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14. ABSTRACT

Nanoparticles can be used to enhance the efficacy of chemotherapeutic drugs while minimizing their side effects. This project aims to utilize nanoparticles for effective delivery of two therapeutic agents. In particular, we aim to use two breast cancer drugs which enhance each other's efficacy (synergistic drugs), and use them as a model drug pair for the development of a novel dual-drug nano-therapeutic system. The research to date on the project has been focused on screening and identification of drug combinations that exhibit synergistic effect in vitro. This report summarizes these studies and the progress to date.

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Nanoengineered Drug Combinations for Breast Cancer Treatment

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I. Introduction

Nanoparticles have been utilized as a superior delivery alternative to free drug solutions. In particular, nanoparticles for encapsulating two drugs have become an attractive area of research. Such a vehicle would not only protect both drugs, but would also allow unified drug accumulation at the disease site. Breast cancer is a particularly appealing disease target due to the vast variety of FDA-approved breast cancer drugs, many of which have already been used in combination treatments. However, clinical trials show that combination chemotherapy can result in increased side effects and little or no increase in therapeutic efficacy. These issues can be attributed to drug accumulation in healthy tissue and unprecedented drug interactions, such as antagonism. A nanoparticle which encapsulates two synergistic drugs and is made to specifically target breast cancer tumors can limit accumulation in healthy tissue and increase therapeutic efficacy. This project aims to identify two breast cancer drugs which enhance each other's mechanisms (synergistic drugs), and use it as a model drug pair for the development of a novel dual drug nano-carrier. The proposed nanoparticle vehicle is one which has already been developed for the compartmental loading of various agents, but has not yet been applied for drug encapsulation: biphasic particles. Biphasic particles contain two separate compartments, which can potentially be loaded with distinct drugs.

II. Progress Report of Research Tasks

Task: Generate and screen libraries of binary combinations of chemotherapeutic drugs for breast cancer.

Synergy between two drugs can be quantified by calculating the combination index (CI), a method developed by Chou et al. [1]. A drug combination is synergistic if CI < 1, antagonistic if CI > 1, and additive if CI = 1. This method was utilized to screen for synergistic drug pairs

Eqn. 1	$\mathbf{CI} = \frac{\mathbf{D_1}}{\mathbf{D_{x,1}}} + \frac{\mathbf{D_2}}{\mathbf{D_{x,2}}}$	CI
Eqn. 2	$\frac{\mathbf{f_a}}{\mathbf{f_u}} = \left(\frac{\mathbf{D}}{\mathbf{D_{50}}}\right)^{\mathbf{m}}$	Dose-effect

between FDA-approved drugs. Four drugs were chosen for the original scope in order to keep the screen at a manageable size. Docetaxel (DOC) is an antimicrotubule agent, lapatinib (LAP) is a tyrosine kinase inhibitor, 5-fluorouracil (5-FU) is an antimetabolite, and doxorubicin (DOX) is a topoisomerase II inhibitor. Each drug has a unique mechanism, which enables cell inhibition by various pathways upon combination.

The CI is defined in Eqn. 1. $D_{x,1}$ and $D_{x,2}$ are the doses of drug 1 and 2 respectively which inhibit the system *x%* when used alone. D_1 and D_2 are the combination doses of drug 1 and drug 2 respectively which also inhibit *x%*. CI can also vary for each drug combination, depending on the doses used. To find $D_{x,1}$ and $D_{x,2}$ values for CI evaluation, the dose D of each drug must be defined as a function of inhibition, x. Thus, single drug dose-effect curves were needed.

To obtain dose-effect data, the human breast cancer cell line BT474 was incubated with the specified drug and subsequently analyzed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in vitro cell cytotoxicity assay. When living cells internalize MTT, they convert MTT to a purple-colored dye. Thus the amount of living cells correlates to the intensity of the dye. Fraction affected, or fraction of cell proliferation inhibition, can be calculated from this and plotted against drug dose. These data points were then fitted to the theoretical dose-effect curve described by Eqn. 2. In this model, f_a indicates fraction affected, f_u is the fraction unaffected, D is the drug dose which elicits the specific f_a, and D_{50} is the dose which causes half of the population to be affected. The shape of the drug-effect relationship is given by m, and it varies from system to system. A linear regression fit to experimental data was utilized to determine m and D_{50} values for each drug.

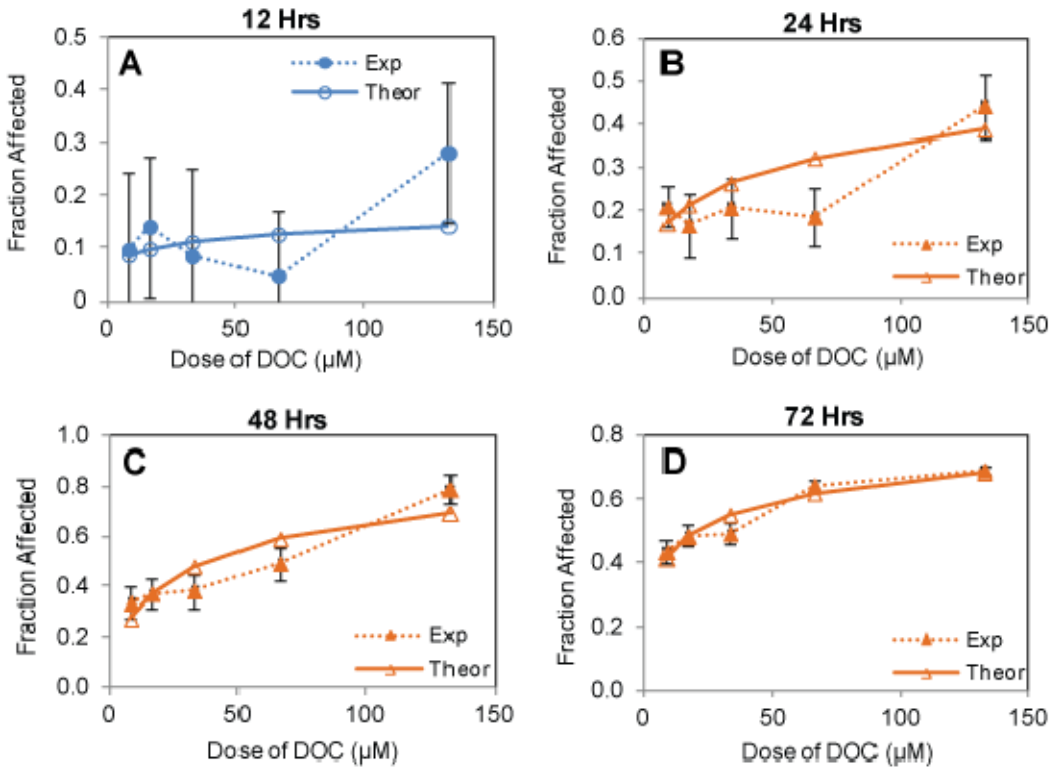

Fig. 1. Dose-effects of DOC on BT474 cell growth after (a) 12 hour (b) 24 hour (c) 48 hour and (d) 72 hour incubations. All data represent averages ± standard deviations of three replicates, except (a), which represents the data for n=1. Solid lines are fits to the median-effect model (Eqn. 3). For (d), m = 0.47 and D_{50} = 23.2 μM.

However, prior to determining the dose-effect curves for all drugs, it was necessary to optimize drug incubation time. Incubation time had to be long enough to allow drug internalization as well as activation. For these studies, cells were incubated with DOC for 12, 24, 48, and 72 hours, and then analyzed with MTT assays. The results are seen in Fig. 1. Reproducibility seemed to increase with incubation time, and the theoretical dose-effect model best fit data from 72 hour incubations. The poor consistency observed at short incubation times was likely due to the lag time between drug internalization and drug action. In addition, the D_{50} value measured after 72 hour drug incubation was 23.2 μM which matched with previously reported values (33.1 +/- 9.3 μM) [2]. Therefore, a 72 hour incubation period was chosen for all dose-effect studies.

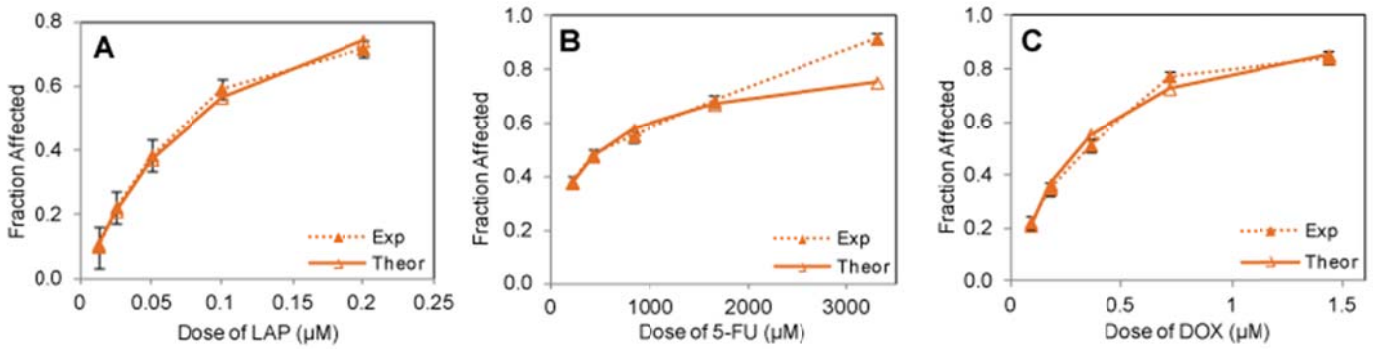

Fig. 2. (a) Dose-effect of LAP, m=1.14, D_{50} = 0.08 μM. (c) Dose-effect of 5-FU, m=0.58, D_{50} =486.8 μM. (d) Dose-effect of DOX, m=1.1, D_{50} =0.30 μM. All data represents averages ± standard deviation of at least three replicates.

Experimental dose-effect data and theoretical dose-effect curves for the remaining drugs are seen in Fig. 2. Generally all theoretical curves fit the experimental data well, and the experimentally determined D_{50} values agreed with previous literature reports [3–5]. One anomaly was seen with 5-FU: deviation from the median-effect model occurred at the largest dose for 5-FU single agent MTT assays (3300 μM). However, the doses to be explored in drug combination studies range from one fourth the D_{50} value to four times the D_{50} value (1950 μM), and the median-effect model fits well for this range. It is clear that all the single drugs are capable of high efficacies (≥ 75%), but only at doses much greater than their D_{50} values. Combining these drugs could potentially decrease the doses needed for high efficacy and thus reduce undesirable side effects.

For initial combination studies, drugs were screened for synergism in equal potencies. For instance, cells were simultaneously exposed to drugs 1 and 2 at each of their D_{50} concentrations. Doses were ranged between one-fourth and four times the D_{50} values for each drug pair. However, this large range of drug concentrations posed potential solubility issues. It was important that the drugs were soluble in water-based cell culture medium both at the large and small dilutions. If drugs were not well-dispersed in medium, varied amounts of drug would be available to cells, leading to varied cytotoxicity results. DOC was the only drug which presented this issue, since it is only soluble in water up until ~0.025 mg/mL (31 μM), very close to its D_{50} of 23 μM. Because of this, DOC had to be excluded from the combination experiments, and the scope was decreased from four to three drugs.

All possible two-drug combinations were evaluated between 5-FU, LAP, and DOX. The CI (Eqn. 1) was calculated for each data point and plotted as a function of f_a. Results for all pairs are shown in Fig. 3. The data suggests that 5-FU with LAP, and DOX with LAP were antagonistic pairs (CI > 1). It seemed that 5-FU with LAP was highly antagonistic regardless of the dose used. On the other hand, DOX with LAP and 5-FU with DOX showed decreasing CI values (increasing synergy) with increasing dose. The most synergistic pair (lowest CI) of these equal potency combinations seemed to be 5-FU with DOX at four-times their D_{50} concentrations.

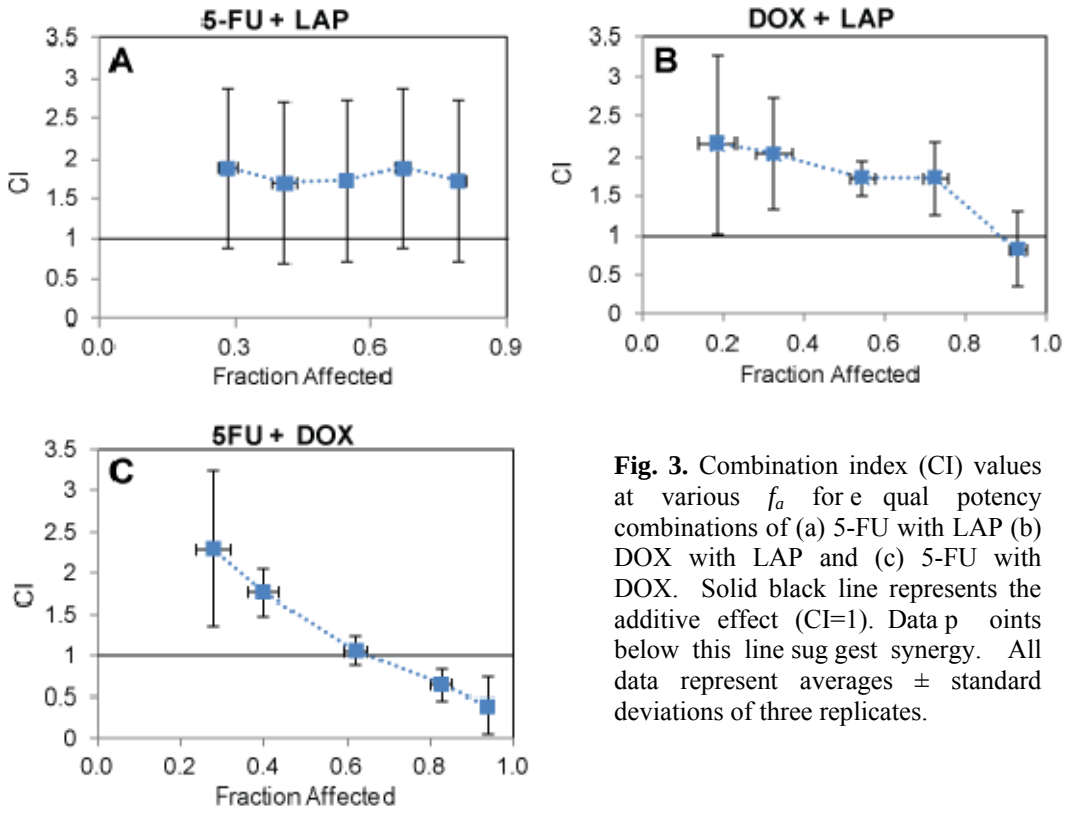

Fig. 3. Combination index (CI) values at various f_a for equal potency combinations of (a) 5-FU with LAP (b) DOX with LAP and (c) 5-FU with DOX. Solid black line represents the additive effect (CI=1). Data points below this line suggest synergy. All data represent averages ± standard deviations of three replicates.

It became apparent, however, that exact concentrations of drugs cannot be controlled with the proposed dual drug carrier since the number of particles which accumulates at target sites can vary widely. Rather, it is drug ratios that can be controlled. Therefore it was necessary to find a drug pair which is consistently synergistic at some optimal ratio. Since 5-FU and DOX seemed to be the most promising drug pair in this scope, various ratios were investigated with this pair until a consistently synergistic ratio was identified. These studies yielded an optimal 5-FU:DOX ratio of 819:1 μM, demonstrated in Fig. 4a. For various concentrations at this ratio, 5-FU and DOX exhibited synergism (CI <1). This contrasts with equal potency combinations of 5-FU and

DOX (Fig. 3c), which is not consistently synergistic. In Figs. 4b-c, single-agent doses and combination doses which achieve the same cell inhibition at 819:1 μM 5-FU:DOX are compared. As expected, large differences in single-agent and combination doses correlate with low CI values (high synergism). For all concentrations tested, the single drug doses well exceeded the combination doses which achieve the same cell inhibition. This verifies that 5-FU and DOX enhance each other's efficacies when combined at an 819:1 μM ratio. Therefore this drug pair will be utilized as a model combination for biphasic particle encapsulation.

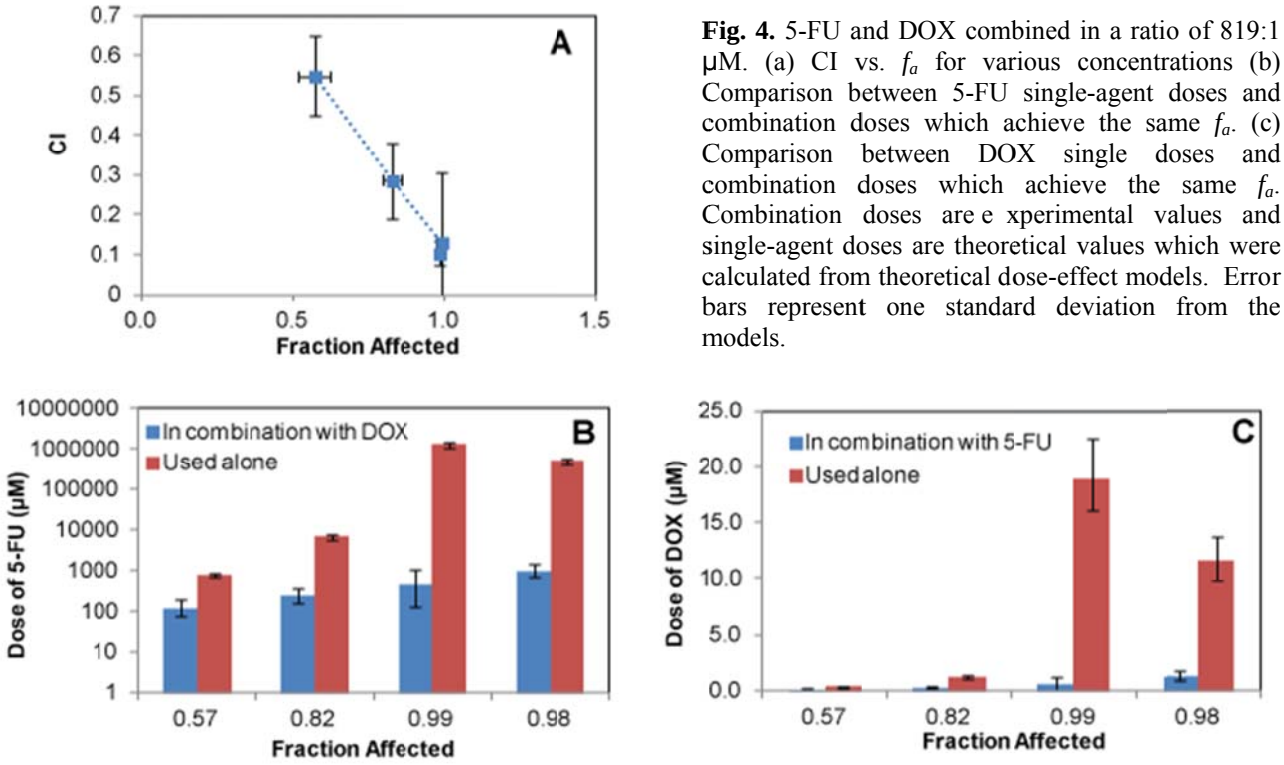

Fig. 4. 5-FU and DOX combined in a ratio of 819:1 μM. (a) CI vs. f_a for various concentrations (b) Comparison between 5-FU single-agent doses and combination doses which achieve the same f_a. (c) Comparison between DOX single doses and combination doses which achieve the same f_a. Combination doses are experimental values and single-agent doses are theoretical values which were calculated from theoretical dose-effect models. Error bars represent one standard deviation from the models.

III. Key Research Accomplishments

Identification of a synergistic chemotherapy drug pair

Dose-effect data was acquired via MTT assays, and demonstrated that single drugs are capable of high efficacies at large doses. The CI method was utilized to screen for

synergistic drug pairs between 5-FU, DOX, and LAP. The combination of 5-FU and DOX resulted in great enhancement for both 5-FU and DOX efficacies, and thus revealed high synergy (CI < 1). However, this effect greatly depended on the incubation ratio of 5-FU:DOX. The ratio of 819:1 μM 5-FU:DOX resulted in consistent synergy, regardless of the drug concentrations investigated. Thus, this drug pair will be used for biphasic particle encapsulation in future work.

IV. Reportable Outcomes

Kathryn Camacho, UCSB graduate student in chemical engineering, has advanced to PhD candidacy based off of her work in this project.

V. Conclusion

Dual drug-loaded nanoparticles can allow for co-localization of drugs at target sites and maintenance of synergistic ratios, leading to more effective and efficient combination therapies. This project aims to develop dual drug-loaded nanoparticles for the co-administration of synergistic chemotherapy drugs. In particular, biphasic particles which were developed at the University of Michigan are suitable particles for multiple drug loading. Studies this past year identified synergism between FDA-approved breast cancer drugs. A highly synergistic drug pair of 5-FU:DOX at 819:1 μM was identified, and will be loaded into biphasic particles for co-administration in the ongoing studies.

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