased at http://www.microsoft.com/en-gb/software-download/windows7
Penicillin-Streptomycin	Gibco	15140-122	100 units/mL penicillin and 100 µg/mL streptomycin in RPMI 1640
Reagent reservoir	Thermofischer Scientific	8094	
Rodent restrainer	Bioseb	TV-150-SM	
RPMI 1640	Gibco	31870-025	
Syringe 1 mL	Henke Sass Wolf	5010.200V0	
Topotecan	Sigma-Aldrich	T2705	

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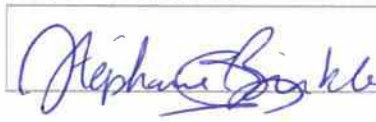
Signature:	
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Rebuttal Letter

Manuscript # JoVE58291 "An effective method to investigate the synergism between anti-cancer antibodies and chemotherapeutic drugs in vitro and in mice."
Authors: Bahri et al.

Editorial and production comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Reply: Done.

2. Please revise lines 69-72, 389-391, 392-421, 484-486, 488-489, 494-497, and 559-562 to avoid previously published text.
Reply: Done.

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Reply: Done.

The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”
Reply: Done.

4. Figure 1: Please change “ml” to “mL”.
Reply: Done.

5. Figure 2: Please ensure that the panels are of the same dimensions if possible.
Reply: Done.

6. Figures 5 and 6: Please change “sem” to “SEM”.
Reply: Done.

7. Please revise the title to be more concise.
Reply: Done. The revised title is “Potentiation of anti-cancer antibody efficacy by anti-neoplastic drugs: detection of antibody-drug synergism using the combination index equation.”

8. Keywords: Please provide at least 6 keywords or phrases.
Reply: Done. We provided the 9 following keywords: cancer research; drug development; antibody-drug combination; antibody-drug interaction; MTT assay; antibody-drug synergy; combination index equation; *in vitro* cell line model; tumor xenograft model.

9. Please rephrase the Abstract to more clearly state the goal of the protocol.
Reply: Done.

10. Please define all abbreviations before use.
Reply: Done.

11. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "CompuSyn" and “Matrigel” within your text. The terms may be introduced but please use them infrequently and when directly relevant. Otherwise, please refer to the terms using generic language.
Reply: Done. After introducing the terms Matrigel and Compusyn we replaced “Matrigel” by basement membrane matrix and “Compusyn” by “simulation software”.

12. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
Reply: Done.

Please include all safety procedures and use of hoods, etc.
Reply: Safety procedures are reminded at the beginning of the Protocol Section.

Please move the discussion about the protocol to the Discussion.
Reply: Done.

13. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.
Reply: Done.

14. 1.1.3: What is used to count cells?
Reply: We used a hemocytometer. This is indicated in the revised manuscript.

15. 1.1.6: Please specify other incubation conditions (temperature, %CO2).
Reply: The temperature condition is 37°C and the % of CO2 is 5%, as added in the revised manuscript.

16. 1.3.4: What is the humidity?
Reply: 95 %, this has been in the revised manuscript.

17. 2.1.3: What happens after centrifugation? Is the supernatant discarded?
Reply: The supernatant is discarded, as corrected in the revised manuscript.

18. Please ensure that the computational steps of the protocol are done in a graphical user interface with explicit user input commands: File | Save | etc.
Reply: Done. We have re-written the corresponding Section.

19. Please revise to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.
Reply: Done. The Representative Results Section has been re-written accordingly.

20. References: Please do not abbreviate journal titles.
Reply: The full journal titles are now indicated.

21. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.
Reply: Antibody 8B6 was generated in our laboratory. Lot numbers and RRID are not available.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the written protocol and the narration in the video. It would be best if the narration is a word for word from the written protocol text.
Reply: We edited the video and homogenized the narration and the written protocol text.

2. Titles of the manuscript and the video do not exactly match.
Reply: Correct. Done, thanks.

3. The details in the video are not the same as the details in the written manuscript. For example:
03:13: The video says incubate overnight while the written manuscript states 18 h.
Reply: Correct. Done, thanks.

04:15: Please use the same figure in video and in the written manuscript.
Reply: Done.

04:24: The video says 38 °C incubate overnight while the written manuscript states 37 °C.
Reply: Correct, fixed, thanks.

4. 04:54: Please remove commercial language from the video (Excel).
Reply: Done.

5. Please upload a revised high-resolution video here:
http://www.jove.com/files_upload.php?src=17778803
Reply: Done.

6. Audio issues

• The audio volume of the narration is a bit low. To match with the other videos on our site, the audio levels should be peaking between -6 and -12 dB.
Reply: Fixed.

• 9:57 - It sounds like the audio fades out while the narrator is still speaking. This should be corrected.
Reply: Fixed.

Reviewers' comments:

Reviewer #1:
This is a straightforward and well-written manuscript. But Bahri et al unable to evaluate the outcome of this study by using any standard approach.
Reply: The outcome of the study has been previously published by our group as cited in the manuscript. Here we focused on the Chou and Talalay method.

Some suggestions for the authors' consideration:
1. The title of this manuscript is not suitable and did not provide any impact of the outcome of this study. It is hard to get away from that fact. Please improve it.
Reply: Done. The revised title is "Potentiation of Anti-Cancer Antibody Efficacy by Anti-Neoplastic Drug: Detection of Antibody-Drug synergism using the Combination Index Equation.

2. It would be good if authors add a schematic representation of this study.
Reply: We added a schematic representation of the study (Fig. 1) is the revised manuscript.

3. Please mention any standard approach using FACS/ Western blot analysis or Immunohistochemistry to prove the hypothesis.
Reply: Good suggestion. This has been added in the Discussion Section. Thanks.

4. Please also check the survival rate after treatment.
Reply: Fair point. Done, see Fig. 5B. Thanks.

5. Protocol for Antibody and drug preparation is too long and ambiguous. Author can easily mention that the used serial dilution of compounds/ Antibody rather than to repeat about the dilution.
Reply: We have shortened protocol Section accordingly.

6. Discussion- Please also correlate the outcome of this study with references from the other published studies in large cohort.
Reply: I am afraid but the combination regimen has not been tested into patients yet.

Reviewer #2:
Major Concerns:
The authors correctly mention that the 8B6 monoclonal antibody can kill tumor cells through

either antibody-dependent cell mediated cytotoxicity or through apoptosis. However, the assay described by the authors does not measure apoptosis directly (MTT assay measures cell viability, and xenograft assay measures tumor size). The authors need to demonstrate that the cells are being killed, and the effect is not just cytostatic, and is not simply a result of cell proliferation decrease due to combination drug load. This is particularly important because the xenograft experiment is in immunodeficient mice, so it is unlikely the tumors are being killed by antibody-dependent cell mediated cytotoxicity. A simple immunohistochemistry for apoptosis markers such as cleaved caspase-3 on xenograft tumors should be sufficient to demonstrate this. Alternatively, Annexin V staining in cell lines treated with the drug combinations should be able to show that the cells are being synergistically killed if the author's conclusions are correct.

Reply: We agree with you. MTT assays only assess the cell viability. In addition, the killing mechanisms of mAb 8B6 have been already published as cited in the Discussion Section. This is clearly stated in the Discussion Section of the first submission. In respect with your comment, we added in the revised manuscript Discussion Section the list of the standard methods we used, in our previously published works, to assess apoptosis *in vitro* and in tumor xenografts biopsies.

Minor Concerns:

-In line 134 the authors mention using 0.05% EDTA solution to dissociate cells. I believe the authors meant to say Trypsin-EDTA solution. Please clarify

Good question. However, there is no typo. 0.05% EDTA solution can dissociate cells such as IMR5 cells. The use of a trypsin-EDTA solution would resulted in a decrease of the cell viability since these cells are very sensitive to trypsin.

-In line 416 the authors need to mention which statistical test was used to calculate the p values

Good suggestion. Done. Thanks

-Figure 2A the authors mention tail vein injection, however the figure indicated shows intraperitoneal injection. Please clarify and use appropriate image

Thanks. We have modified Fig. 2 to match the Figure legend.

-Figure 3 the authors need to include statistics

Good suggestion. Done. Thanks

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