Expert Opinion

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Use of nanoscale delivery systems to maintain synergistic drug ratios *in vivo*

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Importance of the field: Drug combinations have been the standard of care in the treatment of cancer for > 50 years. Typically, combination chemotherapy uses agents with non-overlapping toxicities which are escalated to their maximum tolerated dose. However, emerging evidence indicates that this approach may not be providing optimal efficacy depending on the drug ratios to which the tumor is exposed. Combined drugs can be synergistic whereas other ratios of the same agents may be antagonistic or additive.

Areas covered in this review: In this review, we examine the importance of drug ratios in cancer therapy. We describe how manipulation of the lipid membrane and internal buffer composition maintains synergistic ratios of irinotecan and floxuridine (CPX-1), daunorubicin and cytarabine (CPX-351) or cisplatin and irinotecan (CPX-571). For polymer-based nanoparticles, prodrug hydrophobicity was exploited to coordinate the release of gemcitabine and the more hydrophobic paclitaxel. We present preclinical data for liposomal drug combinations which demonstrate that the most efficacious formulation is not always the highest dose of both agents.

What the reader will gain: An insight into the use of liposomes and polymer-based nanoparticles to deliver synergistic drug combinations to the tumor site and avoid antagonistic drug–drug interactions.

Take home message: The ability to control and maintain drug ratios *in vivo* through the use of nanoscale delivery vehicles results in a significant improvement in therapeutic activity.

Keywords: combination chemotherapy, drug delivery, liposome, nanoparticle, synergy

Expert Opin. Drug Deliv. (2010) 7(12):1329-1341

1. Introduction

The concept of using multiple agents to treat cancer was first evaluated by Freireich and Frei who demonstrated that combining methotrexate, vincristine, 6-mercaptopurine and prednisone induced long-term remissions in children with acute lymphoblastic leukemia [1,2]. Through the addition of asparaginase, daunorubicin and cytarabine, cure rates in children have subsequently increased to ~ 85%. The success of this approach has resulted in the use of drug combination regimens for treating the majority of metastatic cancers today. Most drug combinations have been established by combining drugs with different dose limiting toxicities, pushed to their respective maximum tolerated dose (MTD). In this way, the maximum amount of cytotoxic agent is administered to the patient under the assumption that maximum therapeutic activity will be achieved with maximum dose intensity. More recently, liposome carriers have been used to deliver drug combinations alleviating drug toxicities and enhancing tumor accumulation through the enhanced permeability and retention (EPR) effect. Unfortunately, in some cases, liposomal formulations of combined drugs fail to improve the therapeutic activity over single drug formulations. For example, a study by Abraham *et al.* [3] revealed that the

10.1517/17425247.2010.538678 © 2010 Informa UK, Ltd. ISSN 1742-5247

Article highlights.

- Liposomal delivery of synergistic drug combinations.
- Rationale for the use of low cholesterol liposomes: This section describes the approach used to coordinate the release of two drugs by the incorporation of 10% cholesterol into distearoylphosphatidylcholine/ distearoylphosphatidylglycerol liposomes.
- Simultaneous encapsulation of floxuridine and irinotecan: Our innovative dual encapsulation method is described.
- Rationale for the use of copper gluconate/ triethanolamine: The role of internal buffer composition in modulating drug retention is discussed.
- Singly formulated liposomes: For drug combinations utilizing very reactive agents, each agent is encapsulated in its own formulation. This approach was used to develop CPX-571 a liposomal formulation of cisplatin and irinotecan.
- Importance of controlling drug ratios for optimal drug activity: We examine the effect of various fixed drug ratios on the therapeutic activity of liposomal formulations: CPX-351 (daunorubicin and cytarabine), CPX-1 (irinotecan and floxuridine) and CPX-571 (cisplatin and irinotecan).
- Nanoparticle delivery systems for hydrophobic drugs: An avenue to control prodrug release from polymer-based nanoparticles by varying lipid anchor hydrophobicity is presented.

This box summarizes key points contained in the article.

administration of a co-encapsulated liposomal formulation of doxorubicin and vincristine was not therapeutically superior to liposomal vincristine alone. Efficacy studies in tumor bearing mice indicated that the administration of liposomal vincristine at 2.5 mg/kg resulted in a 16-day delay in tumor growth while only a 7-day delay in tumor growth was obtained with liposomal doxorubicin at 10 mg/kg. Surprisingly, the co-encapsulated liposomal vincristine and doxorubicin administered at the same dose as the single formulated drugs (2.5:10 mg/kg) resulted in a 12-day delay in tumor growth, which was inferior to liposomal vincristine [3]. These surprising results prompted us to investigate the cytotoxic effects of vincristine and doxorubicin when used in combination. *In vitro* cytotoxicity studies for the vincristine:doxorubicin drug combination revealed the presence of strong antagonism for this drug combination. Hence, the method of choosing drug candidates for combination therapy based only on their toxicity profile and mechanism of action does not always result in an improvement in therapeutic activity. Therefore, the identification of synergistic drug combinations is critical to the successful application of combination therapy to cancer treatment. To determine if a drug combination can act synergistically, a panel of tumor cell lines is incubated with the drug combination in a range of drug:drug ratios and concentrations [4-6]. The mathematical analysis of the cell viability data will reveal if the combination is synergistic, additive or antagonistic and if the mechanism of action is drug ratio-dependant.

A variety of mathematical methods have been proposed to evaluate drug combinations for synergy or antagonism. The models described in the literature range from general techniques requiring simple manual calculations to sophisticated algorithms aided by computers. By far the most prevalent model used for drug combination analysis is the median-effect method of Chou and Talalay [7-9]. The advantages of this method include: i) the fundamental equations used were derived from basic mass action enzyme kinetic models; ii) the fitting of data uses an accepted statistical approach, namely, linear regression; iii) the experimental design requires fewer data points than other methods, and iv) the method is available as a software package allowing for easy data entry and modeling. For a comprehensive review of the median effect, isobologram, response surface and other combination synergy assessment methods, refer to 'The Search for Synergy: A Critical Review from a Response Surface Perspective' by Greco *et al.* [10]. The median effect analysis method is used to determine whether specific drug ratios were synergistic, additive or antagonistic as a function of the fraction of cells killed. The cell viability as a function of drug concentration curves are used to generate mathematical algorithms that allow the calculation of the combination index (CI) for each drug combination [5,6]. The results are presented as a synergy heat map where the CI values reflecting synergy (< 0.9), additivity (0.9 – 1.1) or antagonism (> 1.1) are represented by the colors green, yellow and red, respectively (**Table 1**). When CI values are examined at high cell kill (the most clinically relevant), we observe regions of drug synergy as well as regions of antagonism. The ideal synergistic drug ratio zone will involve multiple ratios and show consistent synergy across a variety of tumor types. To capture this synergy *in vivo*, the target drug ratio must be successfully delivered to the tumor site. In this review, we describe the use of nanotechnology to control and maintain synergistic drug ratios *in vivo*.

2. Liposomal delivery of synergistic drug combinations

A major problem associated with the administration of a drug combination as a conventional free drug cocktail is the inability to control drug ratios *in vivo* due to the diverse pharmacokinetic properties of each drug. Liposome-based delivery systems coupled with *in vitro* screening informatics were utilized to avoid *in vivo* antagonism and deliver synergistic ratios to the tumor site. Once the synergistic drug ratios are identified, a carrier that is capable of delivering the combination at these predetermined ratios is designed. Systematic manipulation of liposome parameters such as membrane composition and internal buffer are performed in conjunction with plasma elimination studies to define the role of each component on drug retention. This iterative structure–function analysis produces a delivery vehicle which can fix and deliver the

Table 1. *In vitro* synergy heat map of cytarabine:daunorubicin.

Cell Lines Screened	Tumor	CI @ Fa = 0.9				
		1:10	1:5	1:1	5:1	10:1
HCT-116	Colon	0.8	0.7	0.8	0.8	0.8
SW620	Colon	1.0	0.6	0.8	0.7	0.6
Nalm-6	Leukemia	1.8	1.6	1.1	1.1	1.1
P388	Leukemia	1.2	1.5	0.6	0.7	0.8
HL60	Leukemia	1.3	1.2	0.8	0.8	1.1
L1210	Leukemia	1.4	1.5	1.3	0.8	1.1
A253	Oral	1.1	1.0	1.1	0.8	0.9
BXPC-3	Pancreatic	1.1	1.1	1.2	1.0	0.9
IGROV-1	Ovarian	2.0	1.3	1.5	1.1	0.9
Capan-1	Pancreatic	1.0	0.9	1.0	0.9	1.6

Adapted from data presented in [5].
Green: synergistic (< 0.9); yellow: additive (0.9 – 1.1); red: antagonistic (> 1.1).
CI: Combination Index; Fa: Fraction affected.

desired drug ratio *in vivo*. This review describes the formulation approaches used to successfully develop a liposomal formulation of irinotecan and floxuridine (CPX-1) for the treatment of metastatic colorectal cancer; a liposomal combination of cytarabine and daunorubicin (CPX-351) for acute myeloid leukemia and a combination of liposomal irinotecan and cisplatin (CPX-571) for small-cell lung cancer (SCLC).

2.1 Rationale for the use of low cholesterol liposomes

Historically, cholesterol (Chol) was incorporated into liposomes to increase their stability and decrease liposome binding to plasma proteins [11,12]. The addition of high concentrations of cholesterol to saturated phospholipids has historically provided maximum drug retention *in vivo* [13-18]. Preliminary efforts to co-encapsulate irinotecan and floxuridine were completed in liposomes consisting of distearoylphosphatidylcholine (DSPC)/Chol (55:45 mol/mol). Unfortunately, when this formulation was evaluated *in vivo*, floxuridine retention was very limited [19]. As a result of these poor performances, we investigated the use of cholesterol-free or low cholesterol liposomes which had previously been shown by Dos Santos *et al.* [20] to have unique drug retention properties. This report demonstrated that cholesterol-free liposomes consisting of DSPC/DSPE-PEG_{2000} could enhance retention of idarubicin over conventional DSPC/Chol formulations. The superior retention properties of the low cholesterol liposomes translated into superior therapeutic activity for formulations with low PEG percentages [21]. Although promising, we were concerned about the inclusion of PEG-based lipids in our final formulation for the following reasons: i) the use of PEG containing lipids generally reduces liposome clearance; however, their successful use was reported to vary with the nature of the lipid anchor [22]; ii) accelerated clearance of PEG liposomes has been observed in rats on repeated injection [23,24] and at low lipid doses [25]; and iii) the inclusion of PEG-PE into liposomal formulations of vincristine and idarubicin was shown to enhance drug leakage, resulting in a corresponding decrease in therapeutic activity [20,26,27].

As an alternative to the use of PEG modified lipids, we investigated the use of negatively charged lipids that provide a steric repulsion rather than a steric barrier. The pharmacokinetics of anionic liposomes is highly dependent on the nature of the negatively charged lipid used. Prolonged circulation lifetimes and enhanced drug uptake by tumors with reduced reticuloendothelial system toxicity were observed with liposomes containing monosialoganglioside, phosphatidylinositol (PI) or phosphatidylglycerol (PG) [28-30]. Other anionic lipids such as phosphatidic acid and phosphatidylserine were shown to enhance liposomes clearance [29,31]. These differences in clearance kinetics have been attributed in part to the amount and type of blood proteins bound to the liposomes [29,32]. When PI and PG were compared in our laboratory, the circulation lifetime of liposomes containing between 10 and 30 mol% PG was superior to that of PI containing liposomes. The optimal circulation lifetime was reached at ~ 20 mol% of distearoylphosphatidylglycerol (DSPG).

Due to the superior circulation lifetime of the cholesterol-free DSPG containing liposomes, a DSPC/DSPG (80/20 mol%) formulation was evaluated for co-encapsulation of irinotecan and floxuridine. The results shown in **Figure 1A** reveal enhanced retention of floxuridine compared to irinotecan. The floxuridine:lipid ratio decreased linearly over time with a half-life of ~ 14 h. In contrast, the retention of irinotecan was poor with > 60% drug leakage within the first hour after intravenous (i.v.) injection [19]. The technical hurdle of coordinating the release of these two drugs was overcome by an iterative variation of the lipid composition.

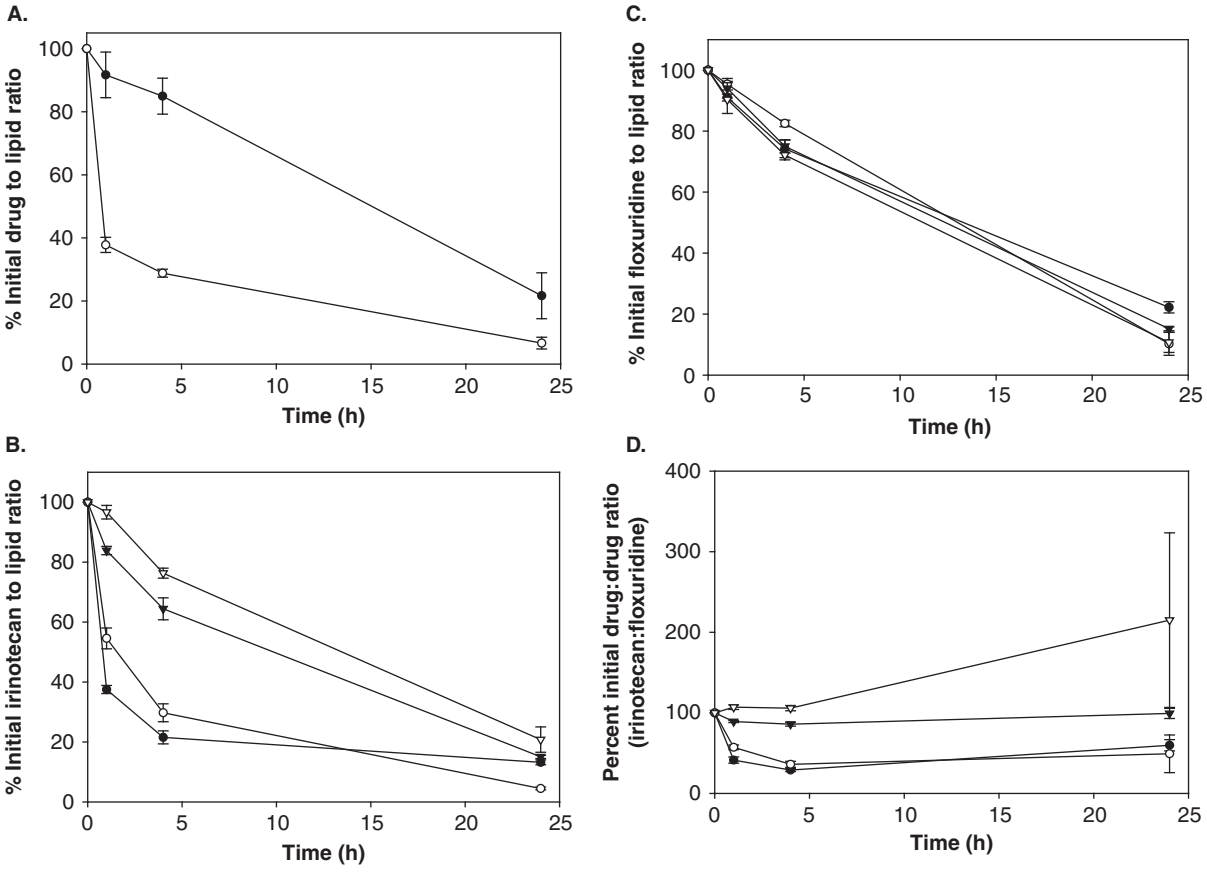


Figure 1. (**A**) The *in vivo* retention of floxuridine (●) and irinotecan (○) co-formulated in DSPC:DSPG (80:20 molar ratio) liposomes. (**B – C**): The *in vivo* retention of floxuridine and irinotecan coformulated in various liposomal formulations. Liposomes composed of DSPC:Chol:DSPG (65:15:20 molar ratio) ▽; DSPC:Chol:DSPG (70:10:20 molar ratio) ▼; DSPC:Chol:DSPG (75:5:20 molar ratio) ○; and DSPC:DSPG (80:20 molar ratio) ●. Changes to irinotecan to lipid levels with time are monitored in the panel **B** while panel **C** plots the floxuridine to lipid levels from the same formulation. Panel **D** charts change in the drug: drug molar ratio in the various liposomal formulations over time.
Reprinted with permission from [19].

Because cholesterol was shown to effect membrane permeability and regulate drug retention properties [19,20], a titration of cholesterol into the DSPC/DSPG (80/20 mol%) liposomes was performed. Various liposome formulations containing cholesterol levels ranging from 0 to 15% were tested for drug retention *in vivo*. Contrary to floxuridine, irinotecan release was found to be dramatically decreased by elevated cholesterol content. Optimal irinotecan retention was obtained with 10 – 15% cholesterol whereas floxuridine leakage was largely unchanged with cholesterol content between 0 and 15% (**Figures 1B** and **C**). As shown in **Figure 1D**, the synergistic 1:1 molar drug ratio was maintained in the formulation containing 10% cholesterol for which the release rates of both agents were coordinated. The successful co-formulation and subsequent control of drug release provide an excellent example of how cholesterol content can be manipulated to modulate drug retention properties of gel phase liposomes.

2.2 Simultaneous encapsulation of floxuridine and irinotecan

The initial co-encapsulation of irinotecan and floxuridine was achieved by hydrating and extruding the lipid films in the presence of floxuridine. The liposomes were subsequently buffer exchanged to remove unencapsulated floxuridine and then actively loaded with irinotecan [19]. The potential hazards associated with extruding large batches of liposomes in the presence of chemotherapeutics led to the development of an alternative entrapment procedure. The ability of irinotecan and floxuridine to readily permeate the liposome membrane allowed for the development of a simultaneous drug encapsulation process. Liposomes are first extruded in the absence of cytotoxics and subsequently incubated with a solution containing both floxuridine and irinotecan at 50°C. This temperature was found to be an optimal balance between the passive accumulation of floxuridine and the high efficiency encapsulation of irinotecan. The two drugs accumulate inside

the liposomes at the target 1:1 molar drug ratio without compromising each others integrity [19]. To our knowledge, this is the first example of simultaneous passive and active drug encapsulation. The pharmacokinetics and drug release properties using the dual loading procedure were similar to those obtained by the sequential encapsulation of floxuridine and irinotecan [19].

Although the example presented above describes the approach used to develop the lipid composition of CPX-1, the unique properties of the low cholesterol liposomal delivery systems were applied to develop CPX-351, a liposomal formulation of cytarabine and daunorubicin, which is currently being evaluated in two concurrent Phase II trials for the treatment of acute myeloid leukemia [33]. The next section describes the iterative process used to develop CPX-351 and the role played by the internal buffer composition on drug encapsulation and coordinated drug release from the liposomes.

2.3 Rationale for the use of copper gluconate/ triethanolamine

Daunorubicin and cytarabine are currently used as the standard of care to treat acute myeloid leukemia. When this drug combination was evaluated in our panel of tumor cell lines for drug ratio-dependent synergy, the 5:1 molar ratio of cytarabine:daunorubicin showed the most consistent synergy while the 1:1 molar ratio was the most antagonistic [6]. Based on these studies, cytarabine and daunorubicin were formulated into liposomes at the synergistic 5:1 ratio (referred to as CPX-351) to fix and coordinate the release of these two drugs at their synergistic ratio using the same lipid composition described above for CPX-1 [34]. Traditional encapsulation approaches for drugs such as daunorubicin have utilized transmembrane pH gradients which are typically generated through the use of citrate or ammonium sulfate [35-38]. A variation on this method has utilized an ionophore and encapsulated manganese sulfate to generate a pH gradient after liposome formation [3,39]. The main disadvantage of the pH gradient method is the potential hydrolysis of the lipids at acidic pH, which can introduce liposome instability during long-term storage [40,41]. Phospholipid hydrolysis was observed for liposomes stored outside their optimum pH range of 6 – 7 [41]. To alleviate potential difficulties associated with lipid degradation at acidic pH, we developed a novel method to encapsulate drugs at neutral pHs.

Anthracycline complexation with transition metals has been previously reported in the literature [42-47]. To investigate if this binding interaction was strong enough to promote active drug encapsulation, we screened a variety of transition metal salts for their ability to promote anthracycline uptake into liposomes. Unbuffered salts of cobalt and manganese were found to facilitate doxorubicin uptake while magnesium was unable to promote significant levels of drug encapsulation (**Figure 2**). Copper was found to be the most effective at promoting daunorubicin encapsulation. Thus, two copper salt forms, sulfate and gluconate, were evaluated in the loading of daunorubicin into DSPC/DSPG/Chol (7/2/1 mol%) liposomes. Sodium hydroxide was initially used to adjust the pH of copper solutions to neutrality; however, copper hydroxide precipitates were readily generated. Alternative buffering agents including phosphate, histidine and triethanolamine (TEA) were evaluated and the latter was found to be the most compatible with copper solutions. Copper gluconate–TEA showed superior daunorubicin encapsulation efficiency compared to copper sulfate–TEA. Based on these results, the gluconate salt form of copper was chosen for the active loading of daunorubicin in the CPX-351 formulation [34]. The rate of daunorubicin encapsulation is rapid with encapsulation efficiencies approaching 100%. The uncharged daunorubicin readily passes through the liposomal bilayer where it subsequently forms a coordination complex with copper gluconate [48]. The formation of this complex can be confirmed by an observed color change from orange to purple or analyzed as a red shift of the absorbance spectrum (not shown). The interactions between encapsulated daunorubicin and cytarabine with copper gluconate–TEA were shown to play a crucial role in coordinating the release of both drugs at their synergistic 5:1 molar ratio [48].

3. Singly formulated liposomes

Our main strategy for the delivery of synergistic drug combinations has consisted of co-encapsulating two drugs inside a single liposome. However, for some drugs, this method must be avoided due to the reactivity between the two therapeutic agents. To circumvent this problem, each drug can be formulated in an individual liposome with subsequent mixing of the two drug loaded liposomes at the desired drug:drug ratio prior to i.v. administration. The lipid membrane composition must be identical for the liposomes to exhibit similar circulation longevity and bioavailability at the tumor site. This approach was applied to the development of CPX-571, a liposomal combination of irinotecan and cisplatin. Liposomal formulations of cisplatin have been reported in the literature for many years. One early formulation variant (SPI-077) was evaluated in clinical trials but found to have no significant therapeutic benefit, probably resulting from the low bioavailability of the drug at the tumor site [49-51]. To investigate the role of cisplatin release rate on therapeutic activity, we formulated the drug in a range of liposomes with varying amounts of dipalmitoylphosphatidylcholine (DPPC) in a DSPC:DSPG:Chol-based membrane. Using a radiolabeled lipid marker, cholesteryl hexadecyl ether, we were able to monitor cisplatin release rate from liposomes circulating in the blood. These same formulation variants were also evaluated for their therapeutic benefit relative to saline in a peritoneal P388 survival tumor model. In **Figure 3**, the half-life of cisplatin release *in vivo* is plotted against P388 survival for various lipid compositions. It is apparent from these data that an intermediate cisplatin release rate in the range of 8 – 12 h provided optimal antitumor activity.

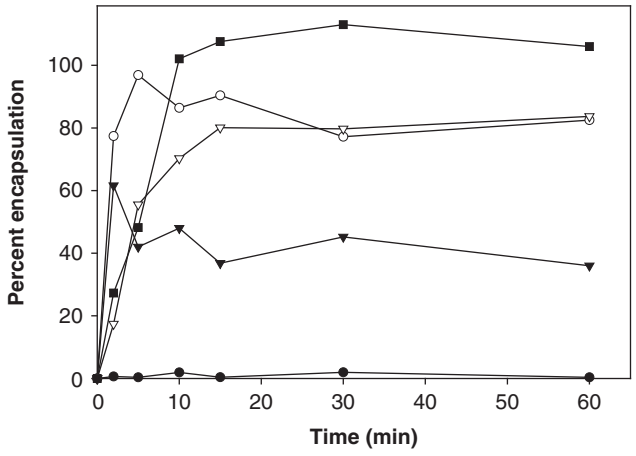


Figure 2. Encapsulation of anthracyclines into liposomes containing various metal salts: $MgCl_2$ (●), $ZnSO_4$ (▼), $CoCl_2$ (○), $MnCl_2$ (▽) and $CuSO_4$ (■).

Based on tolerability and efficacy, the DSPC:DPPC:DSPG:Chol (35:35:20:10 mol%) formulation was chosen for the development of a liposomal cisplatin:irinotecan drug combination (CPX-571).

Irinotecan was actively encapsulated into separate liposomes containing 10 mM sodium gluconate–150 mM TEA, pH 7 while liposomes containing saline were used to passively encapsulate cisplatin. The two formulations were mixed at the synergistic irinotecan:cisplatin molar ratio of 7:1 before i.v. injection into mice. Plasma levels of irinotecan and cisplatin revealed similar clearance rates with an elimination half-life of 5.7 h for irinotecan and 6.9 h for cisplatin [52]. This formulation maintains the irinotecan:cisplatin molar ratio in the plasma near the injected 7:1 ratio over 24 h while the equivalent free drug cocktail ratio fell below detectable levels within 2 h [52].

4. Importance of controlling drug ratios for optimal drug activity

The lipid and buffer compositions of our proprietary drug delivery systems were engineered to coordinate the drug release rates of synergistic combinations. By controlling drug ratios *in vivo*, we have been able to examine the effect of various fixed drug ratios on the overall therapeutic activity of the combination [5,6,53-56]. Based on the data gathered on irinotecan and floxuridine (CPX-1), cytarabine and daunorubicin (CPX-351) and irinotecan and cisplatin (CPX-571), delivery of the synergistic drug ratio *in vivo* results in the most therapeutically active formulation of a given drug combination. Because drug ratios cannot be controlled when administering free agents, the tumor is exposed to a range of ratios, some of which will be antagonistic. Our hypothesis is that this is the reason why some drug combinations may not have met their full potential in clinical trials.

Literature reports indicate that the combination of irinotecan and cisplatin is synergistic when exposed to human tumor cells [57,58] and cancer cells from colorectal patients [59]. However, a recent clinical trial in SCLC patients failed to show a therapeutic benefit with irinotecan–cisplatin over the etoposide–cisplatin regimen [60-62]. This result might be explained by the occurrence of drug ratio dependency where the tumors are exposed to both synergistic and antagonistic drug ratios [4,52,53,63]. Because the pharmacokinetic properties of the free agents cannot be controlled, the true importance of exposing tumor cells to fixed drug ratios can only be addressed with drug delivery. We have evaluated the importance of drug ratio synergy and antagonism in all three drug combinations described above through the use of liposomal formulation.

For the drug combination of irinotecan–cisplatin, *in vitro* drug screening revealed the presence of drug ratio-dependent synergy at molar ratios of 5:1, 7:1 and 10:1. Typically, expanded efficacy evaluation is performed on the ratio that delivers intermediate doses of both agents and has more potential to provide consistent activity against tumor types that exhibit a range of sensitivities to irinotecan and cisplatin [62]. Hence, the liposomal formulation of irinotecan–cisplatin at a 7:1 molar ratio (CPX-571) was selected for further studies and showed superior antitumor activity in several tumor xenograft models compared to the free drug cocktail (**Figure 4A**) or either of the individual liposomal drugs at their respective MTDs [52]. In the H69 human SCLC tumors xenograft models, CPX-571 antitumor activity reflected greater tumor growth inhibition than predicted, based on the log cell kill values of the individual liposomal drugs, consistent with strong *in vivo* synergy [62]. Although no tumor regression was attained in the HCT-116 human colon xenograft model, tumor growth delay of 44.5 days compared with 19.5 days for free drug cocktail was observed [62]. Drug ratios identified as antagonistic provided inferior therapeutic activity *in vivo* compared to a synergistic drug ratio despite using 10-fold greater doses of irinotecan.

For the drug combination of cytarabine and daunorubicin, drug synergy was observed at the 5:1 molar ratio. In a P388 leukemia model, liposomal formulations of cytarabine and daunorubicin fixed at various drug ratios were administered at the MTD. The most therapeutically active formulation was the synergistic 5:1 ratio which resulted in 100% survival even at 80% of the MTD (**Figure 4B**). The next most active ratio was observed at 12:1 where an 83% survival was observed. Interestingly, at the 3:1 ratio, the daunorubicin dose was almost twice as high as that of the synergistic 5:1 ratio with an equivalent dose of cytarabine, yet only 50% survival was observed. The observation that higher drug doses can result in inferior therapeutic activity highlights the importance of formulating and delivering synergistic drug ratios to the tumor site. Similar results were also observed with the drug combination of irinotecan and floxuridine. The efficacy of CPX-1 against the capan-1 solid tumor model showed that the liposomal formulation of irinotecan and floxuridine at a synergistic 1:1 ratio elicits 100% tumor regression and long-term survival whereas the free drug cocktail provided minimal tumor growth inhibition (**Figure 4C** and **D**).

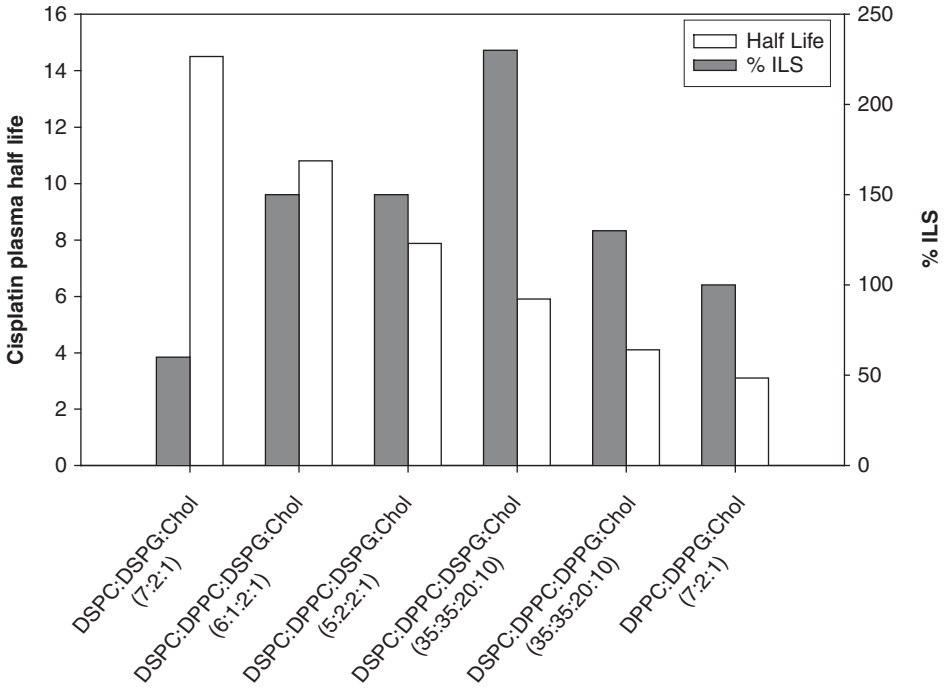


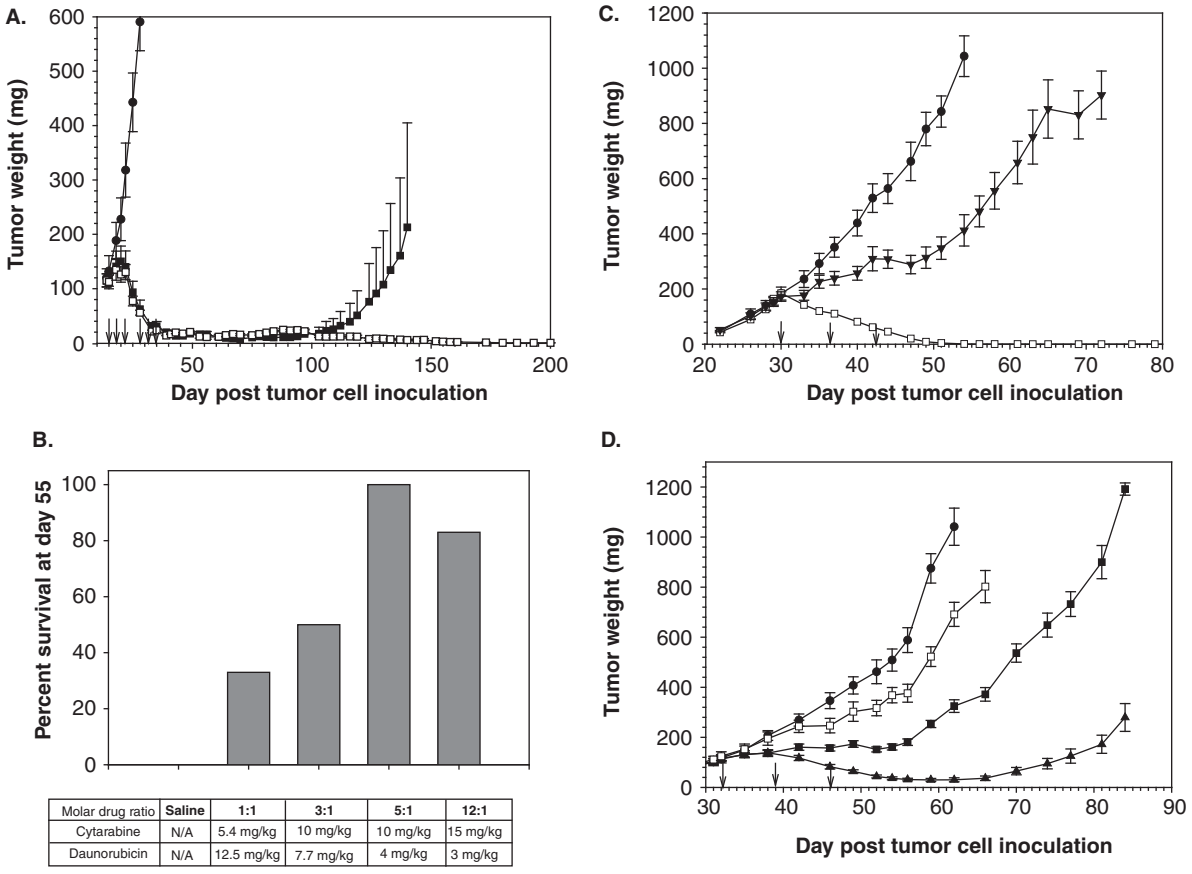
Figure 3. A series of liposomal cisplatin formulations were evaluated for *in vivo* cisplatin release half life and efficacy against the P388 murine leukemia model. Formulations were administered intravenously Q4D × 3 starting one day after tumor cell inoculation. The percentage increase life span was calculated for each treatment group relative to the saline control.

Liposomal delivery of the antagonistic irinotecan: floxuridine 1:10 molar ratio was significantly less active than the synergistic 1:1 molar ratio in solid tumor models [6,19]. Taken together, the full benefit of combination chemotherapy cannot be attained *in vivo* unless the ratios of the drugs are carefully controlled.

5. Nanoparticle delivery systems for hydrophobic drugs

Contrary to water soluble drugs, the use of liposomes to encapsulate hydrophobic drugs such as paclitaxel is more challenging due to the rapid partitioning of these drugs out of the carrier *in vivo* [64,65]. In an attempt to increase the circulation lifetime of hydrophobic drugs, numerous groups have enhanced the aqueous solubility of these drugs by generating hydrophilic prodrugs through chemical conjugation prior to encapsulation into liposomes or polymer-based nanoparticles [66-72]. Unfortunately, there have been limited improvements in efficacy relative to the parent compound using this approach. In our research, we have pursued an alternative approach whereby the hydrophobicity of the drug is increased through conjugation to hydrocarbon anchors. These hydrophobic prodrugs are subsequently incorporated into solid core nanoparticles composed of prodrug, lipid and amphipathic surface stabilizing agents. This technology was used to formulate hydrophobic prodrugs of paclitaxel into nanoparticle vehicles ranging in size from 10 to 30 nm in diameter [73,74]. A range of hydrophobic anchor sizes were conjugated to paclitaxel and assessed for prodrug circulation lifetime following i.v. injection. Nanoparticle levels in the plasma were monitored through the incorporation of the radiolabeled lipid marker cholesteryl hexadecyl ether. As seen in **Figure 5**, the circulation lifetime of the paclitaxel prodrug increased with increasing size of the hydrophobic anchor. Based on particle circulation lifetime, the exchange half-life of the prodrug from the particle could be increased from 1 h in the case of oleyl to ~ 21 h with cholesterol.

To determine the impact of prodrug circulation lifetime on efficacy, the therapeutic activity of the formulations was evaluated in the HT29 human colorectal tumor model. When administered at equivalent paclitaxel doses, the prodrugs with the longest circulation lifetime showed the greatest antitumor effect. Complete tumor regression was achieved in 6 weeks with the cholesteryl diglycolate conjugate (propac8), a considerable improvement over the activity observed with free paclitaxel dosed at its MTD. Hence, the iterative changes to the hydrophobicity of the lipid anchor revealed a correlation between the prodrug partitioning rate and drug efficacy. We hypothesize that the nanoparticles accumulate within the tumors and subsequently release prodrug for hydrolysis back into paclitaxel [74]. Interestingly, no antitumor activity was observed when the paclitaxel was conjugated to lipid anchors



Molar drug ratio	Saline	1:1	3:1	5:1	12:1
Cytarabine	N/A	5.4 mg/kg	10 mg/kg	10 mg/kg	15 mg/kg
Daunorubicin	N/A	12.5 mg/kg	7.7 mg/kg	4 mg/kg	3 mg/kg

Figure 4. (**A**) Antitumor efficacy of CPX-571 compared to free drug cocktail in H69 human SCLC xenograft growth curves using a (Q4D×3) × 2 dosing schedule. Mice received injections of (●) saline, (■) free drug cocktail (7:1 molar ratio) at 34:2.1 mg/kg or (□) liposomal irinotecan:cisplatin (7:1 molar ratio) at 34:2.1 mg/kg. (**B**) Percent survival of P388 ascites tumor-bearing BDF-1 mice at day 55 following intravenous treatment with saline or liposomal cytarabine:daunorubicin (days 1, 4, 7) at different drug molar ratios (n = 6, all formulations dosed at MTD, except CPX-351 (5:1) at 0.8 MTD). (**C**) Efficacy of CPX-1 (□, 25:9.25 mg/kg irinotecan:floxuridine) versus free drug cocktail (▼, 100:37 mg/kg irinotecan:floxuridine) in the Capan-1 human pancreatic tumor xenograft model; (●, saline). (**D**) Efficacy of liposome encapsulated antagonistic ratio versus individual drugs in the Capan-1 pancreatic tumor xenograft model (□, liposomal floxuridine, 18.5 mg/kg; ■, antagonistic liposomal irinotecan:floxuridine (1:10 mol:mol) 5:18.5 mg/kg; ▲, liposomal irinotecan, 5 mg/kg; (●, saline).
Reprinted with permission from (A) [52], (B) [34] and (D) [6].
MTD: Maximum tolerated dose; SCLC: Small-cell lung cancer.

using a slow cleaving succinate linkage. This result highlights the key role that linker chemistry plays in the conversion of the prodrug back into the parent compound at the tumor site.

Varying lipid anchor hydrophobicity provides an avenue for controlling prodrug exchangeability from the nanoparticle. Based on this observation, it should be possible to coordinate the exchange of two distinct prodrugs from the same carrier through manipulation of the hydrophobic anchors. To test this hypothesis, the water soluble drug gemcitabine was made hydrophobic by conjugation to a distearoylglycerol moiety through a diglycolate linker (progem12). Progem12 was subsequently co-formulated with propac7 within the same nanoparticle. When this formulation was administered intravenously into mice, plasma levels of both prodrugs were coordinated for over 24 h (**Figure 6A**). The same formulation evaluated for therapeutic activity in the HT-29 solid tumor model showed superior efficacy of the co-formulated drugs compared to that of the individual agents (**Figure 6B**). The data presented above demonstrate that we have successfully developed a novel approach to deliver hydrophobic anticancer drug combinations in a manner that maintains the drug ratio *in vivo*, resulting in improved antitumor activity.

6. Conclusion

Drug ratio-dependent synergy can profoundly affect the antitumor activity of anticancer drug combinations. Because drug ratios cannot be controlled when administered as a free drug cocktail, we have utilized nanoscale delivery vehicles to maintain and deliver synergistic drug combinations to tumor sites. These

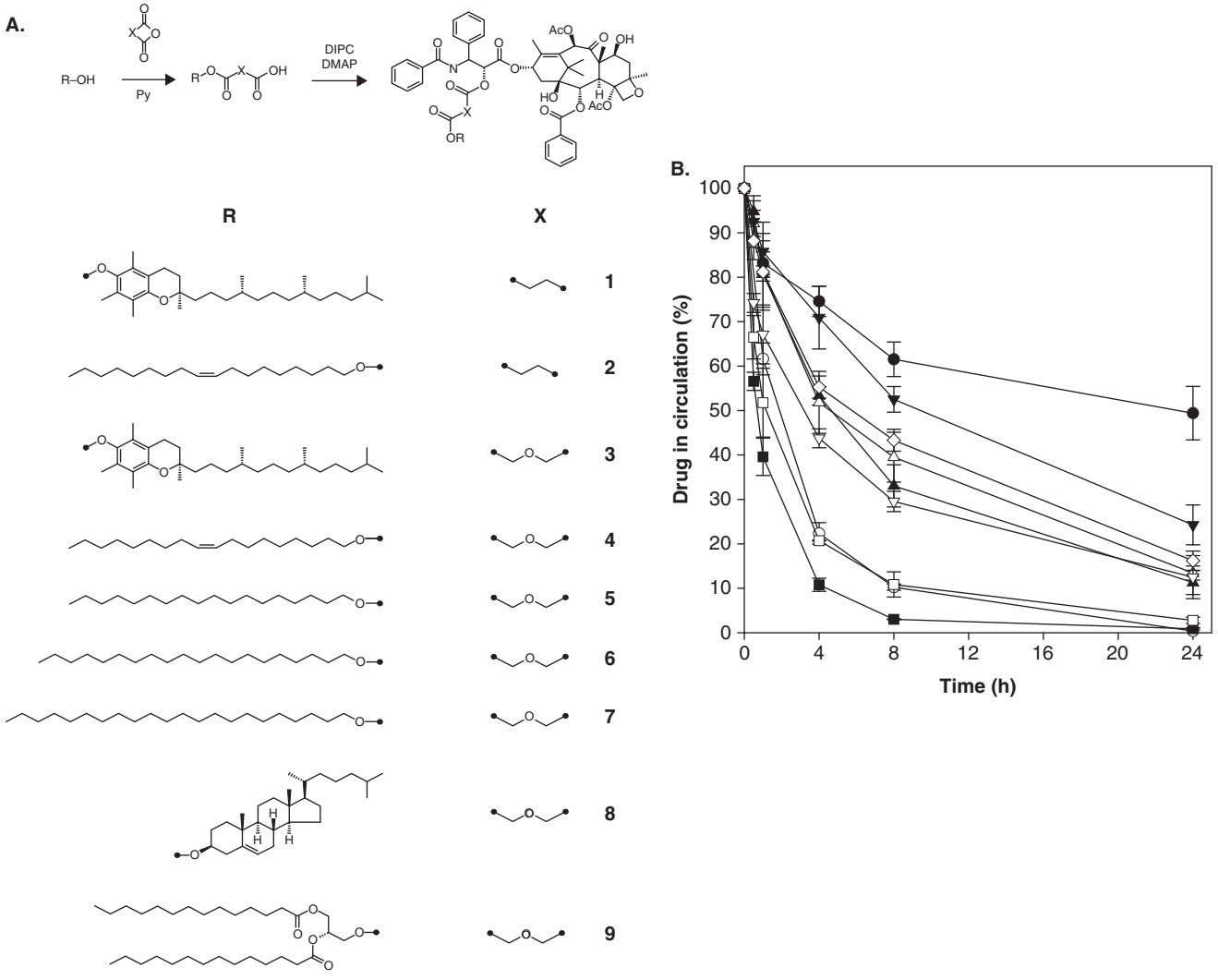


Figure 5. (**A**) Synthesis of lipophilic paclitaxel prodrugs. (**B**) Elimination of prodrug formulated as prodrug/POPC/2kPS3k (1:1:2; w/w) formulations and administered intravenously to athymic nude *Foxn1*^{nu} mice at a dose of 7 mg/kg (n = 3/time point). Drug concentrations were determined by HPLC analysis of plasma isolated at various time points. The prodrugs used were: **1** α-tocopherol (●); **2** oleyl alcohol (○); **3** α-tocopherol (◇); **4** oleyl alcohol (■); **5** octadecanol (□); **6** cosanol (▲); **7** docosanol (△); **8** cholesterol (▼) and **9** 1,2-dimiristoyl-sn-glycerol (▽). The crosslinkers were succinic acid (**1**, **2**) and glycolic acid (**3** – **9**). Error bars represent s.d. (n = 3).
Reprinted with permission from [73].

formulations have allowed us to translate *in vitro* drug ratio informatics into dramatic improvements in therapeutic activity. We have designed nanoparticle formulations for both hydrophilic and hydrophobic drugs to enable this approach for virtually any drug combination. Control of drug release rate for water soluble drug combinations was achieved through membrane and internal buffer manipulation while more hydrophobic drugs were controlled by prodrug hydrophobicity. Preclinical and clinical results indicate that this approach has identified fixed ratio drug combinations which are optimized for therapeutic activity rather than empirically combining drugs in the clinic based on tolerabilities.

7. Expert opinion

For > 40 years, chemotherapeutic agents have been combined together in an attempt to improve the cure rate for various types of cancer. Over time, these drug combinations have evolved to include more agents as well as recently approved targeted therapeutics. Unfortunately, despite the evolution of these treatment regimens, the 5-year survival rates for breast, lung, colon and prostate cancer have not dramatically increased. Why has there not been a larger improvement in cancer therapy through the use of these drug combinations? There may be multiple contributing factors for this

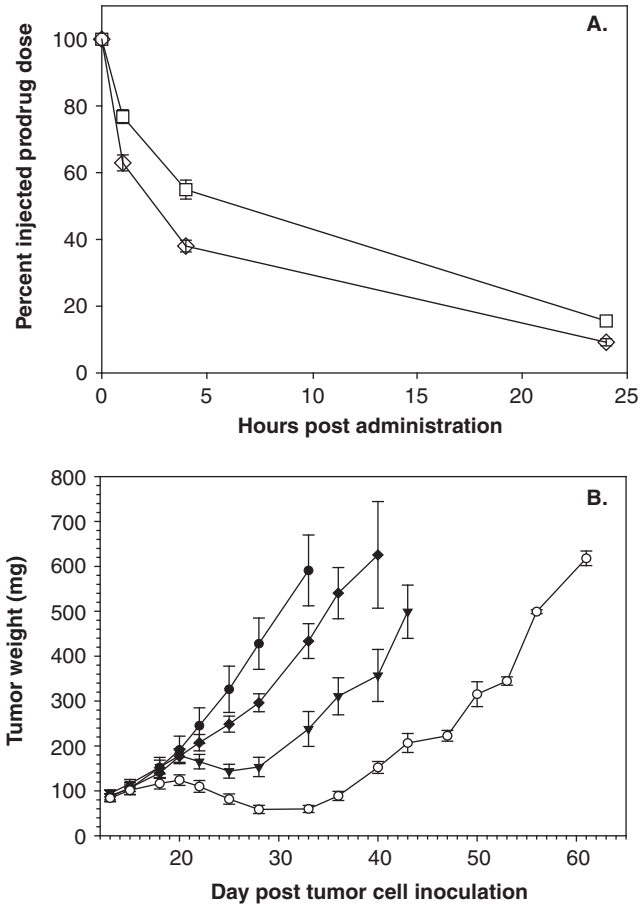


Figure 6. (**A**) Plasma elimination of Propac7 (□) and Progem12 (◇) prodrugs co-formulated as Propac:Progem: POPC:2.5kPS3k (4:1:2:8 w/w). Error bars represent s.d. (**B**) Efficacy of Propac7 (▼, 30 mg/kg), Progem12 (♦, 12 mg/kg) and co-formulated Propac7:Progem12 prodrug nanoparticles (○, 30:12 mg/kg) in the human colon carcinoma HT29 xenograft model (Q2D × 5); (●, saline).
Figure 6A Adapted from data presented in [73].

observation but combination drug delivery may prove advantageous over free drug cocktails in the area of drug–drug interactions and tumor access.

The role of drug–drug interactions is well studied by physicians, pharmacists and pharmacologists. This field of research is critical to the overall performance of an administered pharmaceutical because negative drug–drug interactions can result in unwanted toxicity or limit the therapeutic activity of one or both agents. In the case of drug toxicity, regulatory agencies require strict monitoring of patient health and typically involve blood chemistry, liver enzyme activity or specific indicators of organ function. The results from these data help physicians tailor treatments to minimize patient toxicity. As a result, most combination treatment regimens have been developed based on tolerability. In the case of negative drug–drug interactions which influence antitumor activity, the clinical read-out will be in the form of tumor size, tumor progression or survival. Poor clinical response to the treatment may be attributed to an aggressive tumor phenotype but may also be related to the pharmacology and distribution differences of the drugs within patient tumors. Recently, attention has been given to the observation of drug ratio-dependent synergy and antagonism observed for drug combinations when assessed *in vitro*. Under these ideal conditions, the role of drug–drug ratios on the overall activity of a drug combination can be explored. For drug combinations which can show synergistic or antagonistic activity depending on the ratio of the drugs, dramatic differences in antitumor activity may be observed clinically depending on the drug ratio accumulating at the tumor site. In an attempt to avoid antagonistic drug activity, we have screened anticancer drug combinations *in vitro* to establish their ratio dependency fingerprint. In the examples provided within this review, all three drug combinations were identified as having a synergistic drug ratio 'sweet spot' which was identified as the most synergistic ratio in a panel of tumor cell lines. We believe that formulating and delivering the synergistic ratio to the tumor site through the use of delivery vehicles will be the most effective way of optimizing the therapeutic potential of a given drug combination and avoiding undesirable antagonistic drug–drug interactions.

Our understanding of the molecular pathways involved in tumor progression has increased dramatically in recent years. The existence of multiple cellular mutations and tumor cell survival mechanisms reinforced the limitations associated with treating cancer with one drug. Although tumor cells in culture can be effectively killed with a single agent, the simultaneous use of multiple agents often decreases the concentrations of drug need to kill the tumor cells. To take advantage of drug combination cell killing in a clinical setting, both chemotherapeutic agents must accumulate at the tumor site within the same timeframe. Although most chemotherapy regimens involve drug combinations, they are not typically co-administered but rather dosed sequentially on different days in order to prevent unwanted patent toxicity. As a result, tumor drug levels may not be above their therapeutic threshold during the same time interval and, therefore, limit the potential advantages in cell killing associated with the drug combination. Through the use of combination drug delivery, simultaneous tumor exposure of both drugs should result in cell killing at lower levels than required for a single agent.

A second advantage associated with formulating a drug combination within a delivery vehicle is the EPR effect associated with particulate carriers which allows them to become trapped within the tumor after passing through gaps in the endothelial vasculature. Once localized within the tumor, the drug delivery vehicle can release drugs over time, acting as an *in situ* reservoir for the drug combination. Determining the optimal release rate is critical for optimizing the therapeutic potential of the formulation.

For drug combinations with very low IC_{50} values, a slow sustained release rate may extend the window in which drug is above its therapeutic threshold. For other combinations, peak exposure may be more important. Identifying the optimal release rate for a combination is best achieved by evaluating different release rate formulations in a panel of efficacy models. These results are used to identify the optimized combination formulation composition.

Taken together, enhancing tumor exposure to synergistic drug ratios through the use of drug delivery vehicles resulted in dramatic improvements in the therapeutic activity of the drug combinations described in this review. Although the examples described to date have focused on conventional chemotherapeutic agents, this approach may prove to be even more beneficial when combining targeted agents in which combined pathway inhibition may provide dramatic synergistic antitumor activity. This approach is also not limited to cancer but any disease state where the simultaneous exposure of two agents leads to a therapeutic advantage.

Declaration of interest

All authors are employees of Celator Pharmaceuticals Corp. They declare no other conflict of interest.

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