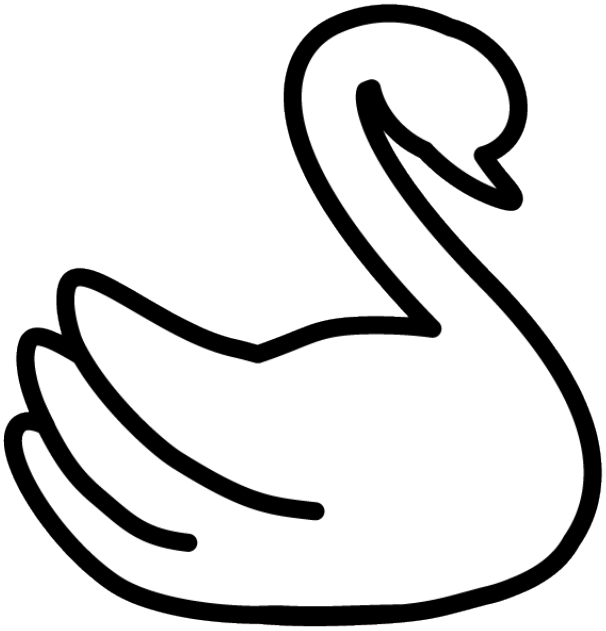

CISNE

User's Guide

Universidad de Oviedo, 2017

Contents

1. Introduction

1.1 What is CISNE?

CISNE is a new program developed at the University of Oviedo by a multidisciplinary group formed by physicists, chemists, biologists and medical doctors, who were unhappy with the available methods to compute synergism phenomena. CISNE stands for Code for the Identification of Synergism Numerically Efficient. Like other available programs, CISNE implements the theory of Chou *et al*. of synergism [1-5], but it differs from the rest in its new mathematical approach, which allows to obtain more accurate results from the experimental data.

Different versions of CISNE for Windows and Mac OS X users are freely available at:

https://cisnecode.github.io

At this webpage you can find:

- The CISNE installer for each different platform.
- The CISNE User's Guide.
- A package with templates and example files for toxicity and synergism studies.

Users are encouraged to cite CISNE in their publications with the following reference:

CISNE: An accurate description of dose-effect and synergism in combination therapies. **A. García-Fuente, F. Vázquez, J. M. Viéitez, F. J. García Alonso, J. I. Martín, J. Ferrer.** ***Sci. Rep.*** **8, 4964 (2018)**

1.2 Installing CISNE on Windows

There are 2 versions of CISNE available at https://cisnecode.github.io for Windows operating systems, one of them is designed for computers with 32 bits architectures and the other one is designed for computers with 64 bits architectures. Nowadays almost all modern computers have a 64 bits architecture and therefore the 64 bits version is highly recommended. Install the 32 bits version only if the 64 bits version does not work in your particular system.

To start the installation of CISNE, open the CISNE installer file that can be found at the CISNE webpage. A window like that of Fig. 1.1 should appear:

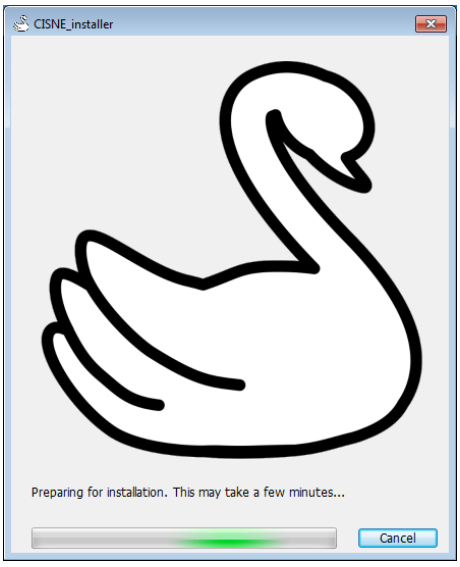

Fig. 1.1: installing CISNE on Windows.

After a few seconds, a second window like that of Fig. 1.2 should appear. Click “Next >” to continue the installation.

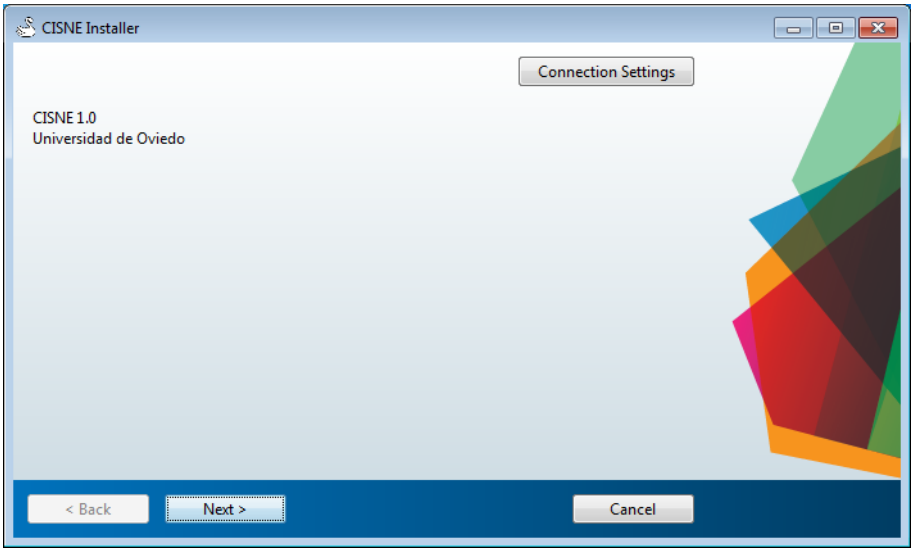

Fig. 1.2: installing CISNE on Windows.

The window of Fig. 1.3 allows to select the installation folder for CISNE. You can also add a shortcut to the desktop to run CISNE. Select a folder or leave the default option and click “Next >”.

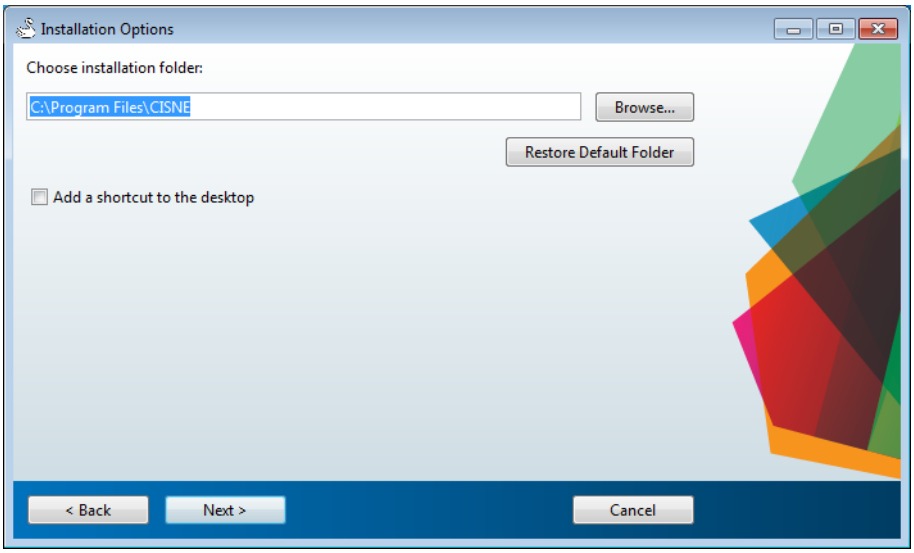

Fig. 1.3: installing CISNE on Windows. Installation folder.

CISNE needs the MATLAB Runtime to work. If it is not installed, the CISNE installer will automatically download it and install it on your computer. The particular required version of the MATLAB Runtime might depend on the particular version of CISNE being installed. A window like that of Fig. 1.4 will appear to select the installation folder for the MATLAB Runtime. Do not change the default folder and click "Next >".

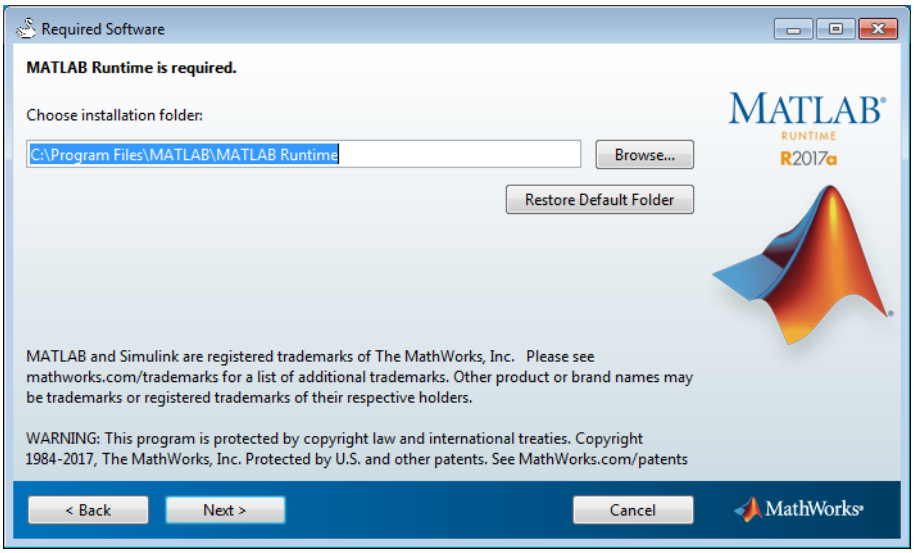

Fig. 1.4: installing CISNE on Windows. MATLAB Runtime installation.

You will be asked to accept the license agreement of MATLAB Runtime in order to install it (see Fig. 1.5). Accept the license agreement and click "Next >".

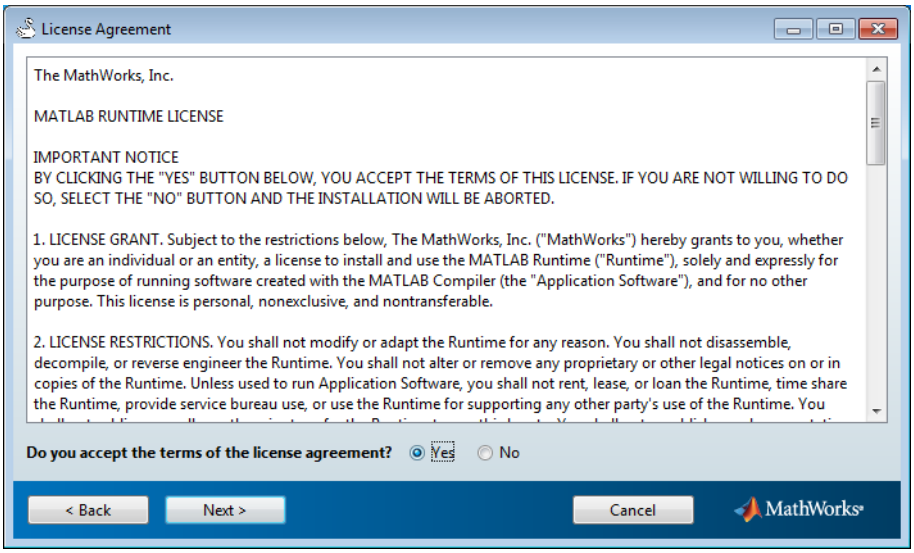

Fig. 1.5: installing CISNE on Windows. MATLAB Runtime license agreement.

Finally, a window of confirmation will appear, like that of Fig. 1.6. Verify that the information shown is correct and click “Install >”. CISNE installer will then download the MATLAB Runtime if needed and install CISNE.

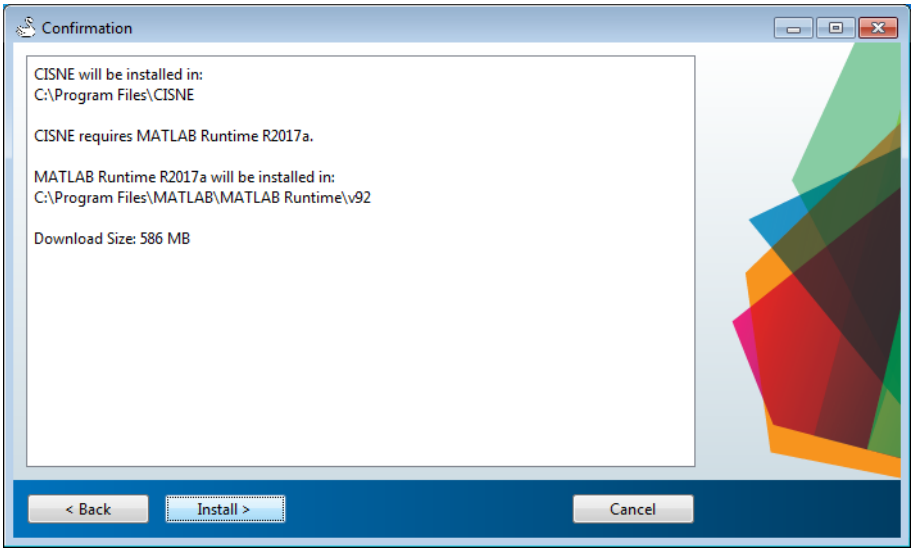

Fig. 1.6: installing CISNE on Windows. Confirmation window.

1.3 Installing CISNE on Mac OS X

At https://cisnecode.github.io there is also a version of CISNE for Mac OS X available. The installation of this version is almost identical to that of the Windows version. Simply open the CISNE_macOS_installer file and follow the same steps described in section 1.2. Once the installation is finished, a

window like that of Fig. 1.7 might appear. In that case follow the instructions shown in that window to make the MATLAB Runtime available for CISNE.

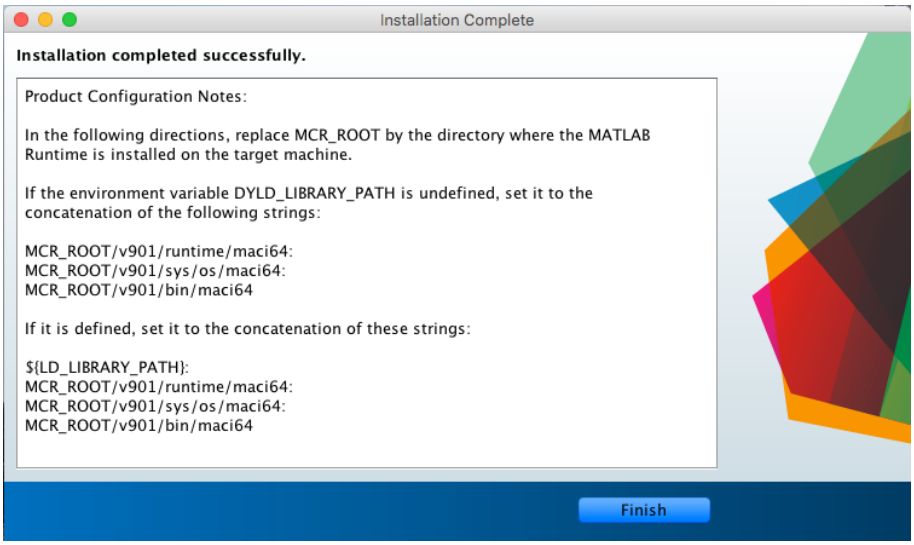

Fig. 1.6: installing CISNE on Mac OS X. Configuration notes.

2. Theoretical Background

2.1 The dose-effect relationship

CISNE describes the relationship between the dose of a drug applied to a system and its effect in terms of the median-effect equation introduced by Chou *et al* [1-3]:

$$\frac{f_a}{f_u} = \left(\frac{x}{D}\right)^m \qquad \text{[Eq. 2.1]}$$

where:

- f_a and f_u are the fraction of the system affected and unaffected by the drug, respectively. Therefore, $f_a+f_u=1$ for any drug concentration.
- x is the dose of the drug that induces the effect in the system.
- D is the drug concentration at which $f_a=f_u=0.5$, measured in the same units of the dose x.
- m is a parameter that indicates the particular shape of the dose-effect curve, where $m=1$ indicates an hyperbolic curve, $m>1$ indicates an sigmoidal curve and $m<1$ indicates a flat sigmoidal curve.

Alternatively, equation 2.1 can be written as:

$$f_u = \frac{1}{1+\left(\frac{x}{D}\right)^m} \qquad \text{[Eq. 2.2]}$$

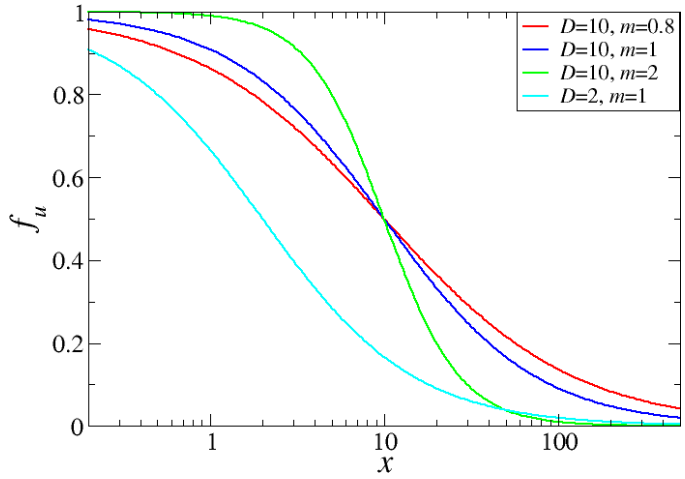

Fig. 2.1: several examples of dose (x) - effect (f_u) relationships.

Therefore, we only need 2 parameters *m* and *D* to completely describe the relationship between the variables *x* and f_u. Several examples of dose-effect relationships are given in Fig. 2.1.

In an experiment designed to determine the dose-effect relationship the objective is to measure the effect (f_a or f_u) of the drug for several values of the dose of the drug *x*, and from that experimental data obtain the *m* and *D* parameters that define the particular shape of the dose-effect relationship.

2.2 Synergism and the Combination Index

The concept of synergism might seem very intuitive at first, but it can lead to some misunderstandings. A combination of drugs is synergistic if their combined effect is larger than the additive effect of each drug separately. We might be tempted to think then that if a concentration *x*(A) of drug A leads to a fraction affected f_a(A), and another concentration *x*(B) of drug B leads to a fraction affected f_a(B), their combination is synergistic when it leads to a fraction affected f_a(A+B)> f_a(A)+ f_a(B). However, this would only be true if the dose-effect relationship was perfectly linear, which is not true (see Fig. 2.1). Instead, we need a definition of synergism that takes into account the non-linear shape of the dose-effect relationship.

To solve this problem, Chou *et al.* defined the Combination Index (*CI*) for a combination of 2 drugs as:

$$CI(f_a) = \frac{x_A^C(f_a)}{x_A^0(f_a)} + \frac{x_B^C(f_a)}{x_B^0(f_a)} \qquad \text{[Eq. 2.3]}$$

where $x_A^0(f_a)$ and $x_A^C(f_a)$ are the doses of drug A that produce an effect f_a individually and in combination with drug B, respectively. This equation can be generalized to the combination of *N* drugs as:

$$CI(f_a) = \sum_{i=1}^{N} \frac{x_i^C(f_a)}{x_i^0(f_a)} \qquad \text{[Eq. 2.4]}$$

A combination of drugs is then additive when its *CI*=1, it is synergistic when *CI*<1 and it is antagonistic when *CI*>1. Lower (larger) values of *CI* indicate a stronger synergistic (antagonistic) effect of the combination. Notice that *CI* depends on f_a, therefore it is possible that combination of drugs are found to be synergistic at some concentrations (leading to particular values of f_a), and antagonistic to others.

Eq. 2.3 can be rewritten in terms of the *m* and *D* parameters of the dose-effect relationship of each individual drug (m_A, m_B, D_A and D_B) as well as of their combination at a given ratio R_A:R_B (m_{A+B}, D_{A+B}) as:

$$CI(f_a) = \frac{D_{A+B}}{R_A+R_B}\left(\frac{f_a}{f_u}\right)^{\frac{1}{m_{A+B}}}\left(\frac{R_A}{D_A}\left(\frac{f_u}{f_a}\right)^{\frac{1}{m_A}} + \frac{R_B}{D_B}\left(\frac{f_u}{f_a}\right)^{\frac{1}{m_B}}\right) \qquad \text{[Eq. 2.5]}$$

2.3 How to design a drug-combination study

In order to apply Eq. 2.3 or Eq. 2.4 to determine the *CI* of a drug combination we need to know the dose of each drug separately and of their combination that leads to an effect f_a. However, in an experiment we can only control the applied dose *x*. Therefore, the dose-effect relationship of each individual drug, as well as of their combination at a fixed ratio, must be determined first. Then, from the obtained parameters Eq. 2.5 can be applied. Some important points must be considered:

- In order to obtain a good dose-effect curve, several doses *x* above and below *x*=*D* should be included in the study.
- The drugs must be combined at a fixed ratio, and the synergism results might be dependent of this ratio. A good first attempt can be to fix the ratio R_A:R_B close to the relation between the parameters D_A:D_B.
- Whenever possible, the determination of the effect of the different drug doses *x* of each individual drug and their combination at a fixed ratio must be performed under the same conditions and at the same time.

Once the dose-effect data has been obtained experimentally for each individual drug and their combination, CISNE allows to obtain directly the dose-effect relationship of each individual drug and of the drug combination. From that, CISNE calculates the *CI* as a function of f_a and its confidence interval.

3. Using CISNE

3.1 The imput graphical interface

The first thing that appears on screen when you open CISNE is the input graphical interface (see Fig. 3.1). This is the interface that allows to introduce all the input data related to your particular dose-effect or combination experiment. The interface is divided into two blocks. In block A the general information of the experiment is introduced, meanwhile the block B collects information of each drug of the study separately.

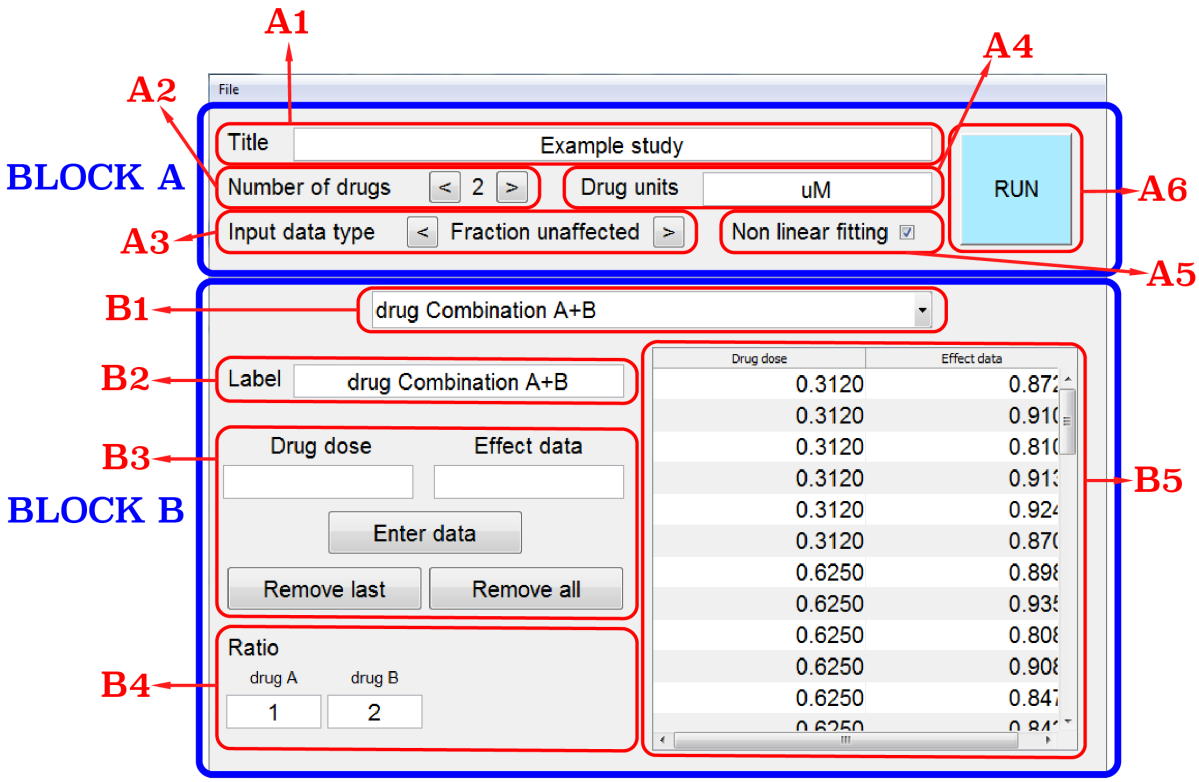

Fig. 3.1: the input graphical interface of CISNE

Within block A we find:

- A1: Space to introduce a name to label the study.
- A2: Input for the number of drugs analyzed in the study. For a simple dose-effect study it would be just 1, meanwhile it can take values between 2 and 4 for combination studies.
- A3: Indicates the type of input that is introduced for the effect data. It can be:
  - Total unaffected: Any value proportional to the number of members of the study unaffected by the drug can be used

as input for the effect data. It requires to introduce input data for zero dose in B3. The average of the values of the effect data introduced for zero dose is used to normalize the rest of the data and calculate the fraction unaffected.

  - Fraction unaffected: The effect is introduced as the fraction of members of the study unaffected by the drug at a given dose. Corresponds to f_u as defined in section 2.1. If non linear fitting is not selected in A5 effect data outside of the range (0,1) are ignored.
  - Fraction affected: The effect is introduced as the fraction of members of the study affected by the drug at a given dose. Corresponds to f_a as defined in section 2.1. If non linear fitting is not selected in A5 effect data outside of the range (0,1) are ignored.

- A4: Space to introduce the units in which the drug doses are measured. This units label is only used for output purposes, and it has no effect in the calculation.
- A5: Indicates whether the new non linear fitting procedure introduced by CISNE should be used or not. It is highly recommended to use the new method. If unselected, a standard least squares linear fitting method is used.
- A6: The run button to perform the calculation. If everything is correct, the output graphical interface will appear after clicking it. If not, an error will appear indicating the problem that is found in the input.

Within block B we find:

- B1: Pop-up menu to select each of the drugs in the study. If it is a combination study (that is, if the number of drugs in A2 is larger than 1), the last option of the menu corresponds to the drug combination.
- B2: Space to introduce the name of each drug of the study, or of the drug combination.
- B3: Controls to introduce de input data. The dose must be a positive value (or also zero if Total unaffected is selected in A3). If the drug combination is selected in B1, the drug dose that has to be introduced here is a reference dose. The total dose of each drug used in the experiment should be the result of multiplying this reference dose by each value of the ratio introduced in B4. There are no restrictions to the values of the effect data. Several effect data can be included for the same dose, and all of them will be used in the fitting procedure. In this case, average values and

standard deviations of the effect for each dose are also calculated.

- B4: Input of the constant ratio in which the drug combination experiment has been performed. This input section only appears when the drug combination is selected in B1. The values introduced for the ratio of each drug multiply the reference doses introduced in B3 to give the total doses of each drug in the combination.
- B5: Table containing all the introduced data from B3.

Once you have finished to introduce the input data of the study, the input data can be saved in a .txt file by going to the File->Save project menu. You can open a previously generated input data file by going to the File->Open project menu. Data from a new experiment can be introduced by going to the File->New project menu.

3.2 Input data from a text file

The format in which CISNE saves an input data file is human-readable, which means that they can be easily edited or generated by a standard plain text editor, such as Notepad. Some example and template files can be found at **http://cisnecode.github.io** that can be used to generate CISNE input files more easily. An example of an input data file in .txt format is shown in Fig. 3.2. The input is formed by flags, identified by a # symbol followed by their name. The name of the flags is case-insensitive, therefore #NAME is equivalent to #name and to #NaMe. There are two type of flags, one line flags and block flags. For one line flags, the input of the flag is just what follows the flag in the line. For block flags, their input is composed by all the lines between the name of the flag and the flag #end.

The one line flags, and their possible input, are the following:

- #Title: the input is any string of text. It corresponds to the input A1 in Fig. 3.1.
- #Ndrugs: the input is a number between 1 and 4. It corresponds to the input A2 in Fig. 3.1.
- #DataType: the input can be ‘tu’ for total unaffected, ‘fu’ for fraction unaffected, or ‘fa’ for fraction affected. It corresponds to the input A3 in Fig. 3.1.
- #Units: the input is any string of text. It corresponds to the input A4 in Fig. 3.1.

```
#title Example study

#Ndrugs   2
#Units     uM
#NonlinearFit YES
#DataType    fu

#label1 drug A
#label2 drug B
#labelC drug Combination A+B

#Ratio  1 2

#data1
  0.312   1.016   0.979   0.865   1.051   0.949   1.029
  0.625   0.964   0.990   0.927   0.906   0.960   0.953
  1.250   0.964   0.832   0.821   1.016   0.831   0.904
  2.500   0.828   0.758   0.872   0.885   0.821   0.745
  5.000   0.697   0.738   0.724   0.745   0.736   0.748
 10.000   0.474   0.468   0.610   0.479   0.473   0.542
 20.000   0.326   0.401   0.353   0.339   0.361   0.391
 40.000   0.232   0.148   0.128   0.194   0.158   0.260
 80.000   0.145   0.097   0.141   0.207   0.184   0.099
#end

#data2
  0.312   0.973   1.035   0.959   0.892   0.942   1.103
  0.625   0.911   1.033   0.959   0.902   0.979   0.878
  1.250   0.949   1.024   1.033   1.024   1.060   0.987
  2.500   0.942   0.975   0.907   0.988   0.872   0.972
  5.000   0.853   0.838   0.909   0.918   0.861   0.887
 10.000   0.719   0.749   0.686   0.737   0.698   0.803
 20.000   0.510   0.537   0.521   0.447   0.513   0.520
 40.000   0.330   0.286   0.374   0.190   0.203   0.162
 80.000   0.093   0.079   0.144   0.085   0.193   0.137
#end

#dataC
  0.312   0.872   0.910   0.810   0.913   0.924   0.870
  0.625   0.898   0.935   0.808   0.908   0.847   0.842
  1.250   0.666   0.728   0.707   0.612   0.614   0.672
  2.500   0.457   0.523   0.467   0.361   0.523   0.478
  5.000   0.336   0.278   0.201   0.200   0.217   0.221
 10.000   0.078   0.188   0.052   0.243   0.175   0.068
 20.000   0.096   0.083   0.052   0.166   0.172   0.052
 40.000   0.040   0.042   0.089   0.036   0.094   0.085
 80.000   0.058   0.041   0.050   0.062   0.116   0.044
#end
```

Fig. 3.2: example input data file in plain text, corresponding to the example_2drugs.txt file included in the examples folder of CISNE

- #NonLinearFit: the input can be 'YES' or 'NO', depending on if the new non linear fitting method introduced by CISNE should be used or not. It corresponds to the input A5 in Fig. 3.1.
- #Label*N*: the input is any string of text. It corresponds to the input B2 in Fig. 3.1, with *N*=1,2,3 or 4 for each of the individual drugs or *N*=C for the drug combination.
- #Ratio: the input is as many values as different drugs the study has. Their values corresponds to the ratio of each of the drugs in the combination. It corresponds to the input B4 in Fig. 3.1. If the number of drugs is equal to 1 this flag is not needed.

The block flags, and their possible input, are the following:

- #data*N*: the input is the data of dose and effect, as introduced in the input B3 in Fig. 3.1, with *N*=1,2,3 or 4 for each of the individual drugs or *N*=C for the drug combination. The first value of each row of data corresponds to the drug dose, and the rest correspond to effect data for that given drug dose. There is no restriction to the order of the rows within the block or the number of effect data values for each drug. The same drug concentration can also appear in several rows.

The input data file also allows to include comments with the % symbol. Anything that follows this symbol in a line is ignored by CISNE. This allows, for example, to test results omitting data at wish. Notice however, that as CISNE ignores these comments, **this information will be lost if the input file is overwritten by CISNE via File->Save project**.

3.3 The output graphical interface

After clicking on the run button of the input graphical interface (A6 in Fig. 3.1), CISNE will perform the calculations and then show the output graphical interface (see Fig. 3.3). From this interface all the data and graphics generated by CISNE can be found. The interface is divided into two blocks. In block A we can select a particular drug, combination or comparative, and the mathematical results of the fitting are given, meanwhile block B allows to check different graphs for the selection in block A.

Within block A we find:

- A1: The back button, to go back to the input graphical interface.
- A2: Pop-up menu which allows to select each individual drug, the drug combination or a comparative mode. This affects to the

information given in A3, as well as to the graphs shown in block B.

- A3: Numerical values of the fitting parameters *D* and *m* of the median effect equation, as described in section 2.1. Confidence intervals of 95 % are given for each parameter. If the non linear fitting option is not selected in A5 of the input graphical interface, the *r* parameter of the linear fitting procedure is also given.
- A4: The report button, that allows to generate a report in .html format with all the graphs in .png format. The report contains all the information that can be found in the input and output graphical interfaces, as well as some numerical values of the information shown in B2 of the ouput graphical interface.

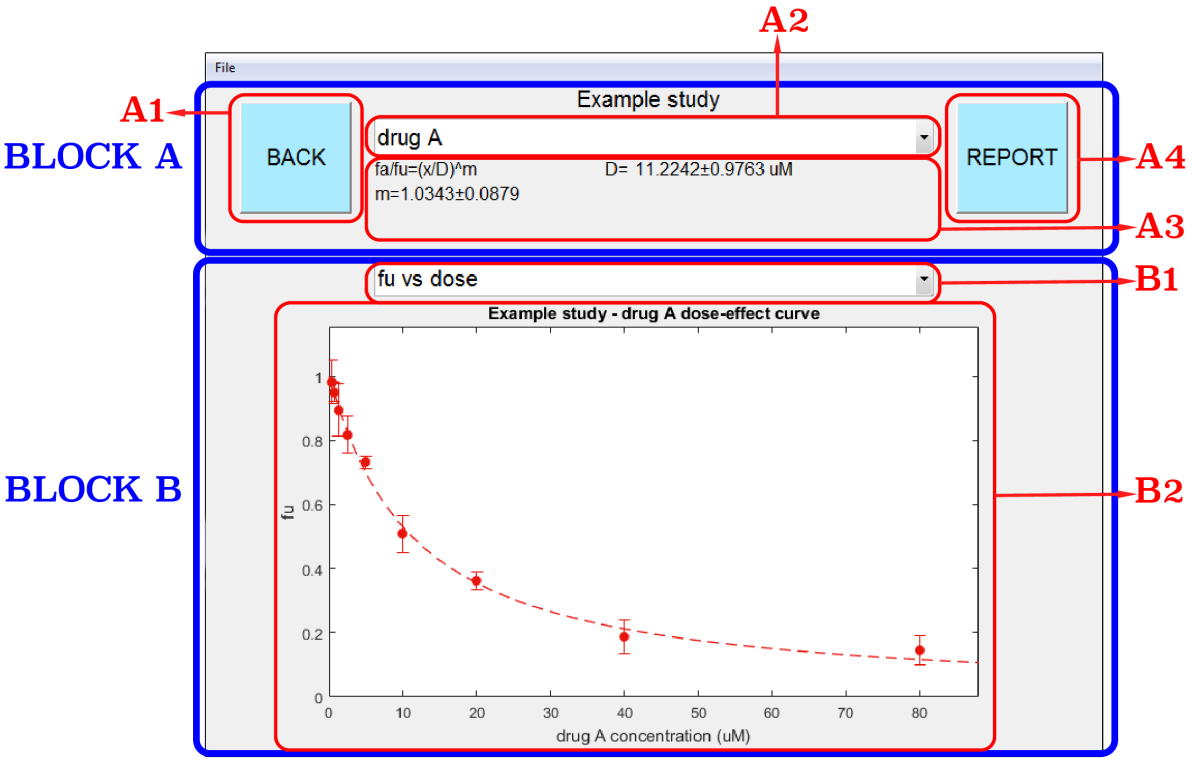

Fig. 3.3: the output graphical interface of CISNE

Within block B we find:

- B1: Pop-up menu to select the graph to show in B2. The available options depend on the selection in A2. More details are given below.
- B2: Graph selected in B1. More details are given below.

We describe now the different graphs that the output graphical interface can generate. If the selection in A2 is an individual drug, the available options in B1 are:

- fu vs dose: represents the fraction unaffected (f_u) against the drug dose. Dots indicate the dose and f_u obtained from the input data. If more than one effect value is given for the same dose value, the dots represent the average of the effects and a vertical bar indicates its standard deviation.
- fu vs dose (semilog x): represents the same information as 'fu vs dose', but the dose is shown in logarithmic scale.
- fa/fu vs dose (log): this plot is only represented when the non linear fitting option A5 in the input graphical interface is not selected. It shows f_a/f_u against the dose, both in logarithmic scale. The dots indicate the values obtained from the input data that come into the fitting. A straight line indicates the result of the linear fitting of the data.

If the drug combination is selected in A2, new graphs can be shown beside the ones for the individual drug. Notice that the dose shown for the 'fu vs dose', 'fu vs dose (semilog x)' and 'fa/fu vs dose (log)' is the total dose (the sum of the dose of each drug in the combination). The new available graphs are:

- combination index: the combination index CI is given as a function of the fraction affected f_a. with a continuous line. Dashed lines indicate the upper and lower limits of a 95 % confidence interval of the combination index. The space within the confidence interval is shaded with dots.
- combination index (log): represents the same information of 'combination index', but the combination index CI is substituted by its logarithm.
- IC50 isobologram: this graph is available only if the number of drugs in the combination is 2. The isobologram represents the concentration of the first and second drugs on the x and y axis, respectively. A straight red line joins the drug concentration that generates f_a=f_u=0.5 for each of the drugs (what is usually called IC50) between the axes. Dashed red lines join the upper and lower limits of a 95 % confidence interval of the IC50 of each drug between the axes. The space within the confidence interval lines is shaded with red dots. Now, the dose of each compound that corresponds to the IC50 in the combination is plotted as a blue point, with the confidence interval of both drug concentrations represented with a dashed blue line with

elliptical shape. The red line represents an additive effect of the drug combination. If the combination blue dot is within the space limited by the red line and the axes, this indicates that the drug concentrations needed to generate the same effect is lower than in the additive case, and therefore the combination is synergistic. If the dot is outside of the triangle, the opposite happens and the combination is antagonistic. If the confidence intervals overlap, no synergistic or antagonistic effect can be guaranteed and the combination might be just additive.

- normalized IC50 isobologram: represents the same information of 'IC50 isobologram', but with the individual drug concentrations corresponding to the IC50 normalized to 1. This allows to compare easily isobolograms between different drug combinations.

If the comparative is selected in B1, the same 'fu vs dose' and 'fu vs dose (semilog x)' as before are available, where now the graphs include the information of each drug and the drug combination together. For each individual drug the following options are also available:

- *label* drug change: where *label* indicates the name of the selected drug. It shows the dose-effect relationship of the selected drug individually or in the combination. Notice that a dose-effect curve indicating more effect of the drug in combination that individually does not necessarily indicate a synergistic effect, but only a reduction of the dose of this drug in the drug combination needed to obtain the same effect as the individual drug.
- *label* drug change (semilog x): represents the same information as '*label* drug change', but the dose is shown in logarithmic scale.

4. References

[1] Chou, T.-C. Derivation and properties of Michaelis-Menten type and Hill type equacions for reference ligands. *J. Theor. Biol.* 59, 253 (1976).

[2] Chow, T.-C. and Talalay, P. A simple generalized equation for the analysis of multiple inhibitions of Michaelis-Menten kinetic systems. *J. Biol. Chem.* 252, 6438 (1977).

[3] Chou, T.-C. and Talalay, P. Generalized equations for the analysis of inhibitions of Michaelis-Menten and higher-order kinetic systems with two or more mutually exclusive and nonexclusive inhibitors. *Eur. J. Biochem.* 155, 207 (1981).

[4] Chou, T. and Talalay, P. Analysis of combined drug effects: a new look at a very old problem. *Trends Pharmacol.* Sci. 4, 450 (1983).

[5] Chou, T.-C. and Talalay, P. Quantitative analysis of dose-effect relationships: the combined effect of multiple drugs or enzyme inhibitors. *Adv. Enzym. Regul.* 22, 27 (1984).