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In Vitro Study and Evaluation of Anti-Proliferation Efficacy of Multicomponent Antitumor Combinations for the Treatment of Non-Small Cell Lung Cancer

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Abstract. Multicomponent anticancer combinations containing various anticancer drugs and nanoparticles with high antitumor activity can play a decisive role as the basis of the efficient anticancer therapies with high biological activity against tumor tissues minimizing acute toxicity to healthy tissues. This can be achieved by providing high super-additive synergy, which requires careful study of dozens or even hundreds of different substances following certain specific principles of selection and combination of mixture components. Taking into account that non-small cell lung cancer (NSCLC) is the most lethal specific form of cancer, the subject of the reported study was selected to be the development and *in vitro* testing of anti-proliferation and apoptotic efficacy of the multicomponent combinations against A549 cancer cell cultures in comparison to healthy NHDF cell cultures. Fifty novel multicomponent combinations containing twelve highly active anticancer substances and saline have been developed and tested using MTT cell proliferation assay, Annexin V-FITC/PI staining apoptosis assay, A549 human lung cancer cell and Primary Normal Human Dermal Fibroblast (NHDF) cell lines with aim to evaluate anticancer efficacy and safety of the developed combinations. Three novel indices showing three characteristics of the specific selectivity to cancerous cells have been developed and quantified. A surprisingly high necrosis and apoptosis inducing efficacy of the developed and tested combinations against the cancerous cells was especially evident with the addition of copper oxide and zinc oxide nanoparticles. © *2025 Bull. Georg. Natl. Acad. Sci.*

Keywords: NSCLC, multicomponent combinations, therapeutic window, selectivity index, nanoparticles

Introduction

Malignant tumors are the second leading cause of death in humans after cardiovascular diseases. In recent years, it was believed that the risk of cancer and other non-communicable diseases and the threat they pose to human life and health would rapidly decrease. However, the constant uncontrolled growth and aging of the population, as well as an irreversible degradation and pollution of the environment are leading to a further rapid rise in

cancer incidence and mortality. Alarming expectations are also caused by the consequences of nuclear weapons testing and incidents at nuclear power plants [1] exacerbated by the construction and commissioning of new nuclear power plants and nuclear facilities in at least 15 countries around the world [2]. In the current situation, one of the most promising responses to these challenges may be the combination of two innovative methods of treating malignant neoplasms, namely proton therapy [3] and the highly localized multicomponent combination therapy, together with more traditional methods of treatment and diagnosis [4, 5]. Multicomponent anticancer combinations can play a decisive role as the basis of anticancer combinations with high biological activity to tumor tissues and low acute toxicity against healthy tissues. Achieving high super-additive anticancer synergy requires a careful study of many dozens, or even hundreds of different substances following certain principles of selection and combination of mixture components [4-6]. It is especially important to study the anti-cancer drugs approved by the FDA and EMA in recent years and to compare the newly developed combinations with the traditionally used ones [4-7].

Selection of the research area and the components of anticancer combinations. Based on the well-known fact that non-small cell lung cancer is the most lethal specific form of cancer, the subject of the reported study was the development and in vitro testing of anti-proliferation efficacy of multicomponent combinations against A549 NSCLC cell cultures and healthy NHDF cells. According to novel principles of selection and combination of mixture components [4-6], and based on the list of the FDA-approved anticancer drugs [4-7], the following substances: rubidium chloride (RbCl), dimethyl sulfoxide (DMSO), highly efficient broad-spectrum anticancer drugs (Gemcitabine + Cisplatin, Carboplatin + Taxol, Tepotinib, Osibertinib, Rybrevant), CuO and ZnO nanoparticle water dispersions were selected as mixture components, while saline was added to adjust the concentration of combinations. It should be emphasized that copper oxide and zinc oxide nanoparticles are putative synergistic anticancer agents, and that Gemcitabine + Cisplatin and Carboplatin + Taxol have been for many years intensively used in clinical practice as anticancer drug combinations approved by FDA for use against NSCLC, while Tepotinib, Osibertinib and Rybrevant have been recently (in 2022-2024) approved by FDA [7] and are much less frequently used in today clinical practice.

The content of combinations is given in Table 1. The first column of the Table shows the numbers of samples grouped according the main anticancer component of the combination approved by FDA.

Selection of the methodology for assessing the effectiveness of developed anticancer multicomponent combinations. Specific selectivity of anticancer combinations to cancer cells. Considering the technical capabilities of our laboratory, we faced the need to choose between the appropriate *in vitro* methodologies: the widely known and used "therapeutic window", or "selectivity index" methods [8, 9], and the recently elaborated and tested method for determining the "specific selectivity" of antitumor agents against cancer cells in comparison to healthy tissue cells [6]. To assess the therapeutic efficacy, the so-called "Therapeutic Window" (TW) criterion (i.e., the dose range from the dose $D_{Ther.}$, at which the therapeutic effect of the drug begins, to the dose $D_{Tox.}$, at which the drug begins to cause the noticeable acute toxicity) is widely used in modern scientific practice [8]. At the same time, the selectivity index (SI) (which is equal to the ratio of the lethal LC_{50} values of the tumorous and healthy cells under a given dose of the drug [9]) is also widely used in the modern practice. The fundamental drawbacks of the both above methods include a negligible probability of detecting synergism in the interaction of components when

Table 1. Composition of the developed combinations

Components / Combination No	Gemcitabine + Cisplatin, mg	Carboplatin + Taxol, mg	Tepotinib, mg	Osibertinib, mg	Rybrevant, mg	RbCl, mg	DMSO, ml	CuO water dispersion, ml	Zn water dispersion, ml
1-5	400	0	0	0	0	400	3	0, 500, 1000 1500, 2000	0
6-10								0	0, 500, 1000 1500, 2000
11-20	0	400						0, 500, 1000 1500, 2000	0
								0	0, 500, 1000 1500, 2000
21-25		0	400					0, 500, 1000 1500, 2000	0
								0	0, 500, 1000 1500, 2000
31-40			0	400				0, 500, 1000 1500, 2000	0
								0	0, 500, 1000 1500, 2000
41-50				0	400			0, 500, 1000 1500, 2000	0
								0	0, 500, 1000 1500, 2000

using a realistically possible number of combinations. It is also doubtful that SI index can *be* used in practice in this scope, especially in the case of *dealing* with dispersed nanoparticle mixtures, since significant changes in concentration can lead to the activation of sedimentation and coagulation processes. That is why the present study is based on varying the content of drugs with fixed concentrations and uses the methodology developed in the frame of this research, which provides the determination and application of three specific selectivity indices (SSI_V, SSI_A, SSI_{VA}). The SSI_V index represents the so-called "anti-proliferation" selectivity of drugs characterizing their efficacy and safety in terms of causing necrotic death of cancerous cells compared to healthy cells: $\mathbf{SSI_V = V_{NCC} / V_{CC}}$, where V_{NCC} is the viability of exposed healthy (non-cancerous) cells, and V_{CC} is the viability of exposed cancerous cells. The SSI_A index represents the so-called apoptosis inducing selectivity of drugs, characterizing their efficacy and safety in terms of causing excess apoptotic death of cancerous cells compared to healthy cells: $SSI_A = \Delta_{ACC} / \Delta_{ANCC}$, where Δ_{ACC} is the specific growth of the number of apoptotic cells in exposed cancer cells, and ΔA_{NCC} is the specific growth of the number of apoptotic cells in exposed noncancerous (healthy) cells. The SSI_{VA} index represents the so-called averaged selectivity of drugs, characterizing their efficacy and safety in terms of both apoptotic and antiproliferative necrotic pathways in cancerous cells compared to healthy cells: $SSI_{VA} = (SSI_V \cdot SSI_A)^{1/2}$.

Materials and Testing Methods

All reagents and chemicals were obtained from Merck (India), Himedia (India), Invitrogen (India), SRL (India) and Sigma-Aldrich (USA). DCFDA (#D6883) was purchased from Sigma-Aldrich (India). Fetal bovine serum (#16000044) was obtainned from GIBCo, USA, and MEM sodium pyruvate, MEM non-essential amino acids L-glutamine and Gentamicin were procured from Hi-Media,

Table 2. Special selectivity index SSI_{VA} of the developed and tested combinations

Combination No	SSI_{VA}	Combination No	SSI_{VA}	Combination No	SSI_{VA}	Combination No	SSI_{VA}	Combination No	SSI_{VA}
1	21	11	19	21	16	31	15	41	14
2	24	12	22	22	18	32		42	17
3	39	13	38	23	31	33		43	32
4	63	14	79	24	67	34		44	48
5	59	15	66	25	61	35		45	52
6	23	16	22	26	18	36	19	46	17
7	28	17	26	27	22	37		47	21
8	47	18	45	28	41	38		48	37
9	84	19	81	29	74	39	77	49	57
10	78	20	75	30	68	40	65	50	61

India. In the current investigation, the A549 human NSCLC cell line and the Primary Normal Human Dermal Fibroblasts (NHDF) cell line were procured from ATCC (USA). To quantify the cell viability, percentage of viable cells in each treatment group was calculated relative to the control group using the tetrazolium dye (MTT) based assay [10]. Quantification of apoptosis induction was carried out through Annexin V-FITC/PI staining flow cytometric analysis of cells treated with control (gemcitabine) and the fifty test samples in accordance with the manufacturer's instructions (BD Bioscience) [11].

Results and Discussion

The obtained experimental data are shown in Table 2. They testify a significantly higher anticancer efficacy of all tested samples in comparison with control samples. Data of selectivity testing clearly showed that: a) the specific selectivity (and hence, efficacy and safety) of the developed and tested combinations is substantially higher than that of the similar gemcitabine-based combinations used as control; b) the surprisingly high anticancer potential of the developed and tested combinations is especially evident with the addition of nanoparticles of copper oxide and zinc oxide, with the effect of adding zinc oxide nanoparticles being significantly higher; c) the dominant contribution of nanoparticle additives to the growth of therapeutic efficacy and safety in the studied concentration range increases smoothly, but in all cases at the end of the interval it shows a clear trend towards a noticeable decrease, which may indicate a more complex interaction of components, up to positive or antagonistic synergy; d) therefore, it is necessary to expand the range of concentration of nanoparticles in combinations by at least one and a half or two times; e) to clarify the issue of the presence or absence of super-additive positive or negative synergy of the components of the combinations, it is necessary to conduct a full-scale testing of so-called “blank” combinations containing aqueous dispersions of nanoparticles and saline, in absence of the anticancer drug components, DMSO and other substances having high anticancer activity.

The highest selectivity was demonstrated by the Gemcitabine + Cisplatine and Carboplatine + Taxol based combinations followed by the Tepotinib, Osibertinib and Rybrevant based ones. In order to more objectively and fully evaluate the practical value of the developed and tested combinations, it is necessary (along with their therapeutic effectiveness and safety) to determine their therapeutic value, which is one of the most important indicators for drug manufacturers, insurers and other important decision makers [12-14]. In our opinion, the simplest indicator of this value can be the ratio of selectivity to the cost of the therapeutic dose of the drug required to obtain a stable therapeutic effect under conditions of optimal concentration of active components.

ადამიანისა და ცხოველთა ფიზიოლოგია

ფილტვის არაწვრილუჯრედოვანი კიბოს სამკურნალო ანტისიმსივნური მრავალკომპონენტიანი კომბინაციების ანტიპროლიფერაციული ეფექტურობის *in vitro* კვლევა და შეფასება

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ანტისიმსივნური პრეპარატებისა და ნანონაწილაკების შემცველი კომბინაციები განსაკუთრებულ როლს ასრულებს როგორც მაღალი ბიოლოგიური ეფექტისა და დაბალი მწვავე ტოქსიკურობის მქონე ანტისიმსივნური სამკურნალო საშუალებანი. მათი ეფექტურობა და უსაფრთხოება შეიძლება კიდევ უფრო გავზარდოთ კომბინაციების კომპონენტების სინერგიული სუპერ-ადიტიური ურთიერთქმედების მეშვეობით, რაც მოითხოვს ათობით ან, შესაძლოა, ასობით კომპონენტის ზედმიწევნით დეტალურ კვლევას, მათი შესაბამისი წესით შერჩევის და კომბინირების პირობებში. ფილტვის არაწვრილუჯრედოვანი კიბო (NSCLC) არის კიბოს ერთ-ერთი ყველაზე უფრო საშიში ფორმა, რაც წარმოადგენს კვლევის ძირითად მიზანს, ამოცანასა და მეთოდოლოგიას. კვლევისთვის შეირჩა მაღალი ანტიპროლიფერაციული და აპოპტოზური ეფექტის მქონე თორმეტი ანტისიმსივნური პრეპარატი, სამედიცინო პრაქტიკაში გამოყენებული გამხსნელი, სპილენძისა და თუთიის ოქსიდის ნანონაწილაკებისა და ფიზიოლოგიური ხსნარის დამატებით დამზადდა 50 მრავლკომპონენტიანი საკვლევი კომბინაცია, განისაზღვრა კომბინაციების ანტიპროლიფერაციული და აპოპტოზური ეფექტიანობა ფილტვის არაწვრილუჯრედოვანი კიბოს A549 და ადამიანის ნორმალური ფიბრობლასტური NHDF უჯრედოვანი კულტურების მიმართ. გაზომილი ეფექტიანობების ფარდობა (სპეციფიკური სელექტიურობა) გამოყენებულ იქნა როგორც კომბინაციების ეფექტიანობის და უსაფრთხოების მახასიათებელი. სამი სახის (ანტიპროლიფერაციული, აპოპტოზური და გასაშუალოებული) სპეციფიკური სელექტიურობის საზომად შემოღებული და გამოთვლილია სამი ახალი ინდექსი. ექსპერიმენტმა დაადასტურა სამივე ინდექსის მოულოდნელად დიდი მნიშვნელობა, რომელიც ძირითადად განპირობებულია ტესტირებულ კომბინაციებში ნანონაწილაკების მაღალი კონცენტრაციით.

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