

Gallic Acid: A Viable Drug for Inducing Apoptosis to Human Neuroblastoma Cancer Cells

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ABSTRACT

Glioblastoma is a malicious brain tumor commonly found in adults. Because of its intrusive behavior and troublesome location, the five-year survival rate for Glioblastoma is meager. Data are collected through Pubmed and Pubchem; 34 different chemicals were chosen to be paired up against the PD-L1, an ICs that causes immune evasion of the tumor cells and receptors; and loaded into PyRx v.08 molecular docking tool to find the binding affinity. Gallic acid and Doxorubicin were chosen as the two mixtures used to conduct the assays. Gallic acid was selected because of its above-average binding affinity with the receptor, it is available at the Lab where I experimented, and the limited research done connecting Gallic acid with tumor cells. On the other hand, Doxorubicin was chosen to compare results with gallic acid; since it is already a conventional tumor drug, having a better impact will mean that Gallic Acid will be a superior option to tumor drugs used today. The two mixtures underwent a serial dilution process, making eight different solutions. LDH, MTT, and Caspase assays were conducted with these mixtures on the HTB-11 tumor cell line; with the result being that Gallic ACid ten thousand times diluted is the best drug as it has caused the lowest percentage of necrosis (2.4% of LDH released) as well as having caused one of the highest percentages of apoptosis (758% in caspase activity).

INTRODUCTION

Glioblastomas tumor

Glioblastoma is a type of malignant and fatal brain tumor found in adults that is well-known for its high recurrence rate and aggressive symptoms. Despite the researcher's efforts to combat it, creating multiple different treatment methods such as Tumor Treating Fields (TTF), Neoadjuvant chemotherapy, radiation therapy, and craniotomy; The 5-year survival rate, according to the national brain tumor society, is an abysmal 6.8 percent. The average length of survival for a patient diagnosed with Glioblastoma is only eight months.

Glioblastoma's impact on health

Glioblastomas tumor cells can weaken antitumor immunity through immune evasion. An Immune checkpoint molecule (ICs) called Programmed death ligand (PD-L1) during this process will connect with programmed death cells (PD-1) on the perimeters of activated t cells and activate Immunological tolerance in T cells inside the tumor micro-environment. This process will lead to stimulation in tumor growth and an immune escape, which is the reason behind poor cancer prognosis. Through extensive research, it is concluded that the expression of programmed death-ligand (PD-L1) in the tissues of the glioblastoma tumor is inversely relative to the overall survival rate of a patient. The expression of PD-L1 on glioma cells is also present in multiple papers. Therefore, to improve the chance of survival for patients, lowering or inhibiting PD-L1 expression is essential. Furthermore, it is also concluded that PD-L1 expression is not affected by other clinicopathological characteristics, such as gender.

Limitations in curing Glioblastoma

Although Immunotherapy, throughout extensive development, has become an excellent cure for multiple types of cancer; due to the intracranial localization of the lesion, the immunosuppressive mechanism that the tumor creates, and molecular heterogeneity, immune checkpoint inhibitors (ICI) for Glioblastoma, in their current state, are limited in their efficacy and insufficient to improve the survival of the patients. The blood-brain barrier (BBB), a semipermeable membrane border consisting of endothelial cells connected by tight junctions, can obstruct the drugs from reaching the inter-tumor tissues, limiting the possibility of successful therapeutic drug concentrations.

There is also a mechanism called macroautophagy, later referring as autophagy, that is necessary to address. Autophagy is a physiological and dynamic process of maintaining metabolic homeostasis by capturing intracellular elements and later recycling them in lysosomes. This mechanism can help suppress tumor initiation and growth during the early stages of tumor development. However, as the tumor enlarges and encounters growth and nutrient stun or hypoxia, autophagy might give tumor cells energy and nutrients through the degradation of cytoplasmic components. It will also allow continuing survival of tumor cells, which will result in drug resistance.

Goal

The extensive goal of this experiment is to discover a potential compound drug that will initiate cancer cell apoptosis and thus eliminate cancer cells effectively. In silico screening will firstly be used: through molecular docking tools, we will be able to determine the binding affinity of certain chemicals to the PD-11 receptor. Molecular visualization and modeling analysis tools will also be used during this process. Then, in vitro experiments will be conducted; the LDH assay is used to determine the cytotoxicity of the chemicals by comparing LDH's concentration levels inside the Cell culture media; The MTT cell viability and proliferation assay will determine the solution's impact on overall cell survival, without direct cell death, through reacting to changes in metabolic activities; the caspase-3 colorimetric assay will determine if GenX can induce caspase-mediated apoptosis. These tests will help determine whether the chemicals selected will be toxic enough to be an effective cancer apoptosis inducer.

METHODOLOGY

In Silico Screening

Molecular Docking:

PyRx v.08 software is the molecular docking tool used to test the binding affinities between each chemical and Pd-L1 receptors. Thirty-four different chemicals were paired with the receptor: Trifluridine, Arundic acid, tipracil, Temozolomide, lomustine, carmustine, cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate, levodopa, radicava, rilutek, Tlglutik, Doxorubicin, Pd-1/Pd-L1 inhibitor, zoptarelin Doxorubicin, cerubiden, triferric Doxorubicin, topotecan hydrochloride, daunorubicin, Idarubicin, valrubicin, 5-fluorouracil, altretamine, mitoxantrone, mitomycin, bleomycin, gallic acid, pirarubicin, Doxorubicin, pixantrone, capecitabine, and entrectinib. All chemicals were downloaded.SDF files using PubMed Compounds database, which was later converted to .PDB files using SMILES Translator. The PD-L1 receptor was retrieved from the Protein Data Bank and loaded onto PyRx to access its binding affinity with the 34 different chemicals.

After opening PyRx, the PD-11 receptor will be loaded and converted into a macromolecule in auto dock format. The chosen chemical will then be loaded through Open Babel universal force and converted into the proper autodock format. After that, select the chemical and the receptor in the vina wizard for it to conduct a docking analysis. The result, which includes the binding affinity between the receptor and the corresponding chemical and root-mean-

square deviation values, will be displayed once the calculating process is finished. Two chemicals will be chosen based on their binding affinity, availability, research, experiment, and records associated with tumor cells. After getting the desired result from molecular docking, the table and the 3d chemical structure will be downloaded; BIOVIA will be used to render the 3d system more detailed.

In Vitro Experiment

Cell And Mixture Preparation:

HTB-11, a human neuroblastoma cell line, is the leading and only cell line used in the cell culture process. The cell line was stored in a conical centrifuge tube and was put inside an incubator with 37C temperature, 95% air, and 5% carbon dioxide. The chemicals chosen were both obtained by Sigma-Aldrich. The substances were then serially diluted in DMSO(Dimethyl sulfoxide) with increments of 10 times (10x μ M, 100x μ M, 1000x μ M, 10000x μ M); and are stored in Eppendorf microcentrifuge tubes kept in a fridge. After the cell culture process, the serially diluted solution and cell lines will be used to conduct various assays in either the 96-well plates (100 nanoliter/well) or six-well plates (2000 nanoliter/well).

The LDH Assay:

The Lactate dehydrogenase Assay is conducted to measure the percentage of cytotoxicity of the solutions on the HTB-11 cells. LDH is a natural enzyme found in virtually every living cell; it is released into the cell culture media once the HTB-11 cell membrane is damaged or destroyed through necrosis. It can then be gauged by combining the media with LDH substrate, which will bind with LDH among the media. The LDH value found inside various wells, when compared with the total LDH, will indicate the number of cell deaths directly from necrosis.

First, after the Cell culture process, transfer the cell-infused media into each well of a 96-well plate. Label each row of the 96-well plate with the corresponding amount of the two serially diluted solutions added. For this experiment, the first row was used as the control, following 5 μ M of solution one 10000x, solution one 1000x, solution one 100x, solution one 10x, solution two 10000x, solution two 1000x, solution two 100x, solution two 10x, solution one + solution two 10000x, solution one+solution two 1000x, and solution one + solution two 100x. This is the "starting plate," a foundation that will be transferred and used in multiple methods. Once everything is labeled and modulated correctly, the plate is placed in the incubator for 24 hours.

Once the 24 hours have passed, remove the "starting plate" from the incubator and grab two new 96-well plates; label the two wells as "released LDH plate" and "total LDH plate," respectively. Transfer 35 μ M of cell culture media from the "starting plate" to the "released LDH plate" tilt the plate so that the pipette tip will not touch and potentially scrap the cells sticking at the bottom of each well. Once the media is transferred, 35 μ M of LDH substrate will be added and mixed in the "released LDH plate." LDH substrate, as the name suggests, binds with the LDH inside the media; and, through using a microplate reader, will measure the exact amount of LDH. once the media and the LDH substrate inside the "released LDH plate" wells are mixed thoroughly, It is placed into a cardboard box deprived of any light for 30 minutes.

For the "Total LDH plate," two rows containing 35 μ M of the cell culture media each are transferred from the "starting plate" to the "total LDH plate." Then, add 10 μ M of the LDH lysis buffer, used to lyse the cells inside the media. The LDH lysis buffer will break off every cell's membrane and release the maximum amount of LDH possible. After that, the plate is placed in the incubator for 45 minutes for the lysis buffer to take effect. Once the 45 minutes pass, the "total LDH plate" is taken out; and 10 μ M of the LDH substrate will be added, serving the same purpose as the "released LDH plate"; except this time, the microplate reader will display the total amount of LDH that could have been released, rather than how much LDH the solutions have caused the cells to release. The plate was also left inside a cardboard box for 30 minutes for the LDH substrate to take effect.

After 30 minutes, remove the "released LDH plate" from the cardboard box and add 30 uM of stop solution into the 96-well plate. The stop solution is used, as its name suggests, it is used to stop different reactions between LDH and the LDH substrate. The same action is performed on the "total LDH plate." Then, bring the two plates, scan them on the microplate reader, and scan them on 655 nanometers. This procedure is used to notice abnormal values the micro reader might display. If any values seem to stand out among the rest, the microplate reader is being interfered with; there are likely bubbles inside the wells that gave out an irregular value. Simply remove the plate and pop these bubbles with an Eppendorf pipette. Then, once the 655-nanometer values are recorded on a Microsoft Excel spreadsheet, scan the two plates under 490 nanometers. Subtract the values obtained from the 655 nanometers reading from the number acquired from the 490 reading to eliminate confounding variables. Find the average number of each row with the same amount of solutions in the 96-well plate through the average command in the spreadsheet. There should be two groups of values, one is the "released LDH" average, and one is the "total LDH" average. Find the percentage of cytotoxicity by subtracting the control "released LDH" average from the sample, i.e., 100x solution one, "released LDH" standard; and divide this number by the result by subtracting the control "total LDH" average from the sample "total LDH" average. Finally, times the value by 100 to find the percentage of LDH the solution has caused to release, which is cytotoxicity.

$$100 \times \frac{\text{sample released LDH} - \text{control released LDH}}{\text{sample total LDH} - \text{control released LDH}}$$

Equation 1: Used to calculate the percentage of LDH released.

MTT Cell Viability And Proliferation Assay:

MTT cell viability and proliferation assay is a colorimetric test conducted to determine cell stress upon interaction with a toxic chemical based on an enzymatic reduction from the faint yellowish MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium salt to its formazan of an intense purple color by the mitochondrial dehydrogenase's activity of exposed cells, which can be quantified spectrophotometrically. Under the right environment and treatment, the Colorimetric analysis result will be directly relative to the overall proliferation rate of viable cells. Compared with the LDH assay, it will determine the amount of cell death resulting from methods other than necrosis.

The first few steps will be identical to the LDH assay. After the cell culturing process:

1. Transfer 35uM of cell-infused media into a new 96-well plate.
2. Label each plate with the name of the chemical solutions added to the 96-well plate.
3. Add 5 uM of each solution into their corresponding well and mix thoroughly.
4. Once all the solution is added, place the plate into an incubator at 37 degrees Celsius temperature and condition with 5% carbon dioxide and 95% air for 24 hours.
5. Once 24 hours have passed, take out the plate, add 10uM of MTT solution to each plate, and place it back into the incubator for an hour and thirty minutes.

Remove the plate from the incubator after the time is up and carefully remove the media from each well. Formazan crystals should be forming at the bottom of the wells. The plate should be tilted to ensure no extra crystals were removed during this process. 70 uM of Dimethyl Sulfoxide (DMSO) will be added after the media is removed; this solution will be used to dissolve the crystals formed at the bottom of each well. There should be a distinct color change to the transparent DMSO liquid immediately after it is in contact with the formazan crystals; however, leave the plate for 10 minutes to ensure the best result. If living cells are inside the mixture, its color will turn purple. The

intensity of the mixture's shade of purple is inversely relative to the overall amount of living cells inhabiting it. This indicates the viability and proliferation of the cells after being treated with the solutions. Then, the plate will be placed into a microplate reader at 595 nanometers. The results will be calculated into an average. Finally, dividing the control average by the sample average times one hundred will calculate the percent change of the decrease in cell survival.

The Caspase-3 Colorimetric Assay:

The Caspase-3 Colorimetric Assay measures Caspase three activity in cell lysates and determines whether the selected cells can induce apoptosis. Caspases (Cysteine-requiring Aspartate protease) are a variety of proteases that mediate cell death and are a crucial element in apoptosis. Caspase-3 belongs to the CED-3 subfamily of caspases and is a more specific enzyme that will be released during apoptosis. By adding caspase substrate, the amount of Caspase will be able to record. The amount of Caspase recorded will be directly proportionate to the number of cells the solution will be able to induce apoptosis.

Grab a 6-well plate and transfer 400uM of cell-infused media into each plate. Then, carefully add 100uM of 5 differently mixed and diluted mixtures into separate wells: Solution one 10k, solution two 10, solution one + solution two 10k, solution one 1k, solution one + solution two 1k; one well will be used as the control. Leave the plate in the incubator for 24 hours. Take the dish out the next day and remove the media; this process is necessary because the media is already mixed with other chemicals. Tilt the plate while drawing the media so the pipette top will not scrape the cells attached at the bottom of the well. Add 500uM of trypsin to each well to detach the cells. Then, add 500uM of new media and transfer the 1000uM of cell-infused media and trypsin to each designated microcentrifuge tube. Leave all the pipes inside a centrifuge for 3 minutes. There should be cell pellets formed at the bottom of each pipe. Carefully remove the media without removing any excessive cell pellet. Finally, add 50 uM of caspase lysis buffer to each tube to break off the inner proteins inside each cell. Store the mixtures inside a fridge until it is needed for the caspase assay.

Once it is time for the caspase assay, take out a new 96 healthy plate and add 50uM of Caspase assay buffer following 45uM of caspase lysis buffer. Then, add 5uM of each sample into their wells; make sure to use the pipette to pump the mixture up and down to remix the cell pellet and the caspase lysis buffer. 5uM of caspase substrate will be added after this; this is added to bind with the Caspase and, in turn, quantify the amount of Caspase in each mixture. Finally, bring the 96-well plate into the microplate reader and read it under 415 nanometers. Record the data and calculate the percentage change in caspase activity or the percentage change in caspase-induced apoptosis using the following equation.

RESULTS

In Vitro Experiment Results

Doxorubicin And Gallic Acid's Effect On Releasing LDH From The Sample Cells:

From figure 1, we can eliminate DH10k, DH1k, GA10k, GA1k, GA100, DH+GA10k, and DH+GA1k; as their % of cytotoxicity in regard to necrosis compared to the control is not significant compared to the other mixtures and is likely a result from errors and factors not related to the primary reaction. We are left with DH100, which had a 20% cytotoxicity in regard to necrosis compared to the control, DH 10 with 9.7% cytotoxicity in regard to necrosis compared to the control, GA10 with 5.3% cytotoxicity in regard to necrosis compared to the control and finally, DH+GA100 with 18.4% cytotoxicity in regard to necrosis compared to the control. We can reach the result with the ones obtained from the mtt cell-proliferation assay and the caspase assay to find mixtures that will induce cell death in other ways besides necrosis. From this experiment, we can conclude that the concentration of each mix will be

directly relative to the overall % of cytotoxicity, as the ones with the highest percentage of LDH released are the ones that are not serially diluted at all. DH100, DH10, GA10, and DH+GA100 are not effective tumor drugs, and more research and assays need to be done to find the right one.

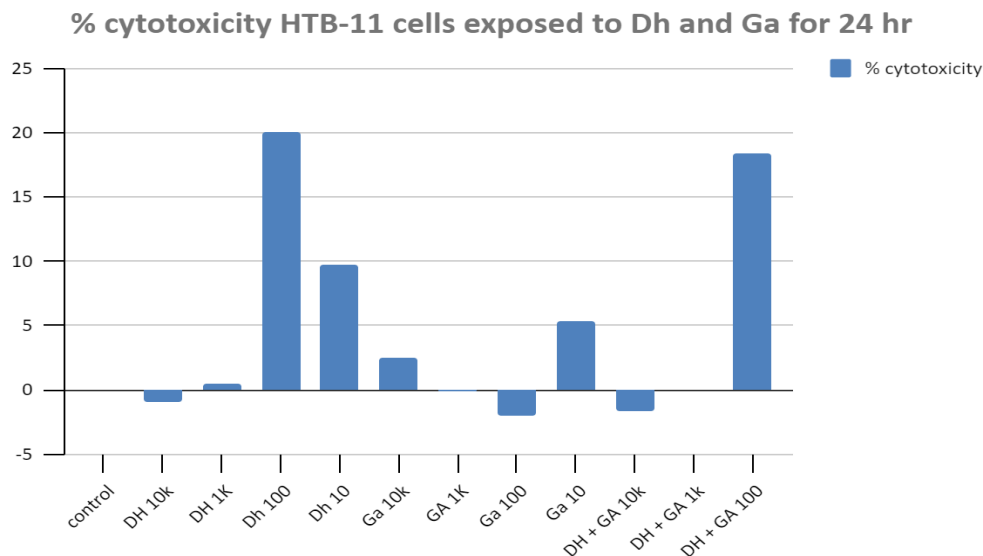


Figure 1: DH10k, DH1k, GA10k, GA1k, GA100, DH+GA10k, and DH+GA1k did not cause a significant amount of LDH to be released from the cell membrane.

Doxorubicin And Gallic Acid's Effect On HTB-Cell's Overall Survival:

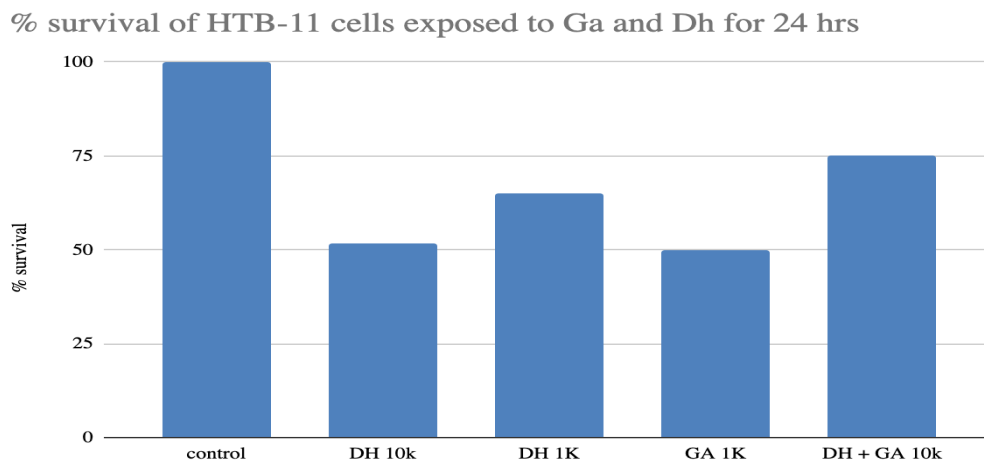


Figure 2: DH+GA1k, DH10k, GA1k, and DH+GA 10k caused the least % survival of the HTB-11 cells

From the data collected, we can conclude that most of the mixtures chosen bring the overall cell survivability to 50% after 24 hours. We then have to eliminate the variables deemed as "unsuitable" from the last experiment, which are DH100, DH10, GA10, and DH+GA100. After that, we can compare the results collected from this assay to those assembled on the previous one, so we can determine mixtures that, although they have been successful at causing cell death, happened through methods other than necrosis. DH+GA1k, DH10k, GA1k, and DH+GA 10k display the best results. From this experiment, we can find out that the more serially diluted mixtures often show better results. A further research target should also be to continue the dilution process, getting combinations such as GA or DH 100k for a different experiment. Currently, one more assay is needed to narrow the list even more.

Doxorubicin And Gallic Acid's Effect On HTB-Cell's Caspase Activity:

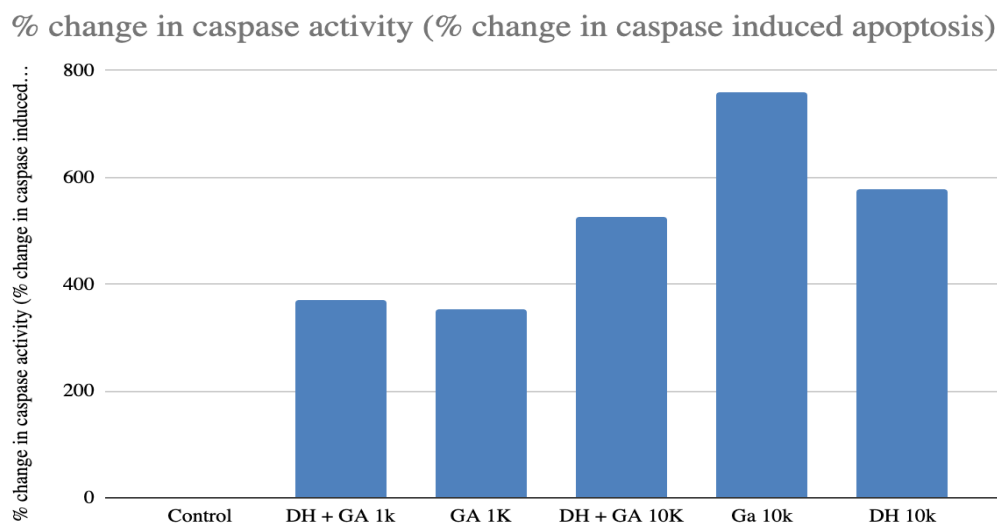


Figure 3: GA10k has displayed the highest % change in caspase activity (% change in caspase-induced apoptosis)

This graph shows the percentage change in caspase activity, which translates to a change in caspase-induced apoptosis. Since apoptosis is the desired result, the higher the percentage, the more influential the mixture is. Looking at the graph, DH+GA 10k, GA10k, and DH10k have the best effect on causing the change in caspase activity, which is expected as these are the three mixtures that released the least LDH while having a low cell proliferation rate. GA 10k had the best result in general, having a 758% percentage change in caspase-induced apoptosis. Overall, this assay can narrow the mixtures further; ultimately, we can find the best assortment for an effective drug for tumor cells, which is GA10k. However, looking at this graph, it will be better if a further experiment is done on mixtures with higher dilutions, as higher dilutions seem to translate to a better result.

Discussion

In Silico Screening:

While researching the chemicals that seem suitable to pair up with the PD-L1 receptor for molecular docking, anti-tumor antibiotics always display an above-average binding affinity. This does make sense as PD-L1 is present in most tumors. This means that if a chemical with a high binding affinity that is not an antitumor antibiotic is found, it is likely to display a similar effect as an antitumor antibiotic, except it's not as researched. This is how gallic acid is chosen.

In Vitro Experiment:

Although the three assays that are used in the experiment are the LDH assay to detect the amount of necrosis that the mixture has caused, the MTT cell viability and proliferation assay that calculates the number of living cells inside a particular environment, and the caspase-3 colorimetric assay which measures the amount of caspase induced-apoptosis seems to be arranged randomly; each assay plays a crucial role in the final calculation. In this experiment, we wish to see the mixture display the least result in the LDH assay, which translate to causing the least amount of necrosis while having the highest percentage in the Caspase assay, which means that it caused the most caspase-induced apoptosis. The process of elimination is used to find the best result. Necrosis is not wanted because it's the direct culprit of the poor prognosis of cancer patients, while apoptosis is crucial in tumor regression.

Conclusion

This study presents a thoroughly tested drug that will effectively combat HTB-11 tumor cells. Through a combination of MTT, LDH, and Caspase assay, we can slowly narrow down the selections, which turns out to be Gallic acid with a 10000 times dilution at the end. The criteria for a good result: release of a minimal amount of LDH, having a low percentage of cell proliferation as well as having a high caspase activity, was matched, and overall, it has been proven to be more effective than some conventional drugs used to fight these tumor cells. However, this is not the end; as I have stressed many times before, although we can, through tests, find a plausible tumor fight drug, experiments with higher diluted mixtures should be done. While gallic acid 10 μ M is exceptionally effective, it's unknown whether mixtures such as gallic acid on a hundred thousand or even one million dilutions will be even more effective.

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