Data Entry Illustrations

"Step-By-Step Instruction" for Using CompuSyn Software for Data Analysis with flexibility and Options: For Single Drug or Drug Combinations

Example: Two Anti-Cancer Drug Combinations, (D_1+ D_2), in Vitro

Start by Clicking "**CompuSyn**" logo, and Click "**New Experiment**"

A typical 2-drug combination *in vitro* at a constant ratio combination usually uses *only 16 data points* for dose and effect, e.g., 5 concentrations for each drug, and their combination, and an uninhibited control, for the constant ratio at or near $(IC_{50})_1/(IC_{50})_2$ ratio, via "2-fold serial dilutions" in a diagonal scheme. The experiment with six or more concentrations each would be even more desirable if the proper concentration-range is not certain.

All *in vitro* assays are carried out on the same day under standardized conditions, and usually in duplicate or triplicate and only the average effect values (fa's) for each drug concentrations were used for the dose-effect data entries into computer for automated computerized analysis. The manual inputs of Experimental Title, Date, Drug Names (including Abbreviations), and Experimental Data may take about 10 min (see below); Computerized Simulation may take 1 seconds; the print-out time and saving the file may take 2 minutes.

Usually drug combination experiments *in vitro* take one to two weeks to complete. The 15 minute data entry and information entry is minor essential effort for the study, and it provides the opportunity to inspect and to delete the extremely low effect or extremely high effect data points which are beyond the accuracy of the assay. *Do not enter*

unreliable data into computer **for data analysis. The negative fa value , or fa=0 or fa=1 would cause the analysis to crash. The r value of the median-effect plot will show how good is your data with r=1 indicating perfect. Usually fa>0.95 (in vitro) and fa>0.92 (in vivo) are considered acceptable.**

More detail information of the CI theory is available in the Chou, T.C. Text and Supplemental Appendices in Pharmacol. Rev. 58:621-681, 2006. *Questions & Answers* are available in Chou T.C. Cancer Res. 70: 440-406, 2010; Integr. Biol. 3: 548-559, 2011; and Synergy 1: 3-21, 2014.

If the theory is difficult to follow, the best way to learn the applications of the Chou-Talalay's CI method is to read the step-by step illustrations given below using examples of real design and real data analysis. These include the specific *CompuSyn Reports* *given below* for *in vitro* 2-drug combos (Zhang et al. Am. J. Cancer Res. 6: 97-104, 2016), and for *in animal* 2-drug combos (Fu et al. Synergy 3: 15-30, 2016). The latter can serve as a model for the drug combination clinical protocol design and execution using only 10 data points.

A. Enter Experimental Information for Your Record

1. Enter the abbreviated experimental "**Name**" (e.g., FD+PXT against MX-1 cells in vitro).
2. Enter "**Date**"
3. Enter "**Description**" of experimental details and conditions: Full name of drugs used, assay and cell used, in vitro or in vivo, and other notes if needed.

B. Data Entries

For Single Drug (**D_1**)

1. Click "**New Single Drug**" [menu pop-up]
2. Enter "full name", "Abbreviation". "Unit"
3. Enter the 1st pair of "Dose" and "effect" data for D_1
   2nd, 3rd, 4th and 5th pairs of D&E data for D_1
4. Enter "Finish"
5. Repeat B1-B4 for **D_2** (instead of D_1)
6. [If D3 data are available, repeat B1-B4 for **D_3**; Later, you may select D_1+D_2, D_1+D_3, D_2+D_3, or D_1+D_2+D_3 combos]

7. Click "**New Drug Combo**": e.g., Combo-1 (1:1 combo for D_1:D_2);
   Combo-2 (1:3 combo for D_1:D_2)
8. Repeat B1-B4 above for $(D_1+D_2)_1$ (instead of D_1).
   Until Enter "Finished" for Combo-1.
9. Repeat B1-B4 above for $(D_1+D_2)_2$; Until enter "Finished", if there is a Combo-2.
10. For constant ratio combos (e.g., Diagonal scheme design); Usually make a mixture at higher concentration, and then 2-fold serial dilutions.
    e.g., D_1:D_2 = 1:1 or 1:3; This type of ***"constant ratio design" is highly recommended,*** since it allows automated simulation for Fa-CI plot and Fa-DRI plot simulations.
    Enter "Ratio", the computer will memorize it.
    When you enter D_1 dose, the computer will automatically pop-up the dose of D_2, and the combo dose of $[D_1+D_2]$.
11. For non-constant ratio combos (e.g., pairs of 1:1, 1:2, 1:5, 1:10, 1:20; 1:3. 3:1, 5:1…etc.
    You need to enter individual pairs of each individual "dose" and "effect", until "Finished". With non-constant ratio design, CI and DRI can still be determined, but the *Fa-CI plot* and the *Fa-DRI plot* can only be constructed, but not be simulated for extrapolations. In addition, classic isobologram cannot be constructed, only the dose-normalized isobologram (*Chou & Chou Isobol*) can be constructed.

C. Data Correction and Deletion

1. "Edit" "dose" or "effect" of single drug or combos. Data points out of range of effect assay sensitivity limits [e.g., too low (fa<0.02) or too high (fa>0.99)] may be deleted). Data of experimental inadvertent errors may be deleted.
2. "Delete" drug (for entire set of data).

D. Other Options (Avail at early menu page, in "Report Option")

1. The default Isobologram effect levels (Fa's) are: 0.5, 0.75, and 0.90.
2. You may change to any preferred effect levels, such as 0.4, 0.5, and 0.8, etc. (By doing so, can sometimes avoid over-crowding of the isobologram which is difficult to read, depending on the actual data points distribution, or out of scale so some data points can be missing from the graph).

[The advantage of the Fa-CI plot is no over-crowding. The Fa range can be, e.g., from 0.05-0.97, etc.]

3. The default effect **(Fa) levels** in the "CompuSyn Report" Summary Table at the end of the Report are: 0.5, 0.75, 0.90, 0.95 and 0.97. These can be changed to your desired levels.
4. [When combination is 3 or more drugs, the "polygonogram" will be automatically constructed (e.g., for the optimal Cocktail Design). When only *2-drug combination*, do not select the "polygonogram" option in the "Generation of Report", since it is *not applicable*.]
5. The default Fa level for the polygonogram is 0.90. You may change to other Fa value if you prefer, with reason(s). [e.g., Hormones, regulatory drugs, hypertensive, or hyperglycemic drugs, etc., the usual Fa range is narrower and lower, although fa=0.9 is Ok for anti-cancer or antiviral drugs.]
6. Other unique options not frequently selected are: (i). The 95% confident limit bars for the Fa-Ci plot. This is usually not necessary, just like when we measure Km or Ki, we don't need to calculate +/-95% intervals, unless it is a very comprehensive study. (ii) The use of **Serial Deletion Analysis (SDA),** introduced by Chou, TC for the CompuSyn). Since there is not exact requirement of how many data points are needed for a dose-effect curve, the recommended data points for in vitro experiments are 4-8, for animal or clinical trials, the data points are 3-5 for each single drug, and their combinations. When we have a dose-effect curve with 5 data points, A, B, C, D and E, based on SDA, we may delete one point at a time and run analysis automatically. Thereby one curve with 5 points for one run becomes additional 5 runs with 4 points. The results of these 6 runs allowed statistical analysis for the 95%-confidence limit bars, etc.

E. The Flexibility and Scope of Applications

For single drug or drug combinations, the same Median-Effect Equation (**MEE**) or Combination Index Equation (**CIE**) is applicable in vitro, in animals and in clinical trials as indicated repeatedly in Chou TC's review articles given in the present web site. The differences for in vitro, in animal, and in clinical trial studies are the practical aspects such as time, cost, equipment,

facility, sample size, dose range and density, variability, and ethical and legal liability.

F. Specific Examples of CompuSyn Report Printouts for Drug Combinations

The detailed drug combination studies in vitro and in animals, as well as illustrations and interpretations are given below. It is suggested that that the MEE and CIE users would be greatly benefited by going through step-by-step running through with real numerical data analysis to familiar with the method.

1. "Synergistic combination of anticancer fludelone with cytoprotective panaxytriol against MX-1 cells ***in vitro***: Experimental design and data analysis using the CI method". Zhang N, Fu J and Chou TC. ***Am. J. Cancer Res. 6: 97-104, 2016***
2. "Drug Combination **in Animals** Using CI Method: Taxotere and T607 against Colon Carcinoma HCT-116 Xenograft Tumor **in Nude Mice**" Fu J, Zhang N, Chou JH, Dong H-J, Lin SF, Ulrich-Merzenich, GS and Chou TC. ***Synergy 3: 15-30, 2016***

These two **CompuSyn Report** examples are given in the last section of this web site.

Important Notes and Precautions

A. Why Data Entries Did Not Use Excel Spread Sheet for Convenience?

1. CompuSyn is a *general* software for *dose* and *effect* Pharmaco-Dynamics (**PD**) and Bio-Dynamics (**BD**) which can do tens of different applications, as indicated by many *options* with great deals of flexibilities. It is not a single purpose software.
2. Drug combination in vitro, in animals and in clinical trials many take weeks, months, and years, respectively, and may

cost thousands to millions of dollars. Combo data entry into computer using CompuSyn software may take 15 to 30 minutes, and the entire analysis as shown in the CompuSyn Report will take about one second, with about 10 pages of report for 2-drung combinations. We don't take data analysis lightly since computer take only digits for analysis in which even a single data point if outlier, out of sensitivity limit of measurements or assays, inappropriate dose-range design, an inadvertent human error, or irrational data input (e.g., Fa =0 or Fa>1.0) the entire combo study may render meaningless or invalidated. For example, when Fa=0, log (0) is negative infinity in the median-effect plot, which makes computer to freeze. Computer take number seriously, there will be no excuse, no leniency and no grace. On logarithmic scale Fa=0.09 to 0.9 to 0.99 to 0.999 has the same distance in difference. We should not enter "unreliable data" into it, otherwise we take the consequences. The conclusion, at best, is only as good as its data. If experimental design or results are not satisfactory, repeat and improve the experiment, do not try to use statistical means to justify the deficiencies.

B. What Combination Ratio or Mechanism Means to the Computer?

1. We know that we are doing drug combinations, the computer doesn't care a bit. It only takes the digital numbers and analyzed them as dictated by the ME Equation/CI Equation algorithms.

2. When we have constant-ratio combinations e.g., 1:1 or 1:3, etc., their combined effect are also the digital numbers as the single drug effects numbers. In a sense, the combined different doses in the mixture (e.g., 1:3) just "behaves like a third drug" that produces the doses and effects. All analysis *only* based on the theory's algorithm.
3. Usually for a potent drugs, the IC_{50} may be in the nM ranges, while others may be in uM ranges. When we say D1:D2 = 1:1, it can be 1 nM: 1 uM, or 1 nM : 1,000 nM. Depending on your unit designation, 1:1 (nM vs uM) or 1:1,000 (nM vs nM), both set of data should give you *exactly identical* conclusions of synergism or antagonism!
4. Not only computer doesn't care what units you use for each drug (e.g., nM, uM, mg/ml, IU, Rad or multiple of infection, etc.). The computer also doesn't care what the mechanisms of each drugs. The PD or BD theory based *only* on the physical, chemical and mathematical general principle of the mass-action law. The algorithm will not tell you how and why synergism or antagonism occurs.
5. Many researchers conducted drug combination studies along with many mechanistic studies. Mechanisms may provide you limited clues for speculations, but mechanisms will *never* quantitate synergism or antagonism.

C. How Many Data Points Are Needed for Drug Combination Studies?

1. The theoretical minimum of two-data points are required to draw a dose-effect curve. This is the breakthrough for the historical conventional thinking that we can only draw a

straight line for two data points. In facts, 2-data points actually have 4-points. The 3rd point is *dose zero*, and the 4th point is the *median-effect dose* (D_m), which is the universal reference point and the universal common link. (*see* Chou TC. Pharmacol Rev 58: 621-681, 2006; Chou TC. Integr. Biol. 3: 548-559, 2011). This is the theoretical basis for Econo-Green Bio-Medical R&D.

2. Therefore, the theoretical minimum for a 2-drug combination for synergism/antagonism is five data points (i.e., 2-points for each drug, and 1 point for combination).
3. However, due to biological variability, limitation in measurement accuracy, and imperfect technical skill, etc. We need duplicate or triplicate assays, and improve practical logistic means. In animal studies and clinical trials, in addition to the cost and time consuming steps, there are also ethical consideration and legal liabilities. Based on the mass-action law based PD and BD econo-green principle, it has been proposed that **the total data points** for drug combination for two drugs and their combinations ***in vitro* is 15-18**, for **animal and clinical combo studies is 9-10** (*see* Chou's review articles above, and the illustrations for the two examples of CompuSyn data analysis, in vitro and in vivo, given above).