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Research Article

Application of sequential factorial design and orthogonal array composite design (OACD) to study combination of 5 prostate cancer drugs

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ABSTRACT

Prostate cancer is one of the most common cancers among men in the United States. It is also a major leading cause of cancer death among men of all races. In order to treat prostate cancer, drug combinations are often applied. Drug combinations target at different pathways of cells can potentially lead to higher efficacy and lower toxicity due to drug synergy. In this paper, we sequentially applied a two-level design and a follow-up orthogonal array composite design (OACD) to investigate combinations of five anti-cancer drugs, namely, doxorubicin, docetaxel, paclitaxel, *cis*-dichlorodiamine platinum and dihydroartemisinin. Our initial screening using a two-level full factorial design identified doxorubicin and docetaxel as the most significant drugs. A follow-up experiment with an OACD revealed more complicated drug interactions among these 5 anti-cancer drugs. Quadratic effects of doxorubicin and paclitaxel appeared to be significant. A further investigation on contour plots of all the two-drug pairs indicated that combination of doxorubicin and docetaxel are the most effective companion, while the combination of *cis*-dichlorodiamine platinum and dihydroartemisinin showed unknown antagonistic effects which diminished the individual drug anti-cancer efficacy. These observations have significant practical implications in the understanding of anti-cancer drug mechanism that can facilitate clinical practice of better drug combinations.

1. Introduction

In the United States, Prostate cancer is one of the most prevalent cancer and approximately 2,20,800 new cases were reported merely in 2015 (Thorn et al., 2016). Surgery and radiation therapy are frequently employed to prevent metastasis in early stages of the tumor (Hong et al., 2010; Peyromaure et al., 2009). However, if the treatment for early stage fails and the cancer spreads out of prostate, chemotherapy and hormonal therapies may also be drawn-out (Miller and Sweeney, 2016; Xie et al., 2015). To combat these intractable circumstances in prostate cancer, the existing therapies (chemotherapy or hormonal therapy) need to be updated and improved urgently.

Drug combination has been gradually considered as a main route for chemotherapy (Ding et al., 2015, 2012; Li et al., 2016; Su et al., 2015). Yet, many challenges remain when researchers try to understand the interactions between multiple drugs and optimize the desired combinatorial drug therapy (Weiss et al., 2015). When the number of drug or dose level increases, the searching space expands exponentially, sometimes make it impractical to browse every single possible drug combination. For instance, 10 drugs at 3 dose levels end up with $3^{10} = 59{,}049$ different drug combinations (Ding et al., 2014). It would be extremely timely and labor consuming to test all of these possibilities. Researchers have reported Feedback System Control (FSC) technique for quick identification of effective drug combinations by testing 1% or less of the total searching space (Honda et al., 2013; Nowak-sliwinska et al., 2016; Tsutsui et al., 2011). While the FSC technique is powerful in identifying desired drug combination, the conventional FSC technique often involves stochastic search algorithms,

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which are designed to quantify drug interactions and drug contributions in the identified drug combination.

Five commonly used anti-prostate cancer drugs are considered in the present study for exploring an optimization combination namely, doxorubicin, docetaxel, paclitaxel, *cis*-dichlorodiamine platinum and dihydroartemisinin. Docetaxel, belonging to taxanes, is an anti-neoplastic drug (Yuan and Li, 2010) and has been widely used in the treatment of prostate cancer with improving median survival of 2 to 3 months (Kinebuchi et al., 2011; Petrylak, 2003). Docetaxel kills cancer cells by disrupting the microtubule network leading to inhibition of cell division (Fabbri et al., 2006; Rody et al., 2007). Doxorubicin is known to intercalate itself into the DNA, with the inhibition of both DNA and RNA polymerase, thus blocking DNA replication and RNA transcription (Tacar et al., 2013). Recently it has been reported that doxorubicin is capable of intercalating with mitochondrial DNA (Lamberti et al., 2014). It is often used in different combination for effective chemotherapy (Felix et al., 2016). Though doxorubicin is proved to have superior tumor response in clinical studies, it is associated with the risk of cardiac failure. On the other hand, paclitaxel can improve the superior tumor response without any cardiac risk (Leonard et al., 2009). In some cases, cancer cells are not sensitive enough to docetaxel, and *cis*-dichlorodiamine platinum is often combined with docetaxel to improve efficacy of chemotherapy (Figg et al., 2013; Sternberg et al., 2009). Therefore, in our current study, *cis*-dichlorodiamine platinum is also chosen for exploring internal interaction with other drugs. Recently, artemisinin is reported to be beneficial for treating solid tumors, including prostate cancer (Efferth et al., 2001; Nakase et al., 2009; Posner et al., 2004). Although the mechanism of artemisinin in suppressing tumor has not been clearly defined, it is now regarded as a potential anti-tumor agent in treating solid tumors. According to the report, dihydroartemisinin is a primary artemisinin present in the blood (Lindegardh et al., 2011).

In the current research work, to study the optimization of 5 selected anti-prostate-cancer drugs, a two-level design was performed and then followed up by an OACD. OACDs which were first introduced by (Xu et al., 2014), are new class of designs that combine factorial designs (two-level) and three-level orthogonal arrays in a single experiment. OACDs are constructed with careful consideration of experimental cost, time, and statistical efficiency. These new designs have many desirable features and are effective for factor screening and response surface modeling. Many successful applications have been reported in chemical and engineering domains for both two-level design and orthogonal array (Jaynes et al., 2013, 2016a,b). However, limited work is reported on successful application of OACD in bio-complex systems, including drug combination stimulations.

2. Material and methods

2.1. Anti-tumor drug combination experiments

For anti-cancer drug experiment PC-3 cells were chosen as representative cancer cell line. PC-3 cells were provided by Stem Cell Bank, Chinese Academy of Sciences. Cells were initially cultured on 75 cm^2 culture flask covered with 12 mL culture medium. The culture medium consisted of DMEM, 10% fetal bovine serum and 1% penicillin–streptomycin. The 75 cm^2 culture flasks were maintained in 37 °C incubator filled with 5% CO_2. Drugs treatment was simultaneously carried out in 96-well plates. Each well contained 10^4 cells in 100 μL of culture medium. Cells were incubated for 24 h before drug treatments occurred. Drug combinations were added to PC-3 in 96-well plates. The plates were incubated at 37 °C incubator with 5% CO_2 for 24 h. Cell count kit-8 was applied to test cell viability after 24 h drugs treatment. Five anticancer drugs doxorubicin (Sigma, USA), *cis*-dichlorodiamine platinum (Acros, Belgium), paclitaxel, docetaxel and dihydroartemisinin (Macklin, China) were selected for the study.

2.2. Initial two-level experiment

2.2.1. Full factorial design

Factorial designs are among the most popular designs for studying two or more factors. The change in response with dose gives an estimate of the effect of that particular factor in a factorial design. This effect is also named as main effect. In some scenarios, the presence of other factors may impact the change of response induced by dose change of the particular factor. This phenomenon is referred to as an interaction effect. Main effects and interaction effects are together termed as factorial effects. A full factorial design is expected to calculate all the main effects and high-order interactions of the researched factors.

A common way to quantify main effects and interaction effects for a two-level factorial design is to fit a regression model. We aimed to study 5 anti-cancer drugs: D1(doxorubicin), D2(docetaxel), D3(paclitaxel), D4(*cis*-dichlorodiamine platinum) and D5 (dihydroartemisinin) with two dose levels each. The total possible combinations would be $2^5 = 32$. A regression model involving all main effects and interactions is:

$$y = \beta_0 + \sum_{i=1}^{5}\beta_i x_i + \sum_{1=i<j}^{5}\beta_{ij}x_i x_j + \sum_{1=i<j<k}^{5}\beta_{ijk}x_i x_j x_k + \sum_{1=i<j<k<l}^{5}\beta_{ijkl}x_i x_j x_k x_l + \beta_{12345}x_1 x_2 x_3 x_4 x_5 + \varepsilon \quad (1)$$

Here, y is the response, or tumor growth rate in our study, $x_1, \ldots, x_5$ are the five anti-cancer drugs. β_0, β_i, β_{ij}, β_{ijk}, β_{ijkl} and β_{12345} are the intercept, linear, two-drug interaction, three-drug interaction, four-drug interaction and five-drug interaction coefficients, respectively. $\varepsilon \sim N(0,\sigma^2)$ is an error term. In this model, the variables x_i could be either coded doses (such as −1 and 1) or actual doses. Twice of the least squares estimates of the β in model (1) are considered as the corresponding factorial effects.

With a full factorial design of 32 runs for 5 drugs, we could fully estimate 1 intercept, 5 main effects, 10 two-drug interactions, 10 three-drug interactions, 5 four-drug interactions and 1 five-drug interaction. In reality, for many drug combination studies, the main effects and two-drug interaction effects almost dominate the contribution to response change. Therefore, higher than three-factor interactions are often considered as less significant and neglected (Xu et al., 2014).

2.2.2. Stepwise regression

Stepwise regression is a process that successively adds or removes variables based solely on the t-statistics of their estimated coefficients. For a large pool of independent anti-cancer drugs, the stepwise regression starts from no variable in the model till adding all potential variables, one at a time, or vice-versa (starts with all potential variables in the model and proceeds backward by removing one variable at a time). Whenever a variable is added to or removed from a model, t-statistic has to be re-performed to evaluate the fitting efficiency of this action (Van der Borght et al., 2011).

For the statistical modeling and analysis of the experimental data acquired from the full factorial design, a second-order regression model with 5 drugs has been utilized. For many drug combination studies, the most effective drug combination may not require the presence of all the drugs. Only a few key drug components are sufficient to provide an effective therapy. This means that we are using many degrees of freedom to estimate

effects that are potentially not significant. Therefore, identifying a minimal model would reduce the requirement of runs and increase the degrees of freedom of the regression model. In the present work we applied stepwise regression to the model generated from the full regression model.

The advantage of stepwise regression, if properly practiced, is in the addition of more power and information to the overall design as compared to the ordinary multi-parameter regression. Amidst a large number of independent variables, the stepwise regression can quickly locate a potential subset with desired response and outcome.

2.3. Orthogonal array composite design (OACD)

Two-level designs are often applied as initial screening to probe for potential subset of factors that may have potential synergy. Three-level designs are more widely used to examine the nonlinear relationship between system output readout and system input stimulations. In our previous publication (Weiss et al., 2015), we had successful application for cancer angiogenesis. Orthogonal array composite design (OACD) is a set of designs which combines a fractional factorial design and an orthogonal array. The OACD has several advantages including the ability to use a resolution IV design, which gives the ability to estimate the two-factor interactions. An OACD can fully decode drug-drug interactions and drug quadratic contributions.

The focus of the current work is to essentially examine the main effects, two-drug interactions and drug quadratic effects in a cascade experimental setup. The applied OACD which had 34 runs, enabled the estimation of all the main effects, quadratic effects and two-drug interactions. The three dose levels were coded as low (0), intermediate (1), and high (2).

To construct an OACD, a two-level design and an orthogonal array need to be chosen. The selection of these designs were acquired from Xu et al. (2014). For the two-level design, a regular fractional factorial design with minimum aberration (or maximum resolution) was selected. For the three-level part, an orthogonal array that accommodated five three-level factors and selected the minimum aberration array was picked up.

3. Results and discussion

3.1. Maximal inhibitory concentration

In the drug combination study, low dose was kept as no drug treatment. In two-level experiment, the high level was coded as 1 and the no acquisition was coded as 0. In the three-level experiment, the higher level was coded as 2 and the no acquisition was coded as 0, with an additional middle level (coded as 1). Table 1 shows the concentration levels for the five drugs. Before this study, screening for single-drug toxicity was performed and a wide range of dosages were tested for each drug to identify the half maximal inhibitory concentration (IC_{50}).

3.2. Full factorial design

As discussed in Section 2.2, a full two-level factorial design could estimate all the 5 main effects, all 10 two-drug interactions and all 10 three-drug interactions, assuming higher interactions are less significant and negligible. Table 2 shows the design and cancer survival readout data for the initial two-level full factorial design experiment with a 2^5 design applied. The choice of the full 32-run was selected because of the present work capability allowed for performing such a large-size experiment along with the same batch of cells and to acquire a full data set, which could potentially increase the credibility and reliability of the statistical analysis. Therefore, more data points could help to relieve the noises coming from the biological system response.

Fig. 1 displays the main effect of each individual drug. It represents the system response where if we change only one drug and keep other drug doses at zero level, the contribution of the particular drug to the system response could be identified. As evident from Fig. 1 for all the 5 drugs, when used alone, the cancer cell survival readout apparently reduced, signifying the effectiveness of all these 5 drugs in cancer disease model. Table 3 presents the least squares estimates, the standard errors, the t values, and the significances of all the effects in a regression model and

Table 2
Design and data for the initial two-level experiment: a 2^5 design.

Run	D1	D2	D3	D4	D5	Survival (%)
1	1	1	1	1	1	0.422205
2	1	1	1	1	0	0.40941
3	1	1	1	0	1	0.413998
4	1	1	1	0	0	0.396392
5	1	1	0	1	1	0.3979
6	1	1	0	1	0	0.466528
7	1	1	0	0	1	0.428256
8	1	1	0	0	0	0.497837
9	1	0	1	1	1	0.534151
10	1	0	1	1	0	0.531262
11	1	0	1	0	1	0.528595
12	1	0	1	0	0	0.555306
13	1	0	0	1	1	0.436332
14	1	0	0	1	0	0.505268
15	1	0	0	0	1	0.571856
16	1	0	0	0	0	0.674148
17	0	1	1	1	1	0.675273
18	0	1	1	1	0	0.693248
19	0	1	1	0	1	0.694103
20	0	1	1	0	0	0.358857
21	0	1	0	1	1	0.495565
22	0	1	0	1	0	0.481028
23	0	1	0	0	1	0.461984
24	0	1	0	0	0	0.499952
25	0	0	1	1	1	0.678805
26	0	0	1	1	0	0.857589
27	0	0	1	0	1	0.772705
28	0	0	1	0	0	0.823383
29	0	0	0	1	1	0.936537
30	0	0	0	1	0	0.871975
31	0	0	0	0	1	0.906058
32	0	0	0	0	0	0.991241

Table 1
Factors and levels for the initial two-level anti-cancer drug experiment.

Drug name	Doxorubicin (μM)	Docetaxel (μM)	Paclitaxel (μM)	cis-Dichlorodiamine platinum (μM)	Dihydroartemisinin (μM)
Coded drug	D1	D2	D3	D4	D5
Low(0)	0	0	0	0	0
High(1)	10	0.01	0.005	15	15

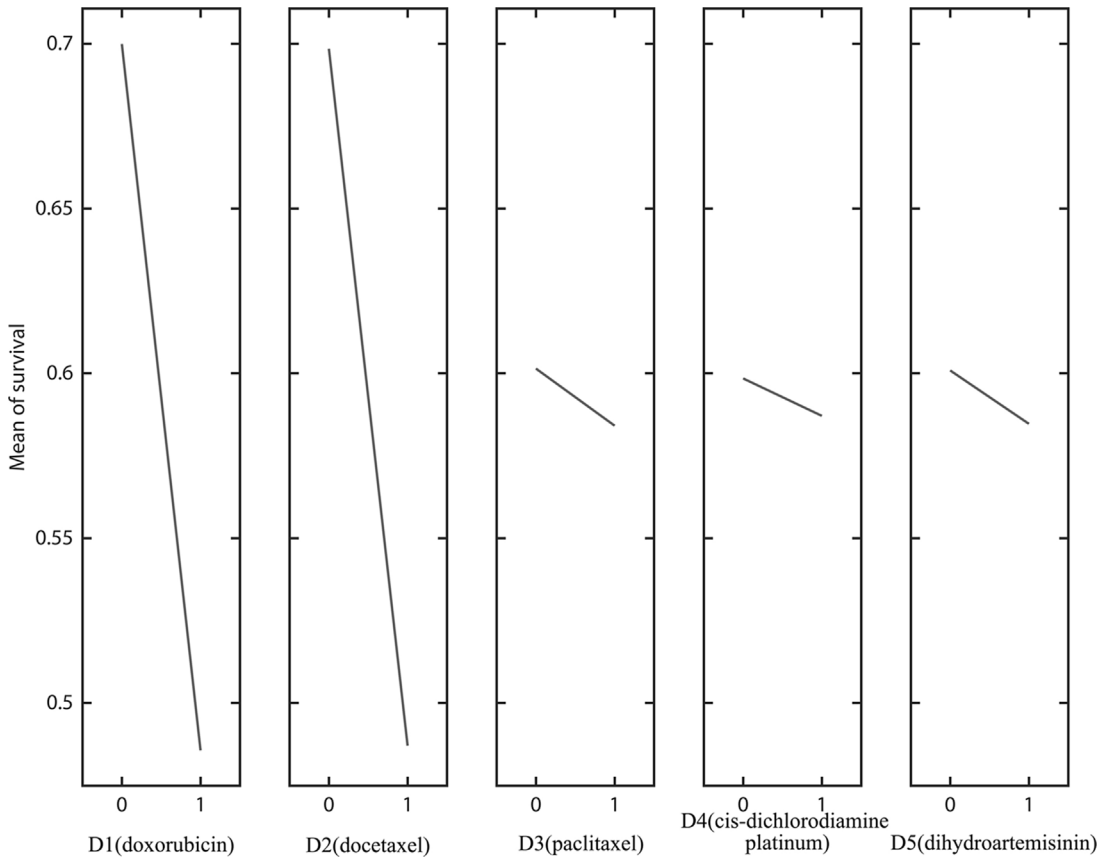

Fig. 1. Main effect plots for 5 anti-cancer drugs from factorial design. For all the 5 single drugs when used alone, dose escalation leads to decreased cancer cell survival. D1 and D2 appeared to be more significant compared to D3, D4 and D5. Five anti-cancer drugs: D1(doxorubicin), D2(docetaxel), D3(paclitaxel), D4(*cis*-dichlorodiamine platinum) and D5(dihydroartemisinin).

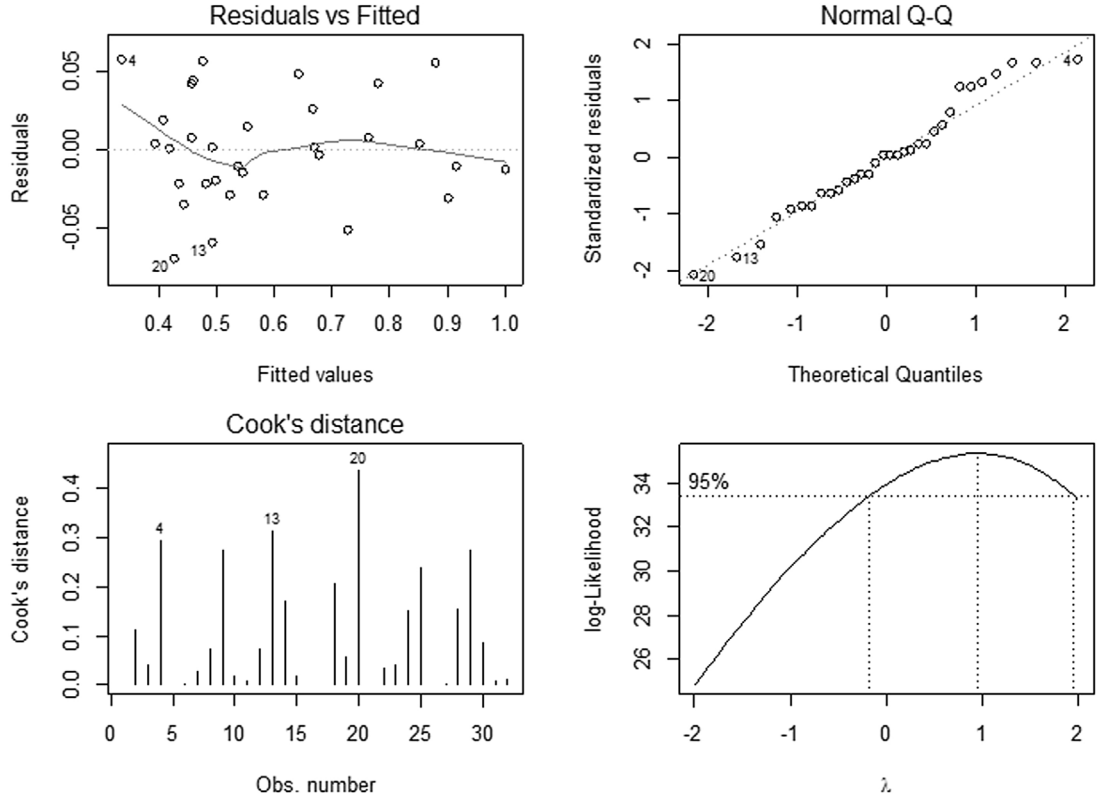

Fig. 2. Statistical analysis of stepwise regression model acquired from factorial design. The model is not biased at any particular fitted value. No obvious outliers are identified in the Cook's distance plot. No indications for data transformation are required for the survival readout.

Table 3
Estimates for the initial two-level experiment.

	Estimates	S.E.	t value	Pr(>|t|)
D1	−0.329558	0.087003	−3.788	0.009096 **
D2	−0.554001	0.087003	−6.368	0.000704 ***
D3	−0.204412	0.087003	−2.349	0.057098 .
D4	−0.106828	0.087003	−1.228	0.265478
D5	−0.076089	0.087003	−0.875	0.415447
D1:D2	0.424166	0.104929	4.042	0.006784 **
D1:D3	0.105889	0.104929	1.009	0.351854
D1:D4	−0.090726	0.104929	−0.865	0.420458
D1:D5	−0.051533	0.104929	−0.491	0.640787
D2:D3	0.184129	0.104929	1.755	0.129828
D2:D4	0.159723	0.104929	1.522	0.178786
D2:D5	0.113284	0.104929	1.080	0.321789
D3:D4	0.160538	0.104929	1.530	0.176899
D3:D5	0.048259	0.104929	0.460	0.661769
D4:D5	0.065514	0.104929	0.624	0.555371
D1:D2:D3	−0.291627	0.104929	−2.779	0.032028 *
D1:D2:D4	−0.049062	0.104929	−0.468	0.656592
D1:D2:D5	−0.114170	0.104929	−1.088	0.318329
D1:D3:D4	0.009702	0.104929	0.092	0.929339
D1:D3:D5	0.046039	0.104929	0.439	0.676194
D1:D4:D5	0.084544	0.104929	0.806	0.451147
D2:D3:D4	0.017196	0.104929	0.164	0.875204
D2:D3:D5	0.142687	0.104929	1.360	0.222759
D2:D4:D5	−0.097292	0.104929	−0.927	0.389589
D3:D4:D5	−0.173274	0.104929	−1.651	0.149759

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '.

Table 4
Stepwise regression estimates for the initial two-level experiment.

	Estimate	S.E.	t value	Pr(>|t|)	% Sum sq.
D1	−0.33123	0.05957	−5.560	0.000241 ***	0.3599054674
D2	−0.54603	0.06319	−8.642	5.95e-06 ***	0.3502754812
D3	−0.22265	0.05957	−3.737	0.003863 **	0.0023519191
D4	−0.10129	0.05957	−1.700	0.119925	0.0010094782
D5	−0.08760	0.06660	−1.315	0.217797	0.0020550735
D1:D2	0.39963	0.07296	5.477	0.000270 ***	0.0758295528
D1:D3	0.13376	0.05957	2.245	0.048563 *	0.0002846456
D1:D4	−0.11041	0.05957	−1.853	0.093538 .	0.0090947791
D1:D5	−0.02851	0.07296	−0.391	0.704137	0.0036777643
D2:D3	0.19273	0.07296	2.642	0.024670 *	0.0273981956
D2:D4	0.14379	0.05957	2.414	0.036452 *	0.0177349854
D2:D5	0.11328	0.08425	1.345	0.208446	0.0121948091
D3:D4	0.17399	0.05957	2.921	0.015285 *	0.0149485377
D3:D5	0.07128	0.07296	0.977	0.351642	0.0061405421
D4:D5	0.06551	0.08425	0.778	0.454790	0.0014813294
D1:D2:D3	−0.29163	0.08425	−3.462	0.006107 **	0.0416546883
D1:D2:D5	−0.11417	0.08425	−1.355	0.205184	0.0063843263
D1:D4:D5	0.08454	0.08425	1.004	0.339280	0.0035008394
D2:D3:D5	0.14269	0.08425	1.694	0.121199	0.0099718667
D2:D4:D5	−0.09729	0.08425	−1.155	0.275010	0.0046362374
D3:D4:D5	−0.17327	0.08425	−2.057	0.066754 .	0.0147054131

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '.

suggests that the main effects of D1 and D2 are most significant, while the main effect of D3 is also marginally important. D4 and D5 are not as significant as D1 and D2. Meanwhile the drug interaction terms between D1 & D2 and between D1, D2 &D3 are also significant.

3.3. Stepwise regression

In order to identify a minimal model that offers a decent fit, we performed a "backward" stepwise regression on the basis of the ordinary full regression model. Table 4 shows the estimates, standard errors, t values, significance and the percentage of total sum of squares of the stepwise regression coefficients. We observe that D1, D2 and D3 are still the most significant drugs in terms of main effects. The two-drug interaction between D1 and D2, D1 and D3, D2 and D3, D2 and D4, D3 and D4 are also significant. The three-drug interaction between D1, D2 and D3 is significant. Together, drug D1 and D2 account for about 75% of the total sum of squares in the data. Overall, D1, D2 and D3 together with their interactions contribute 90% of the total sum of squares. The findings that a subgroup of the factors contributes to majority of the sum of squares in the data are similar to many engineering investigations where factorial designs are applied. This observation provides us confidence that factorial designs could be helpful in studying bio-complex systems such as drug combination optimizations.

Fig. 2 shows the statistical analysis of the stepwise regression model. Residual plot indicates that the residuals are not biased for any given fitted values. Normal Q-Q plot proves the residuals follow a rough normal distribution. Cook's distance plot does not indicate any outlier data point. The boxcox plot suggests no transformation is necessary for survival readout. These plots together confirm the modeling accuracy of the stepwise regression. A natural question is whether the anti-cancer readout is only linearly dependent on the dose change, and whether individual drug quadratic effects are significant. This thought leads to a three-level design of next experimental investigation.

3.4. Orthogonal array composite design (OACD)

Table 5 shows the factors and levels for the three-level anti-cancer drug experiment. A wide range of cancer cell survival readout is observed from as low as 40% up to 100% (control sample with no drug treatment). Fig. 3 shows the main effects of each single drug. D3 and D5 appear to be sensitive to dose increase. At low dose end, D3 and D5 not only fail to reduce cancer cell proliferation, but show cancer promotion to a certain extent. D1, D2 and D4 show continuous tumor cell inhibition as dose level increases. Yet, D1 and D2 do not show obvious anti-cancer efficacy at low or intermediate dose levels, while the efficacy only appears at high level. D4 shows strong anti-cancer efficacy at both middle dose and high dose levels. Table 6 shows the design and cancer survival readout data for the OACD.

Table 7 shows the stepwise regression analysis on the model generated from OACD experiment. Three-level experiment revealed more complex patterns in drug quadratic effects and drug interaction effects. D2 main effect, D1 quadratic effect, D3 quadratic effect, D1 and D2 interaction effect, D4 and D5 interaction effect appear to be significant. In order to have a better visualized relationship between drug interactions, we summarize all the contour plots for two-drug pairs in Fig. 4. Because the interaction between D1 and D2, D4 and D5 are significant, contour plots could serve as more direct ways to

Table 5
Factors and levels for the three-level anti-cancer drug experiment.

Drug name	Doxorubicin (μM)	Docetaxel (μM)	Paclitaxel (μM)	cis-Dichlorodiamine platinum (μM)	Dihydroartemisinin (μM)
Coded drug	D1	D2	D3	D4	D5
Low (0)	0	0	0	0	0
Mid (1)	2	0.002	0.001	3	3
High (2)	10	0.01	0.005	15	15

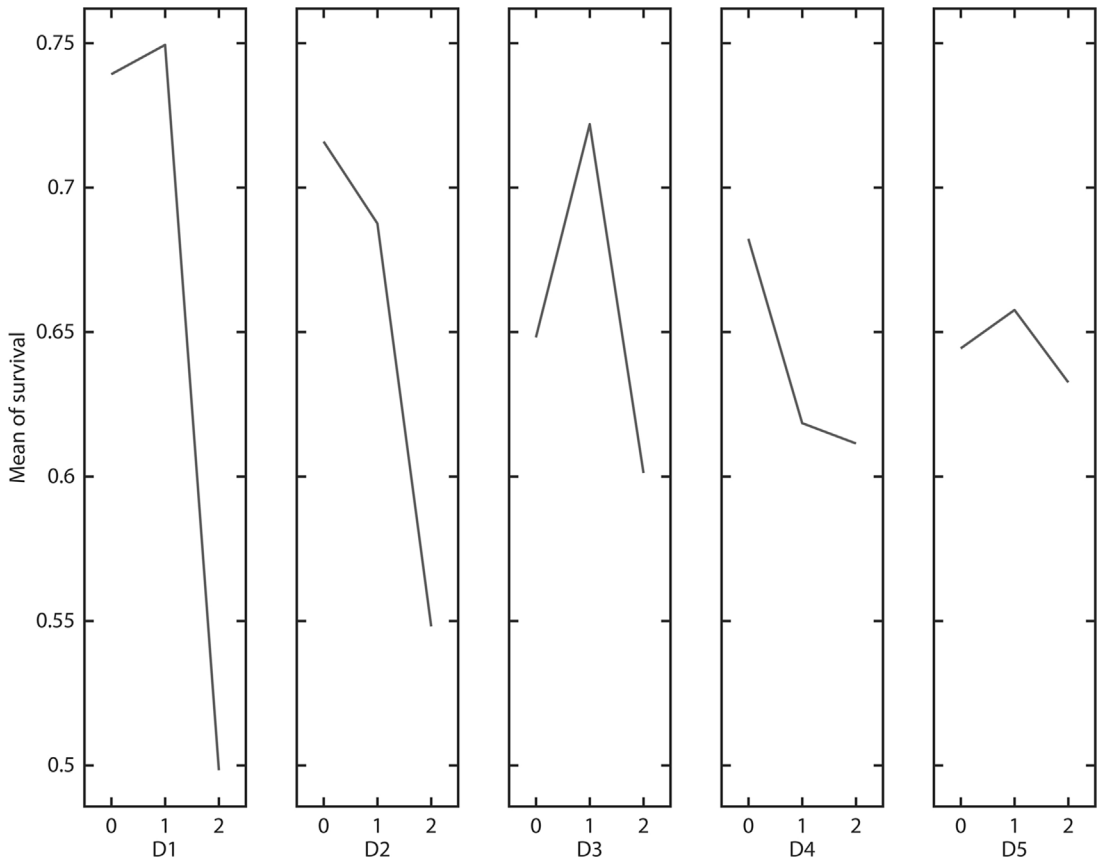

Fig. 3. Main effect plots for 5 anti-cancer drugs from OACD. D1, D2 and D4 show consistent tumor cell inhibitions as dose level increases, while D3 and D5 inhibit tumor cells at a dose dependent manner.

determine settings that minimize the response of our interest (cancer cell survival). In Fig. 4, each panel represents a two-drug contour plot. The x and y axes of the plot represent the dose levels of the first and second drugs. The contour plots show the predicted survival response in terms of the two drugs of interest in the panel, while the other drugs are held at low level (0). This contour plot suggests that minimal cancer survival is achieved when D1 and D2 are both at high dose levels. Meanwhile, D3 and D5, D4 and D5are not recommended at high dose simultaneously. These findings imply that some interesting biological synergies could occur when D1 and D2 are applied together. D3 and D4 could potentially indicated to antagonistic interactions either biologically or chemically, which eventually lead to the offset of anti-cancer efficacy for both drugs. D1 and D3 simultaneous increase to high level, notably leads to most reduced survival response. More drugs usually lead to more accumulative side effects, therefore, if two drugs combined together could deliver a desired efficacy similar as the combination of more drugs, to keep a smaller subset should be rational for future clinical investigations. Thus, we recommend combination of D1 and D2, both at high level, as the optimal therapeutic option.

Similar as the factorial design, we investigate the statistical properties of the stepwise regression model in Fig. 5 for OACD. Residual plot does not show any aberrant patterns at any fitted values. Normal Q-Q plot indicates the residuals closely follow a normal distribution. Cook's distance plot indicates no obvious outlier data point. Boxcox plot suggests that no transformation shall be made toward the response data. Summarizing the results from Fig. 5, the stepwise regression model faithfully depicts the

Table 6
Design and cancer survival readout for the three-level experiment: an Orthogonal Array Composite Design (OACD).

Run	D1	D2	D3	D4	D5	Survival (%)
1	2	2	2	2	2	0.422205
2	2	2	2	0	0	0.396392
3	2	2	0	2	0	0.466528
4	2	2	0	0	2	0.428256
5	2	0	2	2	0	0.531262
6	2	0	2	0	2	0.528595
7	2	0	0	2	2	0.436332
8	2	0	0	0	0	0.674148
9	0	2	2	2	0	0.693248
10	0	2	2	0	2	0.694103
11	0	2	0	2	2	0.495565
12	0	2	0	0	0	0.499952
13	0	0	2	2	2	0.678805
14	0	0	2	0	0	0.823383
15	0	0	0	2	0	0.871975
16	0	0	0	0	2	0.906058
17	0	0	0	0	0	0.991241
18	1	1	1	1	1	0.696531
19	2	2	2	2	2	0.422205
20	0	0	1	1	2	0.879439
21	1	1	2	2	0	0.625209
22	2	2	0	0	1	0.467651
23	0	1	0	2	1	0.835646
24	1	2	1	0	2	0.835499
25	2	0	2	1	0	0.445034
26	0	2	2	1	1	0.444985
27	1	0	0	2	2	0.756139
28	2	1	1	0	0	0.595276
29	0	1	2	0	2	0.862673
30	1	2	0	1	0	0.734656
31	2	0	1	2	1	0.652509
32	0	2	1	2	0	0.672361
33	1	0	2	0	1	0.848452
34	2	1	0	1	2	0.510106

Table 7
Stepwise regression estimates for the OACD.

	Estimate	S.E.	t value	Pr(>|t|)
D1	9.101e-02	7.672e-02	1.186	0.24812
D2	−1.615e-01	2.764e-02	−5.844	7.02e-06 ***
D3	6.593e-02	7.868e-02	0.838	0.41108
D4	9.428e-03	2.251e-02	0.419	0.67943
D5	−4.279e-07	2.697e-02	0.000	0.99999
I(D1^2)	−1.201e-01	3.625e-02	−3.313	0.00317 **
I(D3^2)	−7.406e-02	3.612e-02	−2.050	0.05245 .
D1:D2	3.933e-02	1.658e-02	2.372	0.02687 *
D2:D3	3.094e-02	1.654e-02	1.871	0.07477 .
D3:D5	2.758e-02	1.651e-02	1.670	0.10904
D4:D5	-4.099e-02	1.669e-02	-2.456	0.02240 *

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '.

relationships between drug dose levels and combinatorial drug anti-cancer response.

In Table 8(a and b), the two-level portion and three-level portion in the OACD data are analyzed separately. With the two-level portion (16 runs) in Table 8(a), only main effects and two factor interactions are estimated. With the three-level portion (18 runs) in Table 8(b), only main effects and quadratic effects are estimated. Parameter estimates of either two-level portion or three-level portion may not be exactly the same as full design estimates in Table 8(c) (before model reduction), but the significant coefficients have the same sign and are similar in magnitude.

4. Conclusion

We introduce a new application of OACD to investigate a bio-complex system of combinatorial therapy of 5 anti-cancer drugs. In this study, we started with an initial two-level full factorial design to pre-screen significant drugs at two dose levels. Then we aimed to examine whether linearity assumption in the two-level design holds by sequentially testing an orthogonal array design. The three-level design enables us to investigate whether quadratic effects are significant and to better design the overall drug doses when used in combination. With the sequential study, we found that D1 (doxorubicin) and D2 (docetaxel) are the most significant drugs in the cancer survival model used in our study. An optimized combination of D1 and D2 would lead to the least cancer cell

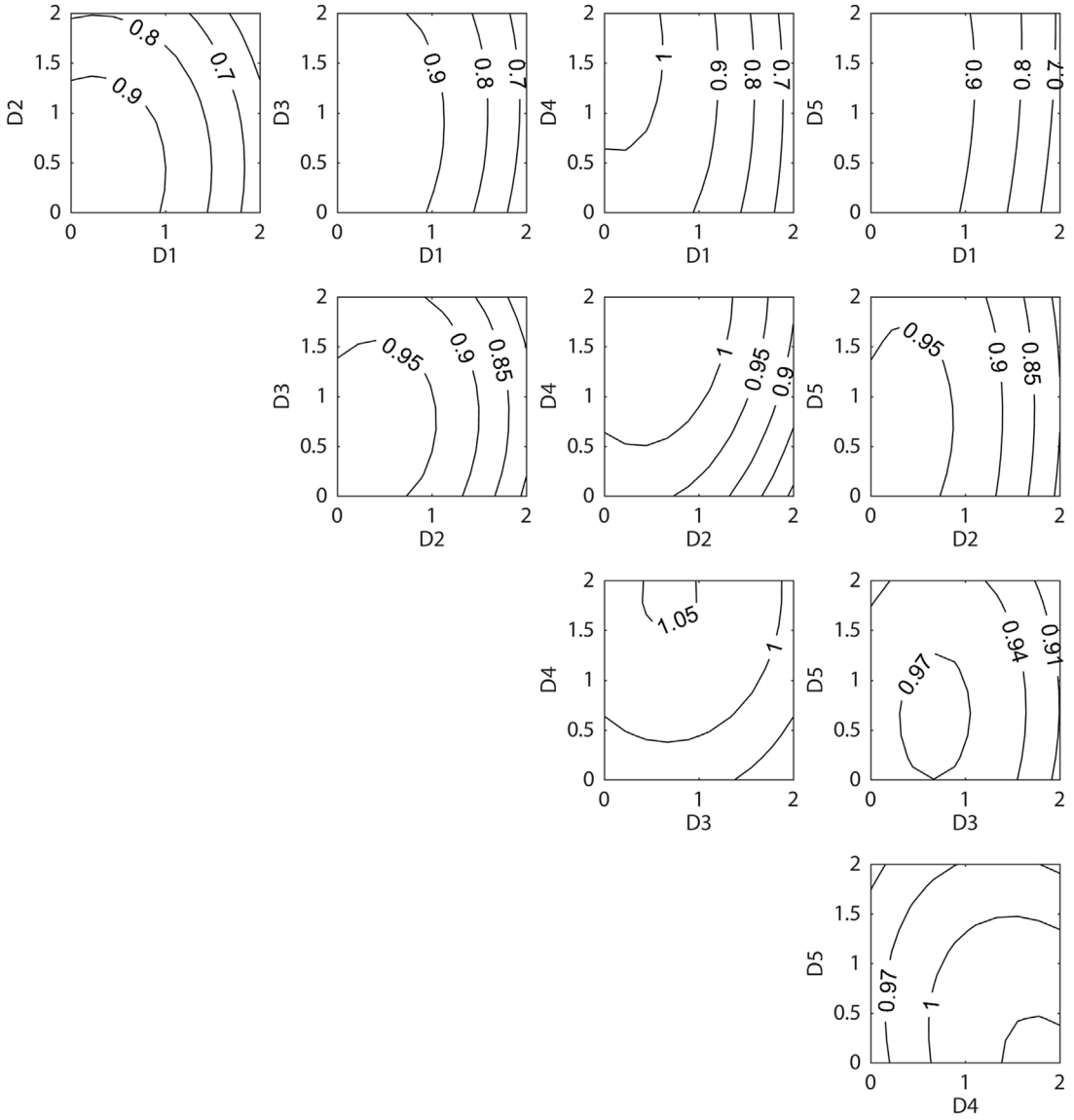

Fig. 4. Drug interaction contour plots. The contour plots provide a direct visualization for the relationship between tumor survival readout and any two pairs of drugs, with the other drug doses set as zero.

survival. Addition of the other drugs to the cocktail statistically does not contribute much to the anti-cancer efficacy. This finding indicates that more drugs may not always lead to better efficacy. The optimization and scientifically guided design of most significant drugs combination is necessary to ensure higher efficacy with lower side effects (by removing the less significant drugs from the combination).

An OACD uses a fractional factorial design and an orthogonal array in one single experiment. When the designs are carefully chosen, OACD can be resolution IV and used to screen for the most influential factors in a biocomplex system. The OACD introduced in the present study serves as a good example of combining a two-level factorial design and orthogonal array in a sequential way, where we could effectively examine the single drug main effects as well as multi-drug interaction effects, simultaneously. The sequential application of the two designs is especially necessary in biological application, since biological experiments, unlike most engineering studies, often possess internal variances and cross validation between different designs would assure more accurate conclusions.

There are thousands of FDA approved drug molecules available for various diseases which are verified for their safety before and after the approval. However, when a patient simultaneously takes the multiple drugs, uncertainty exists on whether the combination will enhance the efficacy or induce the toxicity, as these drugs may eventually trigger unknown biological or chemical interactions.

Table 8
Separate data analysis of two-level portion from OACD (estimate only main effects and two factor interactions).

(a)

	Estimate	S.E.	t value	Pr(>|t|)
D1	−0.129076	0.031128	−4.147	0.1507
D2	−0.216174	0.031128	−6.945	0.0910 .
D3	−0.054459	0.031128	−1.749	0.3306
D4	−0.030163	0.031128	−0.969	0.5100
D5	−0.013121	0.031128	−0.422	0.7460
D1:D2	0.030204	0.014917	2.025	0.2920
D1:D3	−0.012496	0.014917	−0.838	0.5561
D1:D4	0.003482	0.014917	0.233	0.8540
D1:D5	−0.006003	0.014917	−0.402	0.7564
D2:D3	0.042811	0.014917	2.870	0.2134
D2:D4	0.032220	0.014917	2.160	0.2760
D2:D5	0.023616	0.014917	1.583	0.3586
D3:D4	0.010245	0.014917	0.687	0.6169
D3:D5	0.010543	0.014917	0.707	0.6083
D4:D5	−0.040649	0.014917	−2.725	0.2239

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '.

(b) Separate data analysis of three-level portion from OACD (estimate only main effects and quadratic effects).

	Estimate	S.E.	t value	Pr(>|t|)
D1	0.069511	0.091538	0.759	0.4724
D2	−0.066170	0.091538	−0.723	0.4932
D3	0.065965	0.091538	0.721	0.4945
D4	−0.243620	0.091538	−2.661	0.0324 *
D5	−0.056191	0.091538	−0.614	0.5587
I(D1^2)	−0.101154	0.043973	−2.300	0.0550 .
I(D2^2)	−0.008393	0.043973	−0.191	0.8541
I(D3^2)	−0.059936	0.043973	−1.363	0.2151
I(D4^2)	0.095280	0.043973	2.167	0.0669 .
I(D5^2)	0.036524	0.043973	0.831	0.4336

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '.

(c) Data analysis of full design (estimate main effects, two factor interactions, and quadratic effects before model reduction).

	Estimate	S.E.	t value	Pr(>|t|)
D1	0.124820	0.098005	1.274	0.22510
D2	−0.160409	0.098005	−1.637	0.12565
D3	0.105711	0.098005	1.079	0.30036
D4	−0.121592	0.098005	−1.241	0.23665
D5	−0.004902	0.098005	−0.050	0.96087
I(D1^2)	−0.130679	0.042205	−3.096	0.00851 **
I(D2^2)	−0.017954	0.042205	−0.425	0.67749
I(D3^2)	−0.086702	0.042205	−2.054	0.06062 .
I(D4^2)	0.054879	0.042205	1.300	0.21608
I(D5^2)	−0.001064	0.042205	−0.025	0.98027
D1:D2	0.036971	0.018897	1.956	0.07224 .
D1:D3	−0.007413	0.018897	−0.392	0.70122
D1:D4	0.005175	0.018897	0.274	0.78849
D1:D5	−0.007721	0.018897	−0.409	0.68951
D2:D3	0.027819	0.018897	1.472	0.16478
D2:D4	0.023703	0.018897	1.254	0.23181
D2:D5	0.018375	0.018897	0.972	0.34861
D3:D4	−0.005916	0.018897	−0.313	0.75919
D3:D5	0.023559	0.018897	1.247	0.23450
D4:D5	−0.039450	0.018897	−2.088	0.05708 .

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '.

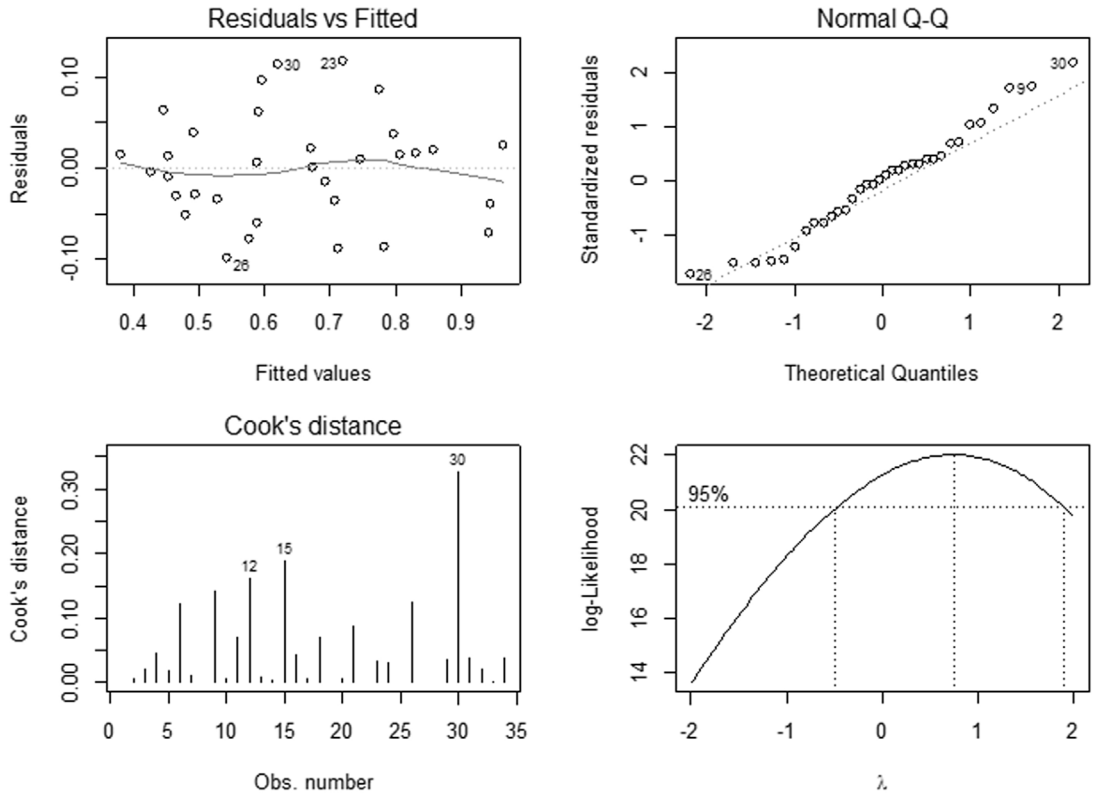

Fig. 5. Statistical analysis of the stepwise regression model acquired from OACD.

Unfortunately, very few literatures are available in this context. Our aim is to identify effective and less toxic drug combinations with minimal amount of experimental efforts. When the number of drugs becomes large (say more than 100), full factorial design will be experimentally impractical. Therefore, more effective designs that tackle this challenge would be extremely useful.

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References

Ding, X., Sanchez, D.J., Shahangian, A., Al-Shyoukh, I., Cheng, G., Ho, C.M., 2012. Cascade search for HSV-1 combinatorial drugs with high antiviral efficacy and low toxicity. Int. J. Nanomed. 7, 2281–2292. doi:http://dx.doi.org/10.2147/IJN.S27540.

Ding, X., Liu, W., Weiss, A., Li, Y., Wong, I., Griffioen, A.W., van den Bergh, H., Xu, H., Nowak-Sliwinska, P., Ho, C.-M., 2014. Discovery of a low order drug-cell response surface for applications in personalized medicine. Phys. Biol. 11, 065003. doi:http://dx.doi.org/10.1088/1478-3975/11/6/065003.

Ding, X., Matsuo, K., Xu, L., Yang, J., Zheng, L., 2015. Optimized combinations of bortezomib, camptothecin, and doxorubicin show increased efficacy and reduced toxicity in treating oral cancer. Anticancer Drugs 547–554. doi:http://dx.doi.org/10.1097/CAD.0000000000000222.

Efferth, T., Dunstan, H., Sauerbrey, A., Miyachi, H., Chitambar, C.R., 2001. The anti-malarial artesunate is also active against cancer. Int. J. Oncol. 18, 767–773. doi:http://dx.doi.org/10.3892/ijo.18.4.767.

Fabbri, F., Carloni, S., Brigliadori, G., Zoli, W., Lapalombella, R., Marini, M., 2006. Sequential events of apoptosis involving docetaxel, a microtubule-interfering agent: a cytometric study. BMC Cell Biol. 7, 6. doi:http://dx.doi.org/10.1186/1471-2121-7-6.

Felix, J., Manchon, M., Dabaghian, Y., Uzor, N., Kesler, S.R., Wefel, J.S., Tsvetkov, A.S., 2016. Levetiracetam mitigates doxorubicin-induced DNA and synaptic damage in neurons. Nat. Publ. Gr. 1–12. doi:http://dx.doi.org/10.1038/srep25705.

Figg, W.D., Chau, C.H., Madan, R.A., Gulley, J.L., Gao, R., Sissung, T.M., Spencer, S., Beatson, M., Aragon-Ching, J., Steinberg, S.M., Dahut, W.L., 2013. Phase II study of satraplatin and prednisone in patients with metastatic castration-resistant prostate cancer: A pharmacogenetic assessment of outcome and toxicity. Clin. Genitourin. Cancer 11, 229–237. doi:http://dx.doi.org/10.1016/j.clgc.2013.04.007.

Honda, Y., Ding, X., Mussano, F., Wiberg, A., Ho, C., Nishimura, I., 2013. and human mesenchymal stem cells 1–9. 10.1038/srep03420

Hong, H., Zhang, Y., Sun, J., Cai, W., 2010. Positron emission tomography imaging of prostate cancer. Amino Acids 39, 11–27. doi:http://dx.doi.org/10.1007/s00726-009-0394-9.

Jaynes, J., Ding, X., Xu, H., Wong, W.K., 2013. Application of fractional factorial designs to study drug combinations. Stat. Med. 30 doi:http://dx.doi.org/10.1002/sim.5526.

Jaynes, J., Wong, W.K., Xu, H., 2016a. Using blocked fractional factorial designs to construct discrete choice experiments for healthcare studies. Stat. Med doi:http://dx.doi.org/10.1002/sim.6882.

Jaynes, J., Zhao, Y., Xu, H., Ho, C.M., 2016b. Use of orthogonal array composite designs to study lipid accumulation in a cell-Free system. Qual. Reliab. Eng. Int. 32, 1965–1974. doi:http://dx.doi.org/10.1002/qre.1900.

Kinebuchi, Y., Wajiki, M., Suzuki, A., Tsuruta, T., K.K, 2011. Docetaxel-based chemotherapy for hormone-refractory prostate cancer in Japanese patients: experience in a single institute. Acta Urol. Jpn. 57, 481–486.

Lamberti, M., Giovane, G., Garzillo, E.M., Avino, F., Feola, A., Porto, S., Tombolini, V., Di Domenico, M., 2014. Animal models in studies of cardiotoxicity side effects from antiblastic drugs in patients and occupational exposed workers. Biomed. Res. Int. 2014 doi:http://dx.doi.org/10.1155/2014/240642.

Leonard, R.C.F., Williams, S., Tulpule, A., Levine, A.M., Oliveros, S., 2009. Improving the therapeutic index of anthracycline chemotherapy: focus on liposomal doxorubicin (Myocet). Breast 18, 218–224. doi:http://dx.doi.org/10.1016/j.breast.2009.05.004.

Li, H., Zhang, Z., Zhou, Z., Ding, X., Zhou, G., 2016. Optimal combination of gemcitabine, sorafenib, and S-1 shows increased efficacy in treating cholangiocarcinoma in vitro and in vivo. Anticancer Drugs 600–608. doi:http://dx.doi.org/10.1097/cad.0000000000000365.

Lindegardh, N., Hanpithakpong, W., Kamanikom, B., Pattayaso, J., Singhasivanon, P., White, N.J., Day, N.P., 2011. Quantification of dihydroartemisinin, artesunate and artemisinin in human blood: overcoming the technical challenge of protecting the peroxide bridge. Bioanalysis 3, 1613–1624. doi:http://dx.doi.org/10.4155/bio.11.158.

Miller, R.E., Sweeney, C.J., 2016. Chemotherapy for metastatic castrate-sensitive prostate cancer 1–6. 10.1038/pcan.2016.10

Nakase, I., Gallis, B., Takatani-nakase, T., Oh, S., Lacoste, E., Singh, N.P., Goodlett, D.R., Tanaka, S., Futaki, S., Lai, H., Sasaki, T., 2009. Transferrin receptor-dependent cytotoxicity of artemisinin – transferrin conjugates on prostate cancer cells and induction of apoptosis. Cancer Lett. 274, 290–298. doi:http://dx.doi.org/10.1016/j.canlet.2008.09.023.

Nowak-sliwinska, P., Weiss, A., Ding, X., Dyson, P.J., Van Den, Bergh H., Griffioen, A. W., Ho, C., 2016. Optimization of drug combinations using feedback system control. Nat. Protoc. 11, 302–315. doi:http://dx.doi.org/10.1038/nprot.2016-017.

Petrylak, D.P., 2003. Docetaxel for the treatment of hormone-refractory prostate. Cancer 5, 14–21.

Peyromaure, M., Valéri, A., Rebillard, X., Beuzeboc, P., Richaud, P., Soulié, M., Salomon, L., 2009. Characteristics of prostate cancer in men less than 50-year-old. Prog. Urol. 19, 803–809. doi:http://dx.doi.org/10.1016/j.purol.2009.04.010.

Posner, G.H., McRiner, A.J., Paik, I.H., Sur, S., Borstnik, K., Xie, S., Shapiro, T.A., Alagbala, A., Foster, B., 2004. Anticancer and antimalarial efficacy and safety of artemisinin-Derived trioxane dimers in rodents. J. Med. Chem. 47, 1299–1301. doi:http://dx.doi.org/10.1021/jm0303711.

Rody, A., Karn, T., Gätje, R., Ahr, A., Solbach, C., Kourtis, K., Munnes, M., Loibl, S., Kissler, S., Ruckhäberle, E., Holtrich, U., von Minckwitz, G., Kaufmann, M., 2007. Gene expression profiling of breast cancer patients treated with docetaxel, doxorubicin, and cyclophosphamide within the gepartrio trial: HER-2, but not topoisomerase II alpha and microtubule-associated protein tau, is highly predictive of tumor response. Breast 16, 86–93. doi:http://dx.doi.org/10.1016/j.breast.2006.06.008.

Sternberg, C.N., Petrylak, D.P., Sartor, O., Witjes, J.A., Demkow, T., Ferrero, J.M., Eymard, J.C., Falcon, S., Calabr, F., James, N., Bodrogi, I., Harper, P., Wirth, M., Berry, W., Petrone, M.E., McKearn, T.J., Noursalehi, M., George, M., Rozencweig, M., 2009. Multinational, double-blind, phase III study of prednisone and either satraplatin or placebo in patients with castrate-refractory prostate cancer progressing after prior chemotherapy: the SPARC trial. J. Clin. Oncol. 27, 5431–5438. doi:http://dx.doi.org/10.1200/JCO.2008.20.1228.

Su, Z., Ye, J., Qin, Z., Ding, X., 2015. Protective effects of madecassoside against Doxorubicin induced nephrotoxicity in vivo and in vitro. Sci. Rep. 5, 18314. doi:http://dx.doi.org/10.1038/srep18314.

Tacar, O., Sriamornsak, P., Dass, C.R., 2013. Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. J. Pharm. Pharmacol. doi:http://dx.doi.org/10.1111/j.2042-7158.2012.01567.x.

Thorn, J.C., Turner, E.L., Hounsome, L., Walsh, E., Down, L., Verne, J., Donovan, J.L., Neal, D.E., Hamdy, F.C., Martin, R.M., Noble, S.M., 2016. Validating the use of Hospital Episode Statistics data and comparison of costing methodologies for economic evaluation: an end-of-life case study from the Cluster randomised triAl of PSA testing for Prostate cancer (CAP). BMJ Open 6, e011063. doi:http://dx.doi.org/10.1136/bmjopen-2016-011063.

Tsutsui, H., Valamehr, B., Hindoyan, A., Qiao, R., Ding, X., Guo, S., Witte, O.N., Liu, X., Ho, C.M., Wu, H., 2011. An optimized small molecule inhibitor cocktail supports long-term maintenance of human embryonic stem cells. Nat. Commun. 25, 167. doi:http://dx.doi.org/10.1038/ncomms1165.

Van der Borght, K., Van Craenenbroeck, E., Lecocq, P., Van Houtte, M., Van Kerckhove, B., Bacheler, L., Verbeke, G., van Vlijmen, H., 2011. Cross-validated stepwise regression for identification of novel non-nucleoside reverse transcriptase inhibitor resistance associated mutations. BMC Bioinf. 12, 386. doi:http://dx.doi.org/10.1186/1471-2105-12-386.

Weiss, A., Berndsen, R.H., Ding, X., Ho, C.-M., Dyson, P.J., van den Bergh, H., Griffioen, A.W., Nowak-Sliwinska, P., 2015. A streamlined search technology for identification of synergistic drug combinations. Sci. Rep. 5, 14508. doi:http://dx.doi.org/10.1038/srep14508.

Xie, H., Li, C., Dang, Q., Chang, L.S., Li, L., 2015. Infiltrating mast cells increase prostate cancer chemotherapy and radiotherapy resistances via modulation of p38/p53/p21 and ATM signals. Oncotarget 5, 1–13. doi:http://dx.doi.org/10.18632/oncotarget.6372.

Xu, H., Jaynes, J., Ding, X., 2014. Combining two-level and three-level orthogonal arrays for factor screening and response surface exploration. Stat. Sin. 24, 269–289. doi:http://dx.doi.org/10.5705/ss.2012.210.

Yuan, J., Li, K., 2010. Research of the effect of recombinant human endostatin and docetaxel on MMP and its following anti-neoplastic effect under different administration sequences. Chin. J. Lung Cancer 13, 580–585. doi:http://dx.doi.org/10.3779/j.issn.1009-3419.2010.06.003.