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Review article

Ratiometric drug delivery using non-liposomal nanocarriers as an approach to increase efficacy and safety of combination chemotherapy

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ABSTRACT

The observation that different drug ratios of the same drug combination can lead to synergistic or antagonistic effects when tested against the same cancer cell line *in vitro* gave rise to a new trend, the ratiometric delivery. This strategy consists of co-encapsulating a specific synergistic ratio of a drug combination into a nanocarrier so that synergism observed *in vitro* will be faithfully translated to *in vivo*, optimizing combination therapy. In this review we focus on how to quantify synergism *in vitro*, followed by how this affected the evolution of nanocarriers culminating in the ratiometric delivery, and finally we summarize the results of the non-liposomal formulations that were built upon this concept.

1. Introduction

The era of chemotherapy began in the 1940s with the first use of nitrogen mustards and antifolate drugs, but surgery and radiotherapy dominated the field of cancer therapy for many years. With the success of treating childhood leukemia by a combination regimen of chemotherapeutics in the 1960s, combination-based chemotherapies became the standard treatment for various types of hematological cancers and solid tumors in the clinics [1,2]. This strategy allows the targeting of different pathways, genes, or cell cycle checkpoints, overcoming the resistance to single agents. Today it is known that the probability of achieving a cure is significantly higher if two or more anti-cancer agents are combined into one regimen [3]. In the clinical setting, combination chemotherapy regimens are still typically developed based on the same principles that Frei and coworkers proposed in the 1960s: 1) cytotoxic drugs with non-overlapping toxicities are selected, which allows for each drug to be administered at the maximum tolerated dose (MTD); 2) cytotoxic drugs having different mechanisms of action are combined, which leads to targeting various aberrant biological processes, minimizing the development of broad-spectrum drug resistance; and 3) the combination is administered as early as possible in the disease. The idea of co-administering each drug at its MTD might seem rational, since the maximum therapeutic activity could be achieved this way. However, recent studies show that the same anticancer drug combinations can act synergistically or antagonistically against the same tumor cells *in vitro*, depending on the ratios of the individual agents comprising the combination. Therefore, this strategy may fail to exploit the full therapeutic potential of many proposed regimens. If a combination regimen is administered to a patient leading to antagonistic ratios, its full benefits are wasted, much to the detriment of the patient [4]. That is the reason why the development of combination therapies based on the concept of drug synergy started attracting significant attention in the first decade of this century. However, once a synergistic ratio between different drugs is identified, there is still the need to control drug ratios reaching the tumor site, which cannot be done upon administration of free drug cocktails. For this reason the interest in ratiometric drug delivery using nanocarriers is rising. Through these carriers, it is expected that the drugs can be simultaneously delivered to the tumor site, ensuring that the effect observed *in vitro* will be faithfully translated to *in vivo*, optimizing combination therapy [5].

Similar to what was observed for nanocarriers encapsulating a single chemotherapeutic drug, liposomes are the nanocarriers leading the studies concerning ratiometric delivery. Studies with these carriers allowed the validation of the ratiometric approach, as clinical studies confirmed the ability of liposomes containing co-encapsulated irinotecan and floxuridine at 1:1 molar ratio (CPX-1) and liposomes containing co-encapsulated cytarabine and daunorubicin at 5:1 molar ratio (CPX-351) in maintaining the drug ratios in the plasma for extended periods of time [6,7]. This last one, now under the trademark of Vyxeos™, had its New Drug Application submitted to the U.S. Food and Drug Administration (FDA) at the end of 2016 and awaits approval. The

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present review concerns the ratiometric delivery using non-liposomal nanocarrier formulations. Despite the fact that none of these formulations has reached clinical trials yet, they are presenting promising preclinical results.

2. Synergism arising from drug combinations

One of the most important benefits of drug combination is the synergistic effect, meaning that the combination effect is larger than the sum of individual effective drugs. Drug synergy allows a therapeutic effect to be achieved with lower doses of component drugs, allowing for improved therapeutic efficacy with fewer side effects [8,9]. Although combination chemotherapy started playing an important role in cancer chemotherapy in the 1960s, the development of combination therapies based on the concept of drug synergy started attracting significant attention only in the first decade of the 21st century [1,2]. Since then, many research efforts have been focused on discovering and exploiting the synergistic interactions of existing and clinically established drugs, and the term "synergy" is being widely used to justify drug combinations [10]. However, it is important to be aware that in most cases the term is used without appropriate understanding of either the underlying concept or the methods necessary to evaluate it. Concerning concepts, it is important to keep in mind that the positive combination effect of two drugs does not mean that they are necessarily acting in a synergistic way. For example, "potentiation" is the right term to use if only one drug is active alone, while "coalism" is the appropriate term if both drugs are not active alone but effective in combination [11]. Concerning methods, one can only claim that two or more drugs are acting in a synergic manner if an appropriate quantitative method was used to demonstrate that the combination effect is greater than that expected from the individual drug's potencies [8]. Various attempts have been made to predict drug synergy by network biology; however, there is still no precise way to predict synergism or antagonism when one combines two or more drugs [9,12,13]. What is known is that, in general, drugs acting on the same pathway through different targets or drugs regulating a relatively small number of highly-connected pathways are more likely to produce synergistic effects [9]. When two cytotoxic agents are combined to kill a cancer cell, it is not possible to know how many events there are from a living cell to a dead cell. There might be several steps or a hundred steps. Quantifying synergism or antagonism for a simple set of drug combinations can be done rather quickly, taking approximately 1–2 weeks. On the other hand, deciphering how and why synergism or antagonism occurs may take months or years, and yet the conclusions may only be tentative, suggestive, or implied [13].

2.1. Quantifying synergy of drug combinations in vitro

In the last century, a variety of methods for the quantitative evaluation of drug combination effects on established cell lines or on primary clinical tumor cultures have been developed. By far the most prevalent method used for quantitative evaluation of drug combinations is the "median effect analysis," proposed by Chou and Talalay in 1984. In that year, they introduced the scientific term "combination index" (CI) in order to describe quantitatively the drug interactions. If $CI = 1$, the combination is non-interactive (additive), if $CI < 1$, a synergism is demonstrated, and if $CI > 1$, the combination is antagonistic [14]. As this analysis requires extensive mathematical calculations, its applications were greatly facilitated in 1989 by the development of the first-generation computer software for dose-effect analysis, with the help of Joseph Chou [15]. Chou devoted his research to this single subject for more than 35 years, refining the median effect analysis approach until it arrived at the state of the art in 2006, when a simple way to end the controversies on how to determine synergism or antagonism was proposed [13]. This led to the development of CompuSyn, a computer software that allows easy automated simulation of synergism and antagonism at all dose or effect levels, now widely employed in drug combination research [16]. The first step in the interaction analysis between two drugs is to test each drug alone *in vitro*, obtaining the "potency" and "shape" of the dose-effect curve. For this first step, usually five to eight concentration points for each drug are recommended to be tested. One can then proceed to the combination studies. At this stage, it is possible to choose between two analysis methods: 1) keeping the combination at an equipotency ratio [e.g., (IC50)1/(IC50)2 ratio], so that the contributions of effects of each drug to the combination would be roughly equal or 2) setting an arbitrarily particular desired ratio [e.g., 3:1 mol/mol or 1:3 mol/mol] to check which ratio presents better synergy. Even a single data point (from a single combination dose) of a drug combination mixture allows the calculation of the CI value [13]. However, it is stated that experiments in which any drug is tested at less than three dose levels are not likely to be sufficient to demonstrate synergy [11]. As *in vitro* drug combination studies can be easily carried out, it is recommended that each ratio combination consist of five to eight data points. Another important point to consider is that the antitumor activity can be dramatically dependent not only on the molecular ratio of the combined drugs, but also on the dose levels reaching cancer cells – the reason why, in many publications, the CI and dose-reduction index (DRI) values are presented in the summary table for the ED50, ED75, ED90, and ED95 levels [13]. When evaluating anticancer drug combinations, the dose range administered should allow the extrapolation of the results up to high levels of activity, i.e. $ED \geq 50$, since tumor growth inhibition below that level is not clinically meaningful [17]. If possible, in drug combination studies, it is recommended that the experiments for a single drug and its combinations be carried out simultaneously. This ensures the same experimental conditions, avoiding variabilities associated with drug decomposition due to the instability, assay conditions, personnel changes, and cells, or animal inconsistency. When all the *in vitro* analyses have been performed, the "dose" and "effect" numerical information for each drug and for the drug combination should be entered into the software. This allows the data obtained for the fixed ratio combination to be compared to that obtained for the individual drugs, automatically calculating the CI [13]. It is important to highlight that the method should not be applied when the dose–response curves are not sigmoidal because of the difficulty of applying linear regression analysis [17]. Detailed procedures for using the program for automated dose-effect analysis for parameters of each drug and its combinations for quantitation/simulation of synergism or antagonism can be found in the CompuSyn user's guide [16].

It is important to keep in mind that assessing synergism might seem easy but can also be tricky. Experimental conditions, drug exposure times, drug exposure sequence, or different approaches to evaluate synergistic, additive, or antagonistic interactions might lead to different conclusions. That is the reason why conflicting results in preclinical studies using the same drugs can be found. A better understanding of the effect of drug combinations demands additional analysis, meaning that the *in vitro* search for synergism should no longer be limited to the measurement of cytotoxic effects [18]. As the analysis of interaction between two or more drugs demands data of "dose" and "effect," not only cell and proliferation but any analysis giving precise quantitative data for "effect" can be used. One example is the measurement of apoptosis induction [8,19].

Despite the many promising results obtained *in vitro* for combinatorial treatments, it is common that the clinical outcomes lead to little enhancement in efficacy and sometimes to higher toxicity. Additional factors, such as drug penetration and metabolism, cell kinetics, and biologic heterogeneity of clinical tumors, are not considered in *in vitro* studies, but must be taken into consideration in clinical studies [18]. Another important point to be considered is that this unsuccessful clinical translation can be related to the uncoordinated pharmacokinetics of the individual drugs tested, failing to exploit the synergistic therapeutic potential. A promising strategy to overcome this issue is the