STUDY DESIGNS

IN BIOMEDICAL RESEARCH

COMBINATION CHEMOTHERAPY

Use of a combination of two different therapeutics (drug-drug, radiation-drug combinations) becomes more and more popular, especially drug-drug combination. Therapy by drug-drug combination is called "**combination chemotherapy**".

Why Combination Therapy?

- To spread out toxicities to different organs with smaller dose of each drug.
- Other reasons: Improved quality of life (one to reduce side effects of the other), less drug resistance.
- Most important rationale: Enhancement of tumor response: It is more lethal.

BASIC QUESTION

- When both of the agents in a combination are active, that is to produce positive tumor response, frequently we wish to compare the therapeutic result of the combination with the results achieved by the component agents.
- Is the effect of the combination equivalent to or greater than the sum of the individual effects?

When the addition of one agent apparently increases the effect of the other, so that the effect of a combination appears to be greater than would be expected; the term synergism is used to describe these situations with enhancement of tumor response

- The term antagonism is used when the effect of the combination less lethal than the sum of the individual effects
- Without synergism and antagonism, the two individual effects are additive (i.e. neutral, no combination effects)

Actually, terminologies are still not yet universal or standardized. In one review article by Golden and Mantel in 1957, seven (7) definitions of "synergism" were given and in a more recent review by Greco et al. (1995), 13 different methods for determining synergism were listed and two methods may not agree with each others. This lecture uses the language and method given in Chou and Talalay (1984), the most popular article in the field.

For example, for some in the field, “Synergism” and “Enhancement” are two different concepts. Synergism (and Antagonism) are two-sided or “mutual” whereas Enhancement (or Augmentation, or Potentiation) is one-sided: One drug is ineffective (so, its ED50 does not exist) but it helps to improve the effect of the other drug (e.g. lower the dose for 50% effects).

- When both of the agents in a combination are active, that is to produce positive tumor response, frequently we wish to compare the therapeutic result of the combination with the results achieved by the component agents.
- For example , for simplicity: when we use a dose equal half of the ED_{50} of drug 1 and a dose equal to half of the ED_{50} of drug 2, is the result equivalent to using either drug alone at its ED_{50} ?

Experiments must be done:

(1) Stage 1: to characterize ability of each drug to kill cancer cells or to shrink tumors; before

(2) Stage 2: to see if the drug combination is more lethal than the sum of individual effects; both are often in the form of early, pre-clinical experiments

- either “In Vitro” or “In Vivo”.

PRE-CLINICAL TRIALS

- In Vitro: “Outside the living body and in an artificial environment” (i.e. in labs).
- In Vivo: “In the living body of a plant or an animal”

The process in Stages 1-2 would depend on the nature of the treatment's "tumor response":

- (1) If the effect of the treatment is expressed by its ability to kill cancer cells, the outcome of "**cell survival**" is binary and studies in both stages are **In Vitro**;
- (2) If the effect of the treatment is expressed by its ability to shrink tumors, the outcome of "**volume reduction**" is continuous and studies in both stages are **In Vivo (animals).**

A STAGE 1 IN VITRO DESIGN

Cells from a tumor-derived cell line are deposited in wells of a cell culture dish in complete growth medium. After phase growth is established (say, 72 hours in a typical cell line), wells are treated with different concentrations of a test agent – including a control (i.e. vehicle) well. Doses are spread over a wide range from very low to very high.

The endpoint is “cell survival” and the aim is to establish “potency parameters” such as ED50. This experiment is often called “Dose-ranging Experiment”

EXAMPLE:

Cells: ALL

Drug: Vincristine

(Extra feature: original and recurrent tumors from the same patient which is not needed here)

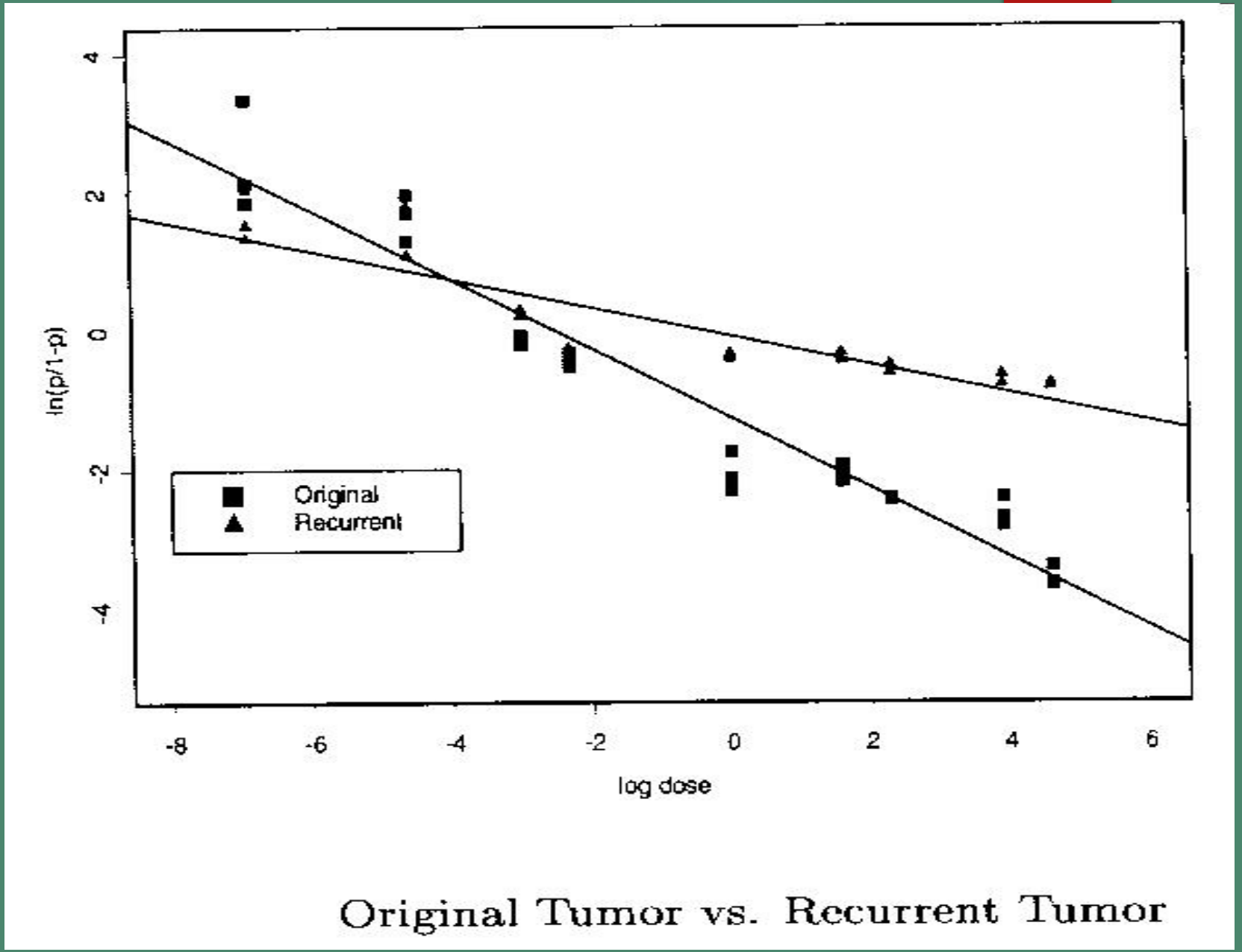
Original Tumor		Recurrent Tumor	
Dose ($\mu g/ml$)	Cell count (x 10^4)	Dose ($\mu g/ml$)	Cell Count (x 10^4)
0	233.8	0	70.8
	212.6		69.3
	221.5		66.6
.001	202.0	.001	58.2
	205.3		54.9
	197.9		58.9
.010	183.3	.010	52.9
	186.1		54.5
	187.2		57.0
.050	104.4	.050	39.1
	100.9		38.4
	106.6		38.2
.100	86.8	.100	30.2
	88.5		26.7
	91.3		29.0
1.000	21.1	1.000	28.1
	31.0		28.3
	23.2		27.8
5.000	24.9	5.000	27.5
	26.8		27.0
	22.4		27.9
10.000	18.9	10.000	24.7
	17.0		26.1
	17.5		25.1
50.000	14.5	50.000	22.2
	17.5		23.8
	12.4		22.7
100.000	5.9	100.000	21.4
	6.9		21.1
	7.2		21.41

Two straight lines:

similar intercepts;

Recurrent tumor:

smaller slope



Original Tumor vs. Recurrent Tumor

AN IN VITRO MODEL

Cells in a well; some die, some survive the treatment – could consider "Logistic Regression".

"d" be one of the doses; x = log (d)

n_0 = # of surviving/viable cells @ control well

n_x = # of surviving/viable cells @ dose "d";

$p_x = n_x/n_0$ % of surviving cells @ dose "d".

$$\ln \frac{p_x}{1-p_x} = \alpha + \beta x + \varepsilon$$

$$\ln \frac{p_x}{1-p_x} = \alpha + \beta x + \varepsilon$$

Note that:

(1) This is the Logistic Regression model;

(2) Drug is used on the log scale;

(3) If we set p = 0.5, we can solve for x;

(4) ED50 = exp(x)

A STAGE 1 IN VIVO DESIGN

A group of mice with induced tumors, say n=50, 10 mice are selected and sacrificed to measure baseline tumor volumes. The other 40 mice are randomized into 10 groups of 4 mice each treated with 10 different doses of a test agent; doses are spread over a wide range from very low to very high.

The endpoint is “tumor volume” and the aim is to establish “potency parameters” such as ED50. This experiment may also be called “Dose-ranging experiment”.

IN VIVO MODEL

Endpoint is “tumor volume” – continuous scale

“d” be one of the doses; x = log (d)

v_0 = average tumor volume of control group

v_x = average tumor volume treated with dose “d”;

$p_x = v_x/v_0$; $(1-p_x)$ is % tumor reduction for dose d;

$$\ln \frac{p_x}{1-p_x} = \alpha + \beta x + \varepsilon$$

$$\ln \frac{p_x}{1-p_x} = \alpha + \beta x + \varepsilon$$

Note that:

(1) This has the form of a Logistic Regression model (with grouped data) but analyzed as a regular Regression Model (term on left hand side is on continuous scale – not binary responses);

(2) Drug is used on the log scale;

(3) If we set p = 0.5, we can solve for x;

(4) ED50 = exp(x)

Most of the times a statistical model is just an assumption- fitting data or not.

In this case, the models - In Vitro & In Vivo models - originate from (or agree with) a well-established principle in pharmacology: **Median Effect Principle of Pharmacology**. It is supported by overwhelming empirical evidence but there still are exceptions.

MEDIAN EFFECT PRINCIPLE

When a dose d of an agent is applied to a pharmacological system, the fractions f_a and f_u of the system affected and unaffected satisfy the so-called "median effect principle" (Chou, Journal of Theoretical Biology, 1976):

$$\frac{f_a}{f_u} = \left\{\frac{d}{ED_{50}}\right\}^m$$

where ED_{50} is the "median effective dose" and "m" is called a Hill-type coefficient. If we set "$p = f_a$", the median effect principle and the logistic regression model are completely identical with a slope $\beta_1 = m$. That is, Drug Dose is on Log scale in a Linear Logistic Model

DETERMINATION OF ED50

$$\ln \frac{p_x}{1-p_x} = \alpha + \beta x + \varepsilon$$

In both In Vitro and In Vivo experiments, we can fit the above model and obtain estimates of “intercept”, “a”, and “slope”, “b”, by MLE (In Vitro) or least squares (In Vivo). Then log of ED50 is obtained by setting p = .5

$$ED50 = \exp(-a/b)$$

Next Question:

In Stage 1 (with one drug), We could “see” – or illustrate - levels of drug potency from different responses to different doses; and we could measure potency or strength by parameter such as ED50.

In Stage 2 (with two drugs), how to illustrate synergistic or antagonistic effects? And, in the presence of synergism, how to measure its strength?

THE ISOBOLOGRAM

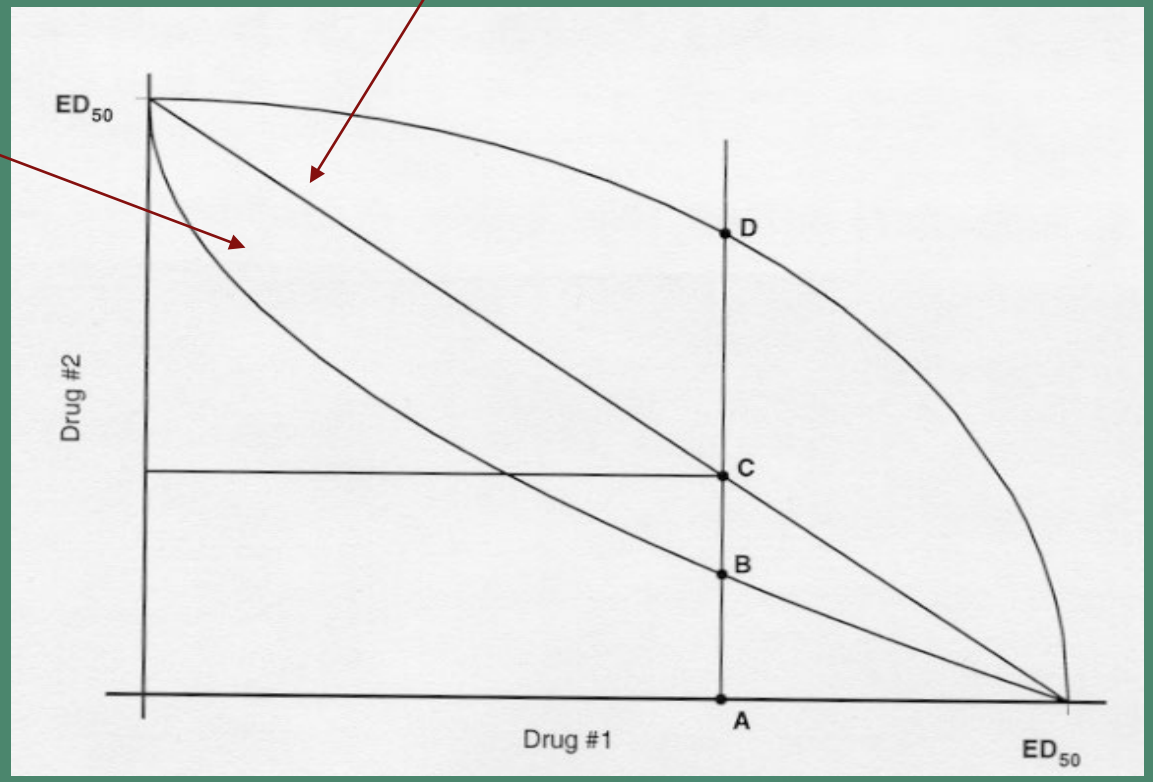
- Steel & Peckham (International Journal of Radiation Oncology, 1979) use a graphical device called "Isobologram" to evaluate and to illustrate what happens when two agents are used in combination
- Effect doses (EDs) of two drugs are put on axes
- A straight line joining two points of the same effect level, say from the ED_{50} of drug 1 on the x-axis to the ED_{50} of drug 2 on the y-axis, is called an iso-effect line: e.g. the 50% iso-effect line.

SYNERGISTIC REGION

You fix dose D of drug 1 and increase the dose of the other drug so as to reach the same response, say 50%: synergism

If you need less Drug #2 in the combination (stop below the line, say point "B"), to reach the same effect; that's synergism. By varying the chosen level "A", the endpoint trace a concave curve .

Additive line

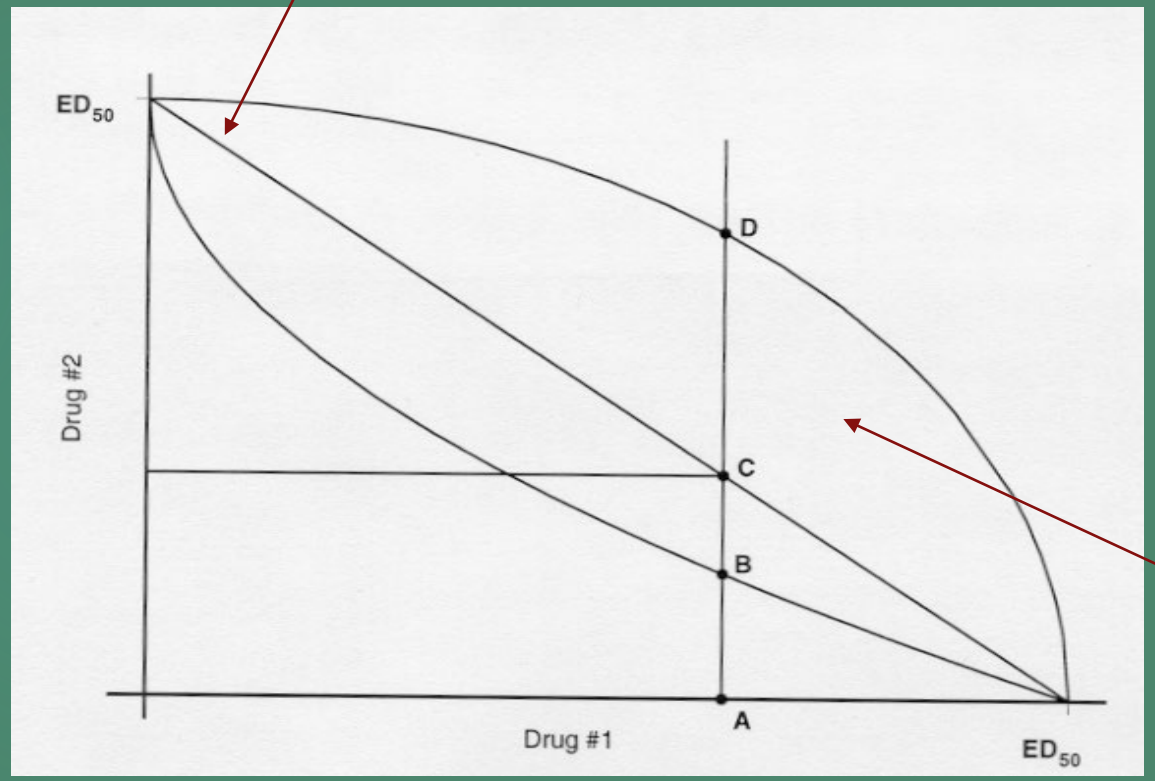


The region below the “Additive Line” is the Synergism Region. Point B, where response reaches 50%, is “deep” in the synergism region, and away from the additive line, when amounts of the two drugs are more similar – say, each is about half of its ED50.

ANTAGONISTIC REGION

additive

You fix dose "A" of drug 1 and increase the dose of the other drug so as to reach the same response, say 50%:



If the two drugs in the combination act *antagonistically*, one needs more drug 2 to reach the same effect and the resulting curve is convex.

antagonism

The region above the “Additive Line” is the Antagonism Region. Point D, where response reaches 50%, is “deep” in the antagonism region, and away from the additive line, when amounts of the two drugs are more similar – say, each is about half of its ED50.

Let d1 and d2 be the amounts of drugs 1 and 2 respectively (on the two axes), the equation of the “Additive Line” is:

$$\left[\frac{d_1}{ED50_1}\right] + \left[\frac{d_2}{ED50_2}\right] = 1$$

“COMBINATION INDEX”

Chou & Talalay define a “combination index” CI as follows; if CI<1, it’s synergism, if CI>1, it’s antagonism, and if CI =1, effects are additive (Chou & Talalay, Advances in Enzyme Regulation, 1984)

$$CI = \left[\frac{d_1}{ED50_1}\right] + \left[\frac{d_2}{ED50_2}\right]$$

The authors maintain that it is based on the “mass-action law”; it has become “the standard” to evaluate combination therapies

HOW TO PROCEED?

You fix dose "A" of drug 1 and increase the dose of the other drug so as to reach 50% (point B)

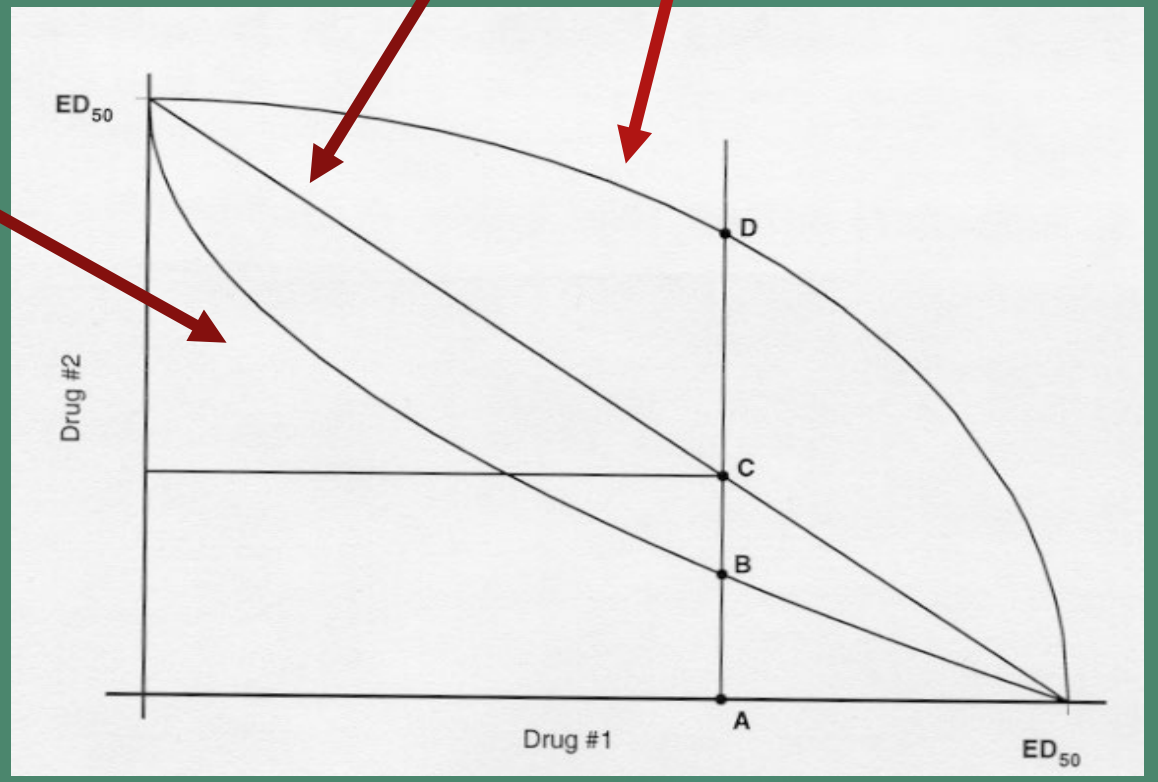
Additive line

antagonism

synergism

Question:

How do we get to point "B" or "D" in the diagram? i.e. how to "calculate" the dose of Drug 2 – the distance from "A" to "B", or "A" to "D"?



The question is how to design an experiment in Stage 2.

Should we “fix” the amount of one drug and try to figure out the amount of the other drug so as to have a mixture with 50% response? If so, does it matter which drug to fix (perhaps not) and at what amount?

Issue #1: EXPERIMENT DESIGN

Should it be a real "Trial by Error": You fix a dose of Drug 1 and increase the dose of the other drug so as to reach a preset response, 50%? & do again if needed ? This is very time-consuming and un-practical – even not possible. Experiments are usually done in one of two possible ways:

(1) Non-constant drug ratio, and

(2) Constant drug ratio

Say, we can fix the dose of Drug #1 at "dose = a" and combine with varying doses of drug #2: b_1, b_2, … b_k; the combined does are: {$d_1 = a+b_1$, $d_2 = a+b_2$, …, $d_k = a+b_k$}. Given this series of combined doses and the corresponding responses (e.g. percentages of cells killed), we then fit the same "logistic model" and obtain $ED50_c$ of the drug combination. Note that the "ratio" of individual doses in a combined dose, say a/d_i, is not constant across the combined doses.

(1) After one such experiment with a series of k doses, we have one CI value

(2) Then you can vary the “a”, the fixed dose of Drug #1, to create another series, or

(3) One can systematically preset a “grid” with different doses of both drugs

(4) Fitting the model to each row and each column to obtain an CI value;

(5) Still, in each series – row or column – the ratio of drug doses is not constant across combined doses.

	Dose of Drug 1					
Dose of Drug 2	0	6	12.5	25	50	100
0	100.00	90.30	77.99	56.72	11.19	4.66
3	98.51	84.33	68.28	50.19	8.96	4.66
6	71.83	55.97	44.40	25.93	9.14	2.43
12.5	19.40	19.98	16.23	12.31	5.60	1.68
25	4.48	8.58	7.65	6.16	7.65	0.00
50	2.24	6.16	7.09	3.92	3.54	0.00

Entries are proportions of surviving cells.

This experiment was kind of poorly designed, i.e. over-dosed; in many configurations, doses of drugs #1 and/or #2 might exceed its/their ED50.

EXAMPLE

	Dose of Drug 1						
Dose of Drug 2	0	6	12.5	25	50	100	**ED50**
0	100.0	90.30	77.99	56.72	11.19	4.66	**21.68**
3	98.51	84.33	68.28	50.19	8.96	4.66	
6	71.83	55.97	44.40	25.93	9.14	2.43	
12.5	19.40	19.98	16.23				
25	4.48	8.58	7.65				
50	2.24	6.16	7.09				
ED50	**9.85**						

(1) Data on column #2 are what we have when dose of Drug 1 is fixed at d_1=6 and varying the dose of Drug 2. We fit data of column #2 to the model and obtain $ED50_c$: This is the combined dose; dose of Drug #2 which would added to d_1 = 6 of Drug #1 to achieve 50% response is ($ED50_c$ - 6).

(2) This is the “distance from A to B” we are looking.

CALCULATION OF CI

The “amounts” of individual drugs in the combination (needed to get 50% response) are: “6” and “ED_c- 6”; therefore:

$$CI = \frac{6}{ED50_1} + \frac{ED50_c - 6}{ED50_2}$$

(Note that if “k”, the number of combined doses, is large one would get ED50c, and hence CI, with negligible or small standard error.)

NATURE OF SYNERGISTIC EFFECTS

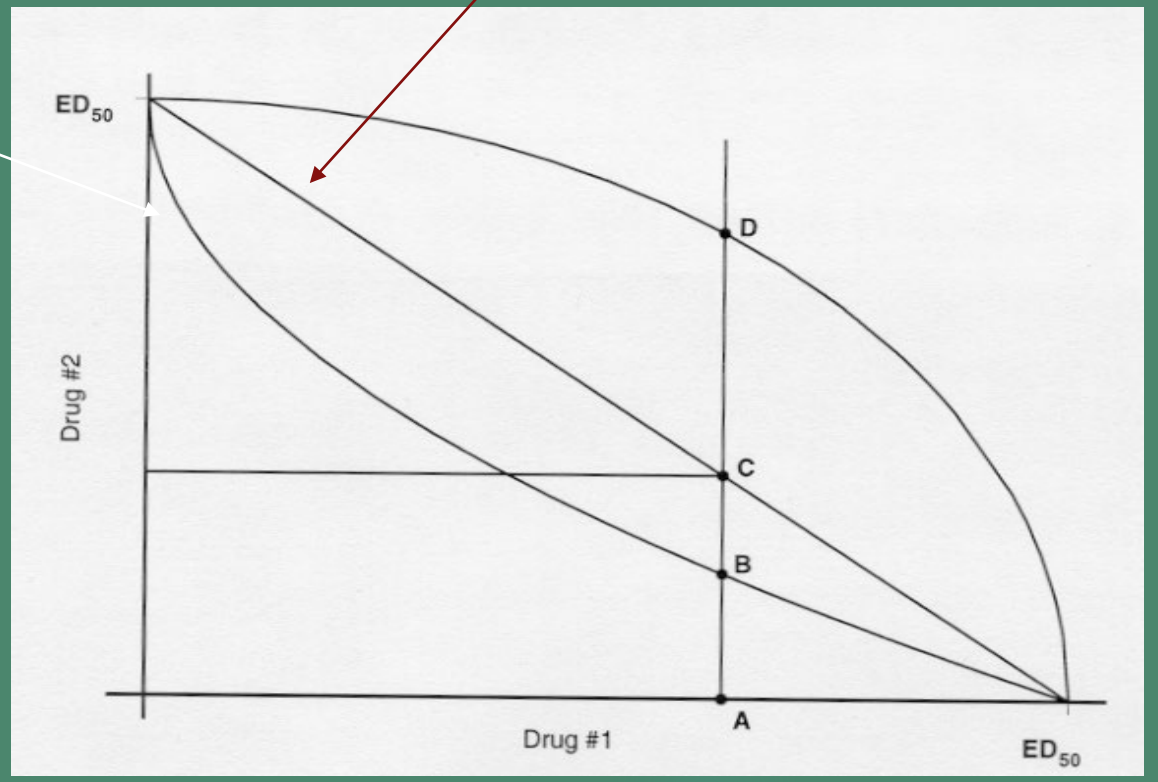
You fix dose "A" of drug 1 and increase the dose of the other drug so as to reach 50% (point B)

Additive line

synergism

A Possibility:

Point "B" may be further away from the "additive line" in the middle part than at the ends: Synergistic effects may vary with drug ratio



In designing experiments with non-constant drug ratio, data in each series carry different levels of synergistic effects; they may not fit the model well.

Even if dada still fit the model; the resulting CI value would have a larger standard error. In a review article (2010), Chou recommended only experiments with constant-ratio drug combinations.

Experiments with constant-ratio drug combinations can be designed as follows: Drugs are pre-mix at certain ratio, say p-to-q (p units of Drug #1 to q units of Drug #2) before dispensing into k combined doses from low to high. The ratio in all k doses is the same, p-to-q. The "units" are not conventional dose units (say, milligrams) because different drugs have different strengths which makes it hard to know how to set the ratio. One can use an unit the ED50 of each Drug.

For example, one can try 5 series of doses (each will yield one CI value):

3 ED50s of Drug #1 to 1 ED50 of Drug #2; and 2 ED50s of Drug #1 to 1 ED50 of Drug #2; and 1 ED50 of Drug #1 to 1 ED50 of Drug #2; and 1 ED50 of Drug #1 to 2 ED50s of Drug #2; and 1 ED50 of Drug #1 to 3 ED50s of Drug #2

i.e. The five ratios are 3:1, 2:1, 1:1, 1:2, and 1:3 in ED50 units.

CALCULATION OF CI

For each series, after the $ED50_c$ of the combination is obtained; the needed amount of each drug and the CI is calculated as follows:

$$d_1 = \left(\frac{p}{p+q}\right) ED50_c$$

$$d_2 = \left(\frac{q}{p+q}\right) ED50_c$$

$$CI = \frac{d_1}{ED50_1} + \frac{d_2}{ED50_2}$$

$$= \left\{ \frac{p}{(p+q)ED50_1} + \frac{q}{(p+q)ED50_2} \right\} ED50_c$$

Issue #2: PRECISION OF CI

An important statistical question is how to determine the Variance or Standard Error of CI (for experiment)? Chou, Talalay and most basic scientists try to avoid the issue (of sampling variation) by calculating the Index only from data sets which fit the model well; those with very high correlation coefficient (say exceeding .995):

$$\ln \frac{p_x}{1-p_x} = \alpha + \beta x + \varepsilon$$

Some other authors/statisticians propose to use “simulation to create “pseudo values” of Intercept, Slope, then CI; then use the pseudo sample of, say, 500 values of CI to form its 95% Confidence Interval. For example if Intercept α is estimated by “a” with standard error s; pseudo values of Intercept are obtained from: $a \pm N(0,1)*s$

In general one can obtained Variance of CI using "Delta Method" (Error Propagation), especially more simple in cases where individual ED50 were obtained from larger samples (so their standard errors can be considered negligible):

$$CI = \frac{d_1}{ED50_1} + \frac{d_2}{ED50_2}$$

$$= \left\{ \frac{p}{(p+q)ED50_1} + \frac{q}{(p+q)ED50_2} \right\} ED50_c$$

$$Var(CI) \cong \left\{ \frac{p}{(p+q)ED50_1} + \frac{q}{(p+q)ED50_2} \right\}^2 Var(ED50_c)$$

Issue #3: IS CI AN "INDEX"?

No matter how we do the experiment, the resulting value of CI is dose-dependent; It depends on the "drug dose ratio". Should we consider such a number an "Index"?

An “Index” is a summarized figure, a statistic representing a system or a phenomenon. CI is a “data point”. If we have a sample of size one (one experiment with a series of doses) then a data point would serve as an index; that was in the early days of combination therapies. If the sample size is greater than one (say, 5 series or more), one must combine all data points to form an “Index”.

A STATISTICAL MODEL

One possible approach is to assume a “Model for Isoboles” (Hewlett, 1969; Machado and Robinson, 1994). There are many possibilities, the following fits in very well with Chou and Talalay’s index:

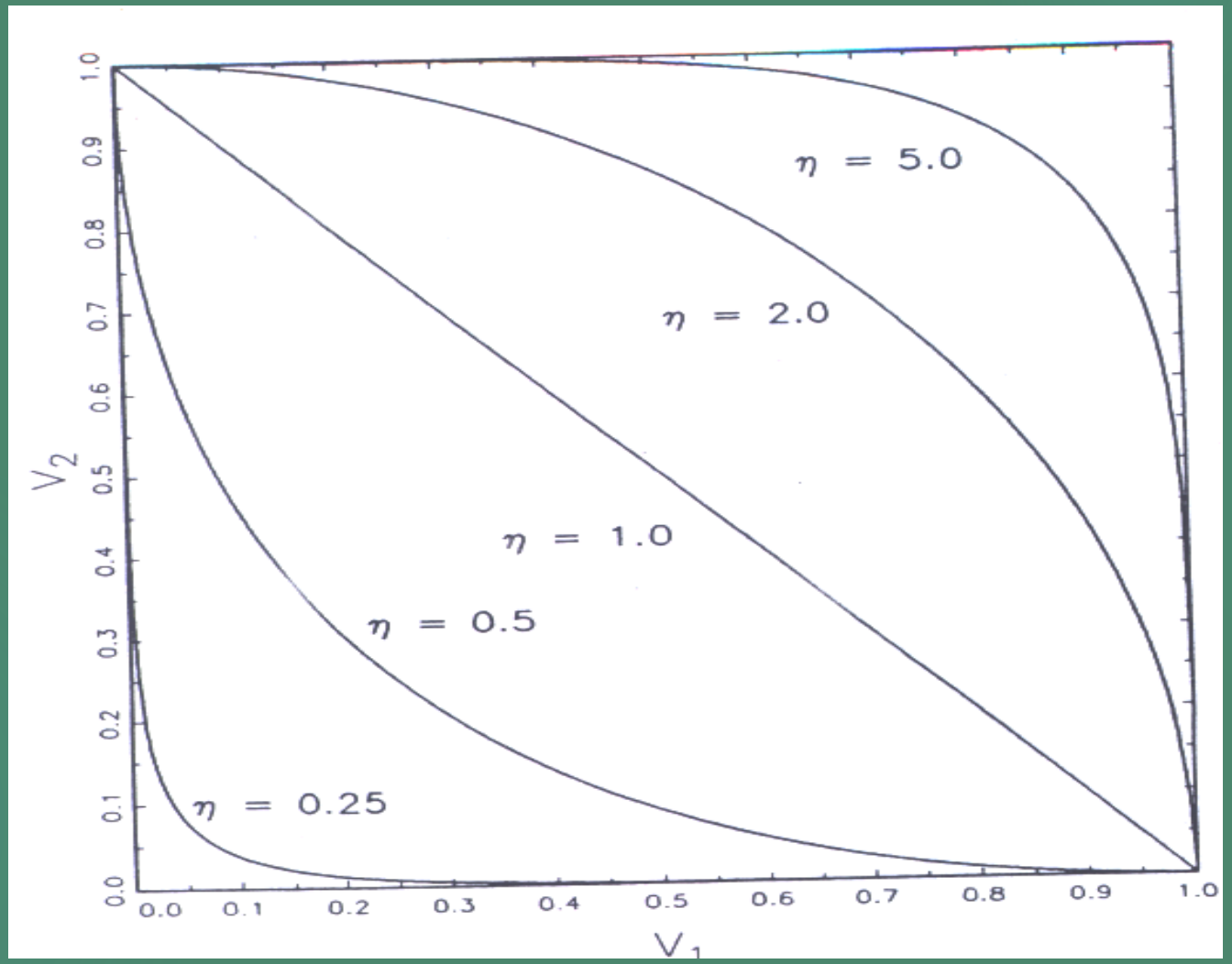
Let consider the "Model for Isoboles":

$$\left(\frac{d_1}{ED50_1}\right)^{\eta} + \left(\frac{d_2}{ED50_2}\right)^{\eta} = 1$$

η is the overall "Combinatio n Index":

$\eta < 1$ for Synergism, $\eta > 1$ for Antago nism,

$\eta = 1$ if Effects are Additive



Various values of η

$$CI = \frac{d_1}{E50_1} + \frac{d_2}{E50_2}$$

No matter how we design the experiment, each series of doses not only giving us a CI value but a point with coordinates (x,y) = (d1,d2); we can “fit” the “isobole model” by “Least Squares” to obtain a value for the Combination Index, a point estimate of η.

$$\left[\frac{x}{ED50_1}\right]^{\eta} + \left[\frac{y}{ED50_2}\right]^{\eta} = 1$$

Note that this overall “Index” might still depend on the “level of response”; the index at 50% maybe different (likely smaller, or stronger synergism) than the index at 80%.

SUGGESTED EXERCISE

	Dose of Drug 1					
Dose of Drug 2	0	6	12.5	25	50	100
0	100.00	90.30	77.99	56.72	11.19	4.66
3	98.51	84.33	68.28	50.19	8.96	4.66
6	71.83	55.97	44.40	25.93	9.14	2.43
12.5	19.40	19.98	16.23	12.31	5.60	1.68
25	4.48	8.58	7.65	6.16	7.65	0.00
50	2.24	6.16	7.09	3.92	3.54	0.00

a) Use data in the first column to determine ED50 for Drug #1
b) Use data in the first row to determine ED50 for Drug #2
c) Use data on the second column to determine ED50 for this combination; then calculating the combination index
d) Use data in the third column to determine ED50 for the new combination; then calculating the combination index and comparing to the result in c).