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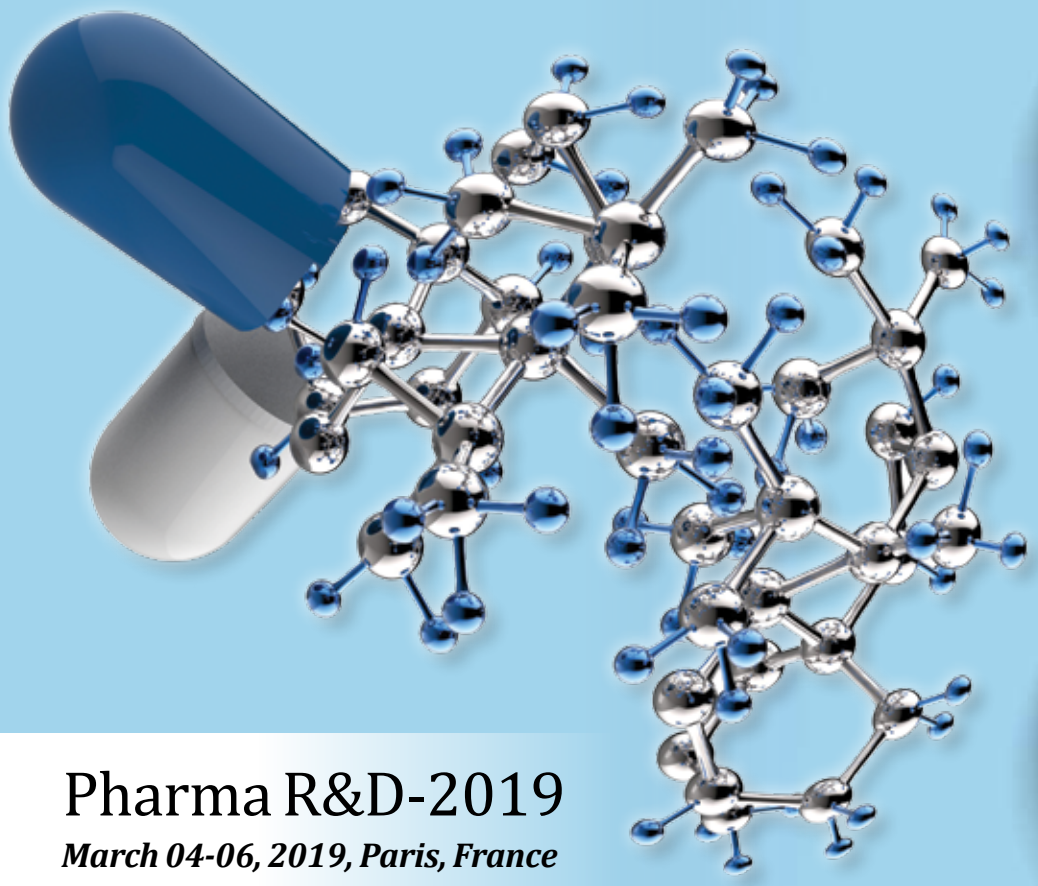

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PHARMAKEFTIKI, 31, 2 (2019) EDITORIAL

The present issue is dedicated to the International Conference on PharmScience Research & Development (Pharma R&D-2019) that took place in Paris, France March 04-06, 2019. It was the first edition of Pharma R&D, aiming to bring together pharmaceutical scientists and engineers from industry, academia, and regulatory agencies to discuss recent developments and future trends in the field of pharmaceutical product and process development. In the conference innovative research spanning the entire spectrum of drug discovery, development, evaluation, and regulatory approval was presented. Original work presented in the frame of Pharma R&D-2019 is included as Conference papers in the present issue.

The 2nd International Conference on PharmScience Research & Development will take place Los Angeles, CA on February 24-26, 2020 . More information is available at https://www.pharma-rd.com/

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ΦΑΡΜΑΚΕΥΤΙΚΗ, 31, 2 (2019) 49-59 PHARMA R&D-2019

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International Conference on PharmScience Research & Development (Pharma R&D-2019)
March 04-06, 2019, Paris, France

Novel Circulating miRNAs Biomarkers and target prediction in triple negative breast cancer patient (TNBC)

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KEYWORDS:
Circulating miRNAs; triple negative breast cancer patients (TNBC); plasma versus tissue; selective secretion; cancer metabolism

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SUMMARY

Women in developing countries are more likely to present with breast cancer only after it has reached an advanced stage. Treatment of advanced stage, malignant breast cancer, LABC and triple negative breast cancer (TNBC) presents many challenges, most prominent being therapeutic resistance, which leads to a high mortality rate. Blood based biomarkers facilitate early diagnosis, prognosis and drug resistance. MicroRNAs (miRs) are defined as non-coding RNAs that regulate post-transcriptional gene expression and cellular processes. This study analyzed circulating miRNAs in blood samples collected from disease-free individuals (n=34), triple-negative breast tumours (TNBC) (n=36) and luminal tumours (n=57) using quantitative RT-PCR. Besides inter-group analyses, the plasma-derived expression levels of miRNA in cancer patients were compared against breast cancer tissue. In breast cancer patient plasma, a differential set of miRs was identified in the plasma. Some of those miRs were uniquely identified on the basis of a prediction model. The most striking findings revealed that some of the tumor suppressor miRs were elevated in the TNBC patients. The circulating miR signatures could serve as biomarkers for detection as well as targets for novel therapies.

1. Introduction

MicroRNAs (miRNAs) are short, non-coding RNAs which regulate the expression of genes via targeted binding of mRNAs[1-3]. Often, in the context of cancer, both aberrant miRNA expression and target signaling is observed[4, 5]. This renders circulating miRNAs as putative biomarkers for cancer diagnoses[6-8]. While tissue biomarkers have been well characterized throughout many types of cancer, the detection of miRNAs in body fluids, particularly blood, offers a source of easily accessible, novel and stable biomarkers[6, 9]. Statistics evaluating datasets from multiple origins, worldwide, indicate that breast cancer in females is the most commonly diagnosed late-stage cancer and also the principal cause of cancer mortality[10]. While physicians are of the consensus that early detection via periodic screening of all females as well as the frequent monitoring of women at higher risk is necessary, more cost-effective and human approaches are desired[11]. Currently, mammography is the gold standard of breast cancer screening and it is associated with pain which hinders and discourages early detection[12]. Therefore, physicians and scientists have are dedicated to the search for non-invasive, safe and precise tumor biomarkers[13]. Numerous scientific research groups have investigated blood-based biomarkers as potential clinical diagnostic, prognostic and theranostic markers[14-19]. Unfortunately, thus far, the large number of studies has resulted in a lack of consensus among circulating miRNA breast cancer biomarkers[20]. The lack of consistency is multifactorial and likely attributed to patient heterogeneity as well as methodological challenges[20-25]. As illustrated by Moldovan *et al*[23], sample size, the total number of miRNAs studied, blood collection protocols and isolation methods are minor contributors. In fact, it is more likely that the methods of detection, the sample type (whether it is plasma or serum), and differences in methods of normalization such as whether spike-in or endogenous controls are used, are the predominate factors in outcome, regardless of which pathological condition is being assessed[25].

2. Materials and Methods

The clinical study was approved by the Ethics Committee on Clinical Research at KFSHRC, Riyadh, Saudi Arabia (Approval number: ORA#2160029) and executed under Helsinki Declaration terms.

Clinical sample processing and miRNA extraction[19]: Blood samples were collected from breast cancer patients naïve to treatment, as well as non-cancerous, healthy individuals in EDTA blood collection tubes (BD Vacutainer, UK) and kept refrigerated at 4°C. Within 2hrs blood was centrifuged at 1,500g at 4°C for 15min. Next, plasma was collected, centrifuged slowly at 4°C, 2,500g for 15min (Heraeus multifug 3S-R, UK) and stored at -80°C until RNA extraction. Each RNA isolation was performed in duplicate. Briefly, 1mL of QIAzol lysis buffer (Qiagen, Germany) was added to plasma (200uL) along with 1μg of carrier MS2 RNA (Roche), vortexed briefly and incubated for 5min at RT. In order to control for quality of RNA isolation, 3.5μl ($1.6x10^8$ copies/μl) of C.*elegans*-miR-39 miRNA was spiked-in along with 12μg of the co-precipitate glycogen. Next, 200μL of chloroform was added, samples briefly vortexed and incubated for 2-3min at RT. Then, centrifugation for 15min at 4°C at 12,000g was performed. Next, to maximize the extracted RNA concentration from the plasma samples, 900μL of 100% ethanol was added to 600μl of the sample (aqueuous phase). Contents were added to the RNeasy MinElute spin columns and spun for 15s at ≥8000g. Columns were washed first with 700μL RWT buffer, followed by 500μL RPE buffer (Qiagen, Germany), then spun 15s at ≥8000g. Next, columns were washed with 500μL of 80% ethanol and centrifuged for 2min at ≥8000g. After 5min of high-speed centrifugation, columns were eluted with 14μL of RNase-free water. RNA was frozen at -80°C until quality and integrity could be assessed (Nanodrop ND-1000, USA). In addition, quality was analyzed via RNA 6000 Nano LabChip, (Agilent Technologies, Germany), Agilent 2100's Bioanalyzer system (Agilent Technologies, USA) as well as the corresponding 2100 expert software tool (Agilent Technologies, USA). To make cDNA for quantitative RT-qPCR for mature miRNA expression

profiling, 250ng of RNA template was used in the Qiagen miScript RTII kit (Qiagen, Germany). The thermal cycler was programmed to 60min at 37°C followed by 5min at 95°C. The cDNA was diluted with 200μL of RNase-free water. For RT-qPCR a total of 2750μL was prepared, made up of 10X miScript universal primers, 2X QuantiTect SYBRgreen PCR master mix, cDNA template and RNase free water. miRNA screening was performed using miScript miRNA PCR Array Human Breast Cancer (Qiagen, Germany), which included miRNA PCR assays for 84 breast cance specific miRNAs. Final reaction volume was 25μL correlating to 1ng of cDNA/well. 96-well plates were run on an iQ5 instrument (Biorad, USA), with a cycling protocol of: 95°C for 15min (allowing for HotStar Taq DNA polymerase activation), 15s at 94°C, 30s at 55°C, 30s at 70°C (40 cycles) followed by a melting curve.

Data Processing and Statistical Analysis: Determination of threshold cycle values (CT) values were performed according to the manufacturers' guidelines. Differential signatures were observed following the implementation of the ddCt calculation method by Livak *et al*[26]. Furthermore, the Benjamini-Hochberg multiple testing corrections and Mann-Whitney unpaired test were used to determine whether miRNA expression differed significantly between groups[27]. All sample reference standard miRNA Ct values were approximately 19±2. Reverse transcription controls (RTC) and positive PCR controls (PPC) were also used to assess RT reaction inhibition. For each sample, Avg C_T^{miRTC} –Avg C_T^{PPC} was calculated. Differences of less than seven indicated RT reaction inhibition. Cell contamination was evaluated using the mean C_T values of snoRNA and only non-zero values were considered. SnoRNA C_T values greater than 32 indicated contamination. SNORD72 performed poorly across samples and was excluded. For each target miRNA analyzed, only samples showing C_T values between zero and 35 were considered. Technical variation during RNA extraction was corrected for by the incorporation of spike-in controls from C. elegans. The Qiagen miScript miRNA PCR Array Human Breast Cancer Array Panel contained 2 cel-mir-39 spiked-in controls. Normalizing factors were calculated by subtracting the median of the average C_Tc^{el} values from the average C_T^{cel} value for the samples. The dC_T of each target miRNA was obtained by subtracting the normalization factor from the individual C_T values. To calculate ddC_T, the average dC_T was first obtained and all values below 15 and above 35 were discarded. ddC_T of a given miRNA for a given pair of groups was computed by taking the difference between the average dCTs of the respective groups; for example, ddCT(TNBC vs. cancer-free)=[Avg dCT (TNBC)]–[Avg dCT (Cancer-free)]. Relative expression between any two groups was assessed with the 2^{-ddCT} calculation. Where relative expression was lower than one, the negative fold change was deduced by using the equation: "1/relative expression" or $1/2^{-ddCT}$. Positive 2^{-ddCT} values were considered fold changes. The Benjamini-Hochberg multiple testing and Mann-Whitney unpaired test statistical corrections were applied in order to determine significance.

Bioinformatics Analysis: Plasma levels of all miRNAs collected exclusively for the present study were compared with breast cancer sample biopsies analyzed for the The Cancer Genome Atlas (TCGA) library [28]. miRNA precursor levels in tissues were listed as RPKM (reads per kilobase of transcript per million) and were acquired for 120 luminal, 38 TNBC and 87 cancer free samples. While circulating biomarker values measured in this study were representative of the active forms of miRNA, TCGA values represented miRNA precursors. Because an miRNA precursor may produce both 3p and 5p active forms, they were matched to the identical precursor. Then the RPKM precursor and expression values (2^{-dCT} using cel-corrected CT values) of the corresponding active circulating miRNAs were auto-scaled using z-transformation. A microT-CDS algorithm and mirPath v.2.0 available on the web-based server DIANA were utilized for pathway analyses[29, 30]. Receiver operating characteristic (ROC) curves were generated using a web-based tool on the cross-validation performance of linear SVM employing ROCCET[31]. Hierarchical clustering was performed using GeneSpring

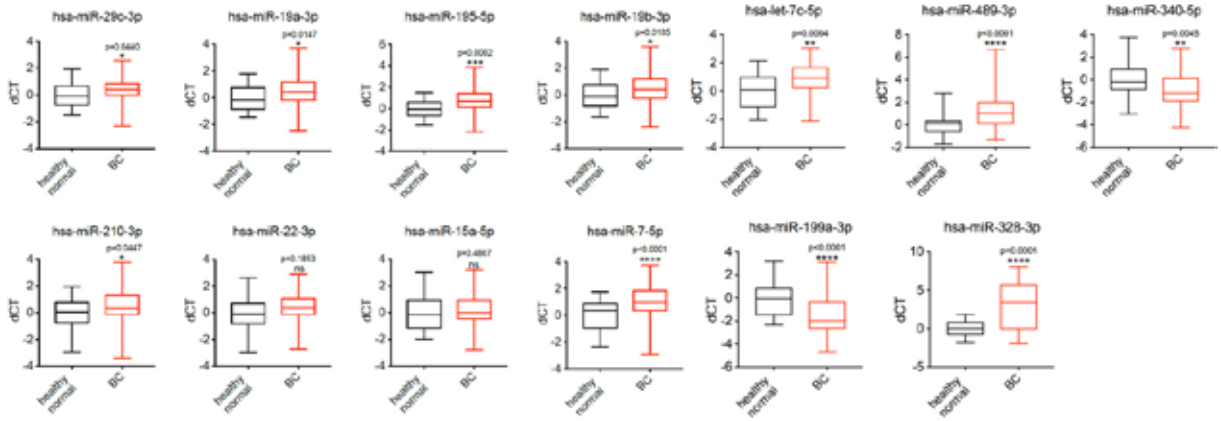

Figure 1: *Comparative analysis of circulating miR for breast cancer patients and healthy control. *p<0.05, **<0.005, ***p<0.0005,p<0.00005, ns: not significant.*

GX 14.5. The miRNA targets and biological pathways were predicted using the microT-CDS algorithm and mirPath v.2.0 available on DIANA (Papadopoulos, Alexiou, Maragkakis, Reczko, & Hatzigeorgiou, 2009). Finally, the micro-T threshold for target prediction was set to 0.8 and considered significant when p-value<0.05. ROC curves were produced with ROCCET (Xia, Broadhurst, Wilson, & Wishart, 2013). Multivariate feature selection was performed using linear Support Vector Machine approach for finding two sets of miRNAs that could best differentiate (i) TNBC tumors from normal and (ii) luminal tissue tumors from cancer free samples. ROC curves were generated by Monte-Carlo cross validation (MCCV). To determine a feature's rank, in each MCCV, two thirds of the total number of samples were evaluated. Then, classification models were constructed of the most significant features and the remaining samples were validated. The MCCV cross validation analyses was repeated many times in order to calculate confidence intervals and performance.

3. Results and Discussion

Differential expression of circulating miRNAs between normal and cancer patients

Enrolled in this study were a total of 127 women, including: 34 who were cancer and disease-free, 36 who had received a triple-negative breast tumors (TNBC) diagnosis and 57 that received a diagnosis of luminal breast cancer type tumors. In order to evaluate expression levels of circulating miRNAs, RNA was extracted from plasma collected. The technical methods of collection, extraction and analyses were supported by many controls including the use of snoRNA in the Human Breast Cancer miRNA PCR Array Panel, reverse transcription inhibition controls (RTC) and positive PCR controls (PPC). All samples collected and included in the analyses were of excellent quality. From the 84 miRNAs analyzed, 54 had detectable CT values which allowed consideration for an in-depth follow-up analysis. Moreover, because of their significant differential signatures, a preliminary set of 18 miRNAs were obtained between patient groups and healthy controls as illustrated in (**Figure 1**). These 18 miRNAs were further investigated as potential biomarkers for TNBC or luminal disease and they were grouped according to their differential expression level across sample groups: (a) triple negative patients (TNBC) and healthy controls; (b) luminal patients and healthy controls; or finally, (c) breast cancer patients unrelated to subtype and healthy controls. For both TNBC and controls, circulating levels of eight miRNAs were unique, while five miR-

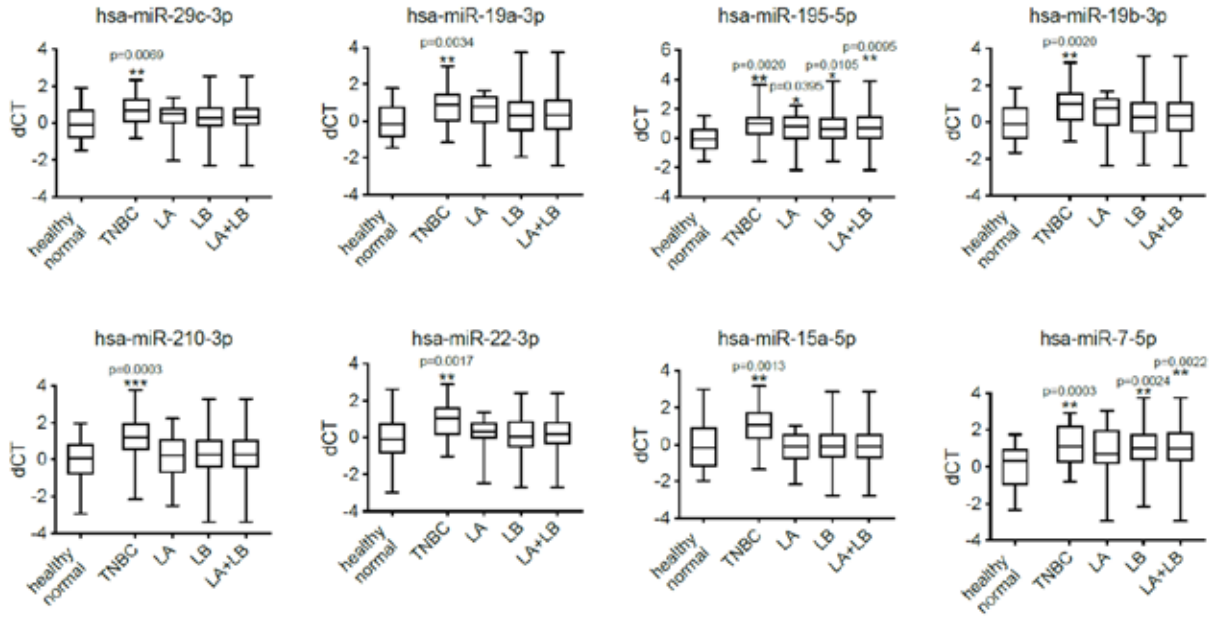

Figure 2: *Differential expression across molecular subtypes of breast cancer and healthy controls. *p<0.05, **<0.005, ***p<0.0005, p<0.00005, ns: not significant.*

NAs were significantly different for luminal tumor patients compared to healthy normal controls. Plasma levels of the remaining five miRNAs were very similar across both breast cancer patient subgroups but were distinct from non-cancer healthy controls. Differentially expressed miRNAs in TNBC and luminal breast cancer compared with the non-cancer controls are illustrated in (**Figure 2**). To assess their potential use as diagnostic biomarkers, we generated ROC curves and the confusion matrix for each miRNA. This analysis revealed 7 miRNA panel: hsa-miR-199a-3p, hsa-miR-15a-5p, hsa-let-7c-5p, hsa-miR-7-5p, hsa-miR-195-5p, hsa-miR-489-3p and hsa-let-7i-5p which showed the maximum discriminatory potential between TNBC and cancer-free women. Likewise, an additional panel of 5 circulating miRNAs: hsa-miR-328-3p, hsa-miR-199a-3p, hsa-let-7i-5p, hsa-miR-195-5p and hsa-miR-25-3p were indicative of women diagnosed with luminal breast cancer.

In this study, we discovered a panel of plasma miRNAs that can distinguish TNBC and luminal breast cancer from cancer-free individuals which may be of great diagnostic value as illustrated in (**Figure 2**). Although previous qRT-PCR based studies have already described various signatures, a lack of consensus exists due to discrepancies in miRNA sampling and processing protocols. However, this lack of consensus was found to be more related to analytical methods, especially normalization. While qRT-PCR values are commonly normalized either by global means or the use of reference genes, these methods were not suitable for the present study. These limitations were surpassed by correcting for the CT value with spiked-in cel-mir-39 array controls. By employing this important control method, this study identified plasma-derived circulating miRNAs differentially expressed in TNBC patients (13 miRNAs) and luminal patients (10 miRNAs) as compared to healthy individuals. Except for the following miRs: hsa-miR-199a-3p, hsa-miR-199a-5p and hsa-miR-340-5p all typical breast cancer tissue miRNAs were significantly increased in plasma. A set of five: hsa-let 7b-5p, hsa-let 7i-5p, hsa-miR-16-5p, hsa-miR-25-3p and hsa-miR-199a-3p were

elevated in both subtypes samples and therefore did not indicate any specific breast cancer subtype. However, when averaged and compared to disease-free samples, specific trends were observed. For example, hsa-let 7b-5p, hsa-let 7i-5p, and hsa-miR-25-3p were higher in control plasma while TNBC and luminal sample expression were similar. Interestingly, in TNBC, hsa-miR-16-5p levels were higher than luminal subtype samples and hsa-miR-199a-3p demonstrated a reversal of this trend (was decreased) in both cancer subtypes. One reason for the observation of decreased levels of tumor suppressor RNA can be found in studies composed by Witwer *et al* [20] and Chen *et al*[32] who observed that the signature of circulating miRNAs in cancer patients may appear aberrant due to both active secretion and passive leaking of tumor cell components. Moreover, increased cellular metabolism and enhanced selective secretion may result in changes in miRNA stability. Likewise, plasma miRNA down-regulation may be indicative of attenuated secretion as well as representative of a neo-plastic condition[20].

Comparison of circulating miRNA expression in plasma and tissue samples

Plasma miRNA expression levels do not always correlate to tissue level expression[33-36]. Our panel of 18 breast cancer indicative plasma miRNAs were compared to publicly available data from TCGA (The Cancer Genome Atlas)[28]. The panel of 18 were mapped to 17 precursor forms according to TCGA. Next, this panel was compared to corresponding miRNA precursors tissue levels reported by TCGA. However, as the RPKM values (Reads Per Kilobase of transcripts per Million) from the TCGA database were not directly comparable to CT values generated by our study, expression trends across disease free and cancer subtypes were observed and analyzed. For hsa-miR-7, hsa-miR-16, hsa-miR-19a, hsa-miR-19b, hsa-miR-210, and to a certain extent hsa-miR-15a, similar values were recorded for tissue and plasma. Other miRNAs reversed this trend. Therefore, attenuated tissue expression of hsa-let-7c and hsa-miR-195 were observed in both TNBC and luminal patients but plasma levels were increased. Minor variations of these trends were observed for hsa-let-7i, hsa-let-7b, hsa-miR-22, hsa-miR-25, hsa-miR-29c, hsa-miR-199a, hsa-miR-328, hsa-miR-340 and hsa-miR-489. Given observations by Witwer *et al*[20], the trends for circulating plasma levels observed in this study were compared to miRNA tissue expression level data reported by TCGA. With this approach, three general categories of miRNAs signatures were observed. First, hsa-mir-7, hsa-mir-15a, hsa-mir-16, hsa-mir-19a, hsa-mir-19b, hsa-mir-210, and whose trends are similar across plasma and tissue. Expressions of this group of miRNAs were increased in TNBC tissue as well as plasma. Therefore, this particular signature may directly indicate and reflect the presence of a TNBC type tumor. A smaller panel of miRNAs: hsa-mir-199a and hsa-mir-340, demonstrated very distinct trends. These two miRNAs were robustly expressed in the plasma of healthy controls but down-regulated in TNBC and luminal subjects. An additional pattern emerged with distinct subgroups. For example, has-mir-7i, hsa-mir-25, and has-mir-328 were distinctly expressed in TNBC plasma and tissue versus luminal samples while has-let-7c and has-let-7i were displayed regardless of subtypes. In both signatures all miRs were more highly expressed in plasma than in tissues. Cell lines from breast cancer[34] as well as other cancer cell types[35] report the release of particular miRNAs from the tumor cells which flow into extra-cellular fluids. Studies have elaborated that miRNAs that are present in extracellular fluids are not just by-products left over from dying tumor cells but rather important mediators of tumor development and the metastasis process as they are able to promote crosstalk between healthy cells and tumor cells[37]. In summary extracellular miRNAs are actively secreted by tumor cells as intercellular messengers[38, 39], which are enveloped in vesicles by primary tumor cells and endocytosed by healthy non-tumorous cells[32, 40]. Once transferred, these miRNAs modulate the healthy cellular machinery which result in aberrant alterations of cellular phenotypes[41]. As elucidated by Falcone *et al*[41], tumor cells create a metastatic niche using a multi-pronged approach

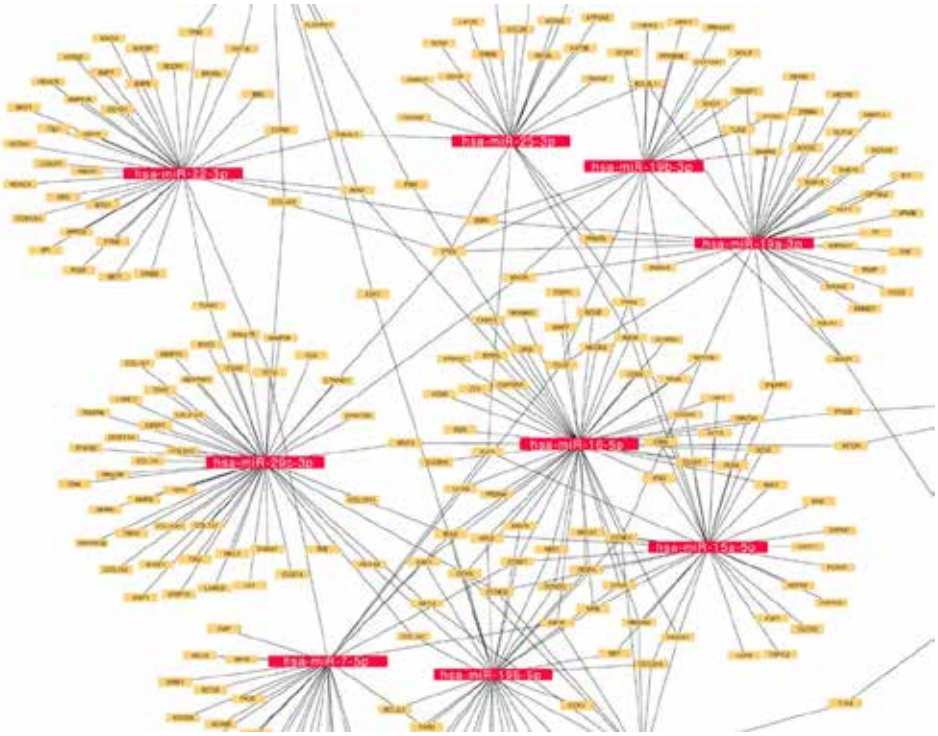

Figure 3: *Interaction network of circulating miRNAs and their validated target genes (MTGs). Lines indicate direct interactions between miRs and MTGs. Circular nodes represent miRs and MTGs.*

that employs the secretion of tumor suppressor miRNAs. Tumor suppressor miRNAs have the roll of attenuating immune observation by actively repressing the immune components and via the promotion of angiogenesis. In the context of these studies it is notable that our patient cohort demonstrated significantly increased levels of the tumor suppressor miRNA family let-7; including: hsa-let-7b, hsa-let-7c and hsa-let-7i. Although an independent data source was used, when compared to non-cancer plasma, TNBC and luminal patient plasma levels were significantly higher than tissue levels. Let-7 family miRNAs were observed to be attenuated in cancer tissues[42]. Taken together, as suggested by Ohshima *et al*[33], it is likely that breast cancer tumor cells selectively secrete tumor suppressor miRNAs in order to maintain their oncogenesis. Interestingly, several studies have suggested that attenuation of let-7 levels may be a viable therapeutic approach for cancer patients[42]. The tumor suppressor mir-15 family including hsa-miR-15a-5p hsa-miR-16-5p, and hsa-miR-195-5p, showed varied patterns. As described above hsa-miR-15a-5p and hsa-miR-16-5p have display similar intracellular and extracellular trends. They are known to be involved in the cell cycle, differentiation, proliferation as well as immune and homone reposnses[43]. It has been widely reported that most tumors repress expression of the miR-15 family of miRNAs[44]. In addition, menstruation was shown to increase miR-15 miRNA expression in circulating plasma[45]. Therefore, given the variety of regulation of the miR-15 family, their use a part of a cancer diagnostic signature remains uncertain.

Functional pathways regulated by the circulating miRs

The predicted target genes for significant miRs (**Figure 3**) was mapped to significant pathways which in-

clude: estrogen, fatty acid biosynthesis (FASN), FoxO, Hippo, MAPK, mammalian target rapamycin (mTOR), PI3K-Akt, p53, TGFβ, and Wnt-signaling. In the context of TNBC, tumor metabolism is attained via complex signaling networks involving mTOR, PI3K-Akt, and PTEN so their upregulation in this study was not surprising. Likewise, extracellular matrix-(ECM)-receptor interaction pathways as well as FASN pathways were upregulated. The pathway data fit with our miRNA data, as gene targets of the let-7 family are implicated in ECM receptor communications and FASN pathway is stimulated by hsa-miR-15a-5p, hsa-miR-195-5p and hsa-miR-16-5p. Notably, TNBC plasma showed selective upregulation of hsa-miR-15a-5p and hsa-miR-195-5p. This data suggests the plasma miRNA signature may reflect a blend of selectively secreted and passively leaked miRNAs[32]. Pathway analysis performed with DIANA[29, 30], suggested that miRNAs as well as their targets involved in signaling pathways critical to carcinogenesis such as PI3K-Akt, TGF-beta, (mTOR) and MAPK pathways, all critical for carcinogenesis. However, one of the striking observations was the involvement of members of the has- mir-15 family leading to an enrichment of the fatty acid metabolism pathway in cases of TNBC. Though miRNAs are implicated in cancer metabolism, their role in breast cancer cell metabolism is not well characterized[19, 46, 47]. This is especially interesting because of the evolving newer approaches to therapy which involve manipulating fatty acid metabolism in an effort to limit the supplies of fatty acid to cancer cells[48]. FASN is one of the key enzymes involved in lipid biosynthesis in cancer cells[49]. FASN is highly expressed in tumor cells and many small molecules targeting elements of the FASN pathway are in development as cancer therapeutics[48, 50-52]. Furthermore, many preclinical models have demonstrated that pharmacologically limiting lipid biosynthesis has resulted in reduced tumor activity[53]. FASN and other lipid biosynthesis pathway genes involved in de novo lipogenesis such as acetyl-CoA carboxylase (ACACA) and 3-Hydroxy-3-Methyl glutaryl CoA reductase (HMGCR), are regulated by hsa-mir-195[54]. Over-expression of hsa-mir-195 attenuates ACACA, FASN and HMGCR expression. Reduced expression of these genes has a net effect of reducing cellular cholesterol and triglycerides as well as inhibition of proliferation, invasion and the epithelial-mesenchymal transformation (EMT) process. Chen *et al*[55] demonstrated that hsa-mir-195 directly upregulates GLUT3. Overexpression of GLUT3 transcripts support aberrant tumor biology such as high glucose intake and accelerated metabolism. However, in vitro assays performed by Singh *et al*[54] demonstrated that over expression of hsa-mir-195 may potentially be therapeutic. In this study circulating hsa-mir-195 levels in TNBC plasma were higher than those in cancer free samples. As tissue levels of hsa-mir-195 reported by TCGA are elevated in healthy tissues but attenuated in TNBC tissue biopsies, suggests that hsa-mir-195 may actively secreted out of tumor cells.

4. Conclusion

In conclusion this study observed that in TNBC plasma, hsa-mir-195 levels are significantly higher than in cancer-free women. Although some studies have suggested that let-7 and mir-195 restoration may be therapeutic, our results supported other literature indicating that tumor cells export hsa-mir-195 and let-7 miRNAs. While our data did not generally support the use of these miRNAs as therapies, it did suggest that these markers may be the most robust markers to use in a blood-based screen for the early detection of TNBC and luminal breast cancer. □

Abbreviations

- Ct – Threshold cycle
- miRNA – microRNA
- qRT-PCR - Quantitative Real Time Polymerase Chain Reaction
- RNASeq – Ribonucleic acid sequencing
- ROC - Receiver Operating Characteristic
- RPKM - Reads Per Kilobase of transcript per Million mapped reads

Declarations

Ethical approval and consent to participate
Approval and written consent was obtained from all

study participants for the use of their blood samples for research purposes. The study was approved by the Ethical Research Committee and Basic Research Committee (Approval number: RAC#2160029) on Clinical Research at KFSHRC, Riyadh, Saudi Arabia and was carried out under the terms of the Helsinki Declaration.

Competing interest

The authors declare that they have no competing interest.

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Synthesis of a spiro quinazoline compound as potential drug useful in the treatment of Alzheimer's disease

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KEYWORDS:
Quinazoline derivatives; Acetylcholinesterase inhibitors; Alzheimer's disease; Cholinergic hypothesis; Spiro compounds

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SUMMARY

Compounds having a quinazoline nucleus are widely distributed in nature and possesses important biological activities, such as antimalarial, anti-tumor, anti-fungal, anti-inflammatory properties and so on. Furthermore, quinazoline derivatives contain important pharmacophore and exhibit several pharmacological effects.

Because cholinesterase enzyme is considered as an important drug target in the treatment of Alzheimer's disease, we considered it interesting to synthesize a novel spiro quinazoline derivative, such as compound 7, as a candidate inhibitor of the cholinesterase enzyme.

1. Introduction

Alzheimer's disease is one of the most often occurring and most devastating neurodegenerative disease. With improvements in health care, longer life expectancy and continued rise of the average age of the population, the prevalence of Alzheimer's disease is projected to increase dramatically over the coming years. The global prevalence of dementia is estimated currently affects nearly 50 million persons worldwide, and is predicted to increase to more than 130 individuals through 2050[1,2].

Elucidated dysfunction of cortical transmitter systems with Alzheimer's disease, namely the deficiency of acetylcholine, was first described. In addition, many observations of substantial neocortical lacks in the enzyme responsible for the synthesis of acetylcholine, choline acetyltransferase, were reported[3,4]. Subsequent discoveries of reduced choline uptake[5], acetylcholine release, reduced number of nicotinic receptors and neurofibrillary degeneration in the basal forebrain confirmed a considerable presynaptic cholinergic deficit[6]. Furthermore, it has well established that cholinergic deficiency plays a fundamental role in the pathogenesis of Alzheimer's disease[7]. These observations together with the well defined rule of acetylcholine in memory, attention, learning and when both hippocampal and cortical areas of the brain receive major cholinergic input from nucleus basalis of Meynert, coined the cholinergic hypothesis, therefore, it was suggested that degeneration of cholinergic neurons in the basal forebrain and the correlated loose of cholinergic neurotransmission in the cerebral cortex and limbic

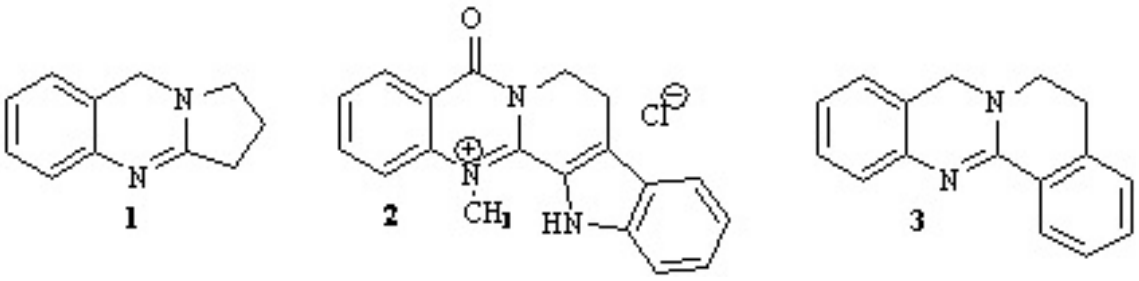


Fig. 1. *Structures of lead compounds deoxyvasicine (1), dehydroevodiamine (2) and 6,8-dihydro-5H-isoquinolino[1,2-b]quinazoline (3).*

system conducted to the impairment in cognitive function, observe in patients with Alzheimer's disease[8,9].

Since the cholinergic hypothesis was acknowledged[10], the dominating treatment strategy to relief Alzheimer's disease symptoms is based on the revival of cholinergic transmission in the central nervous system, utilizing compounds that inhibit cholinesterase enzymes which decomposes acetylcholine[11]. Lately, it has been demonstrated that cholinesterase can influence a series of other processes, such as β-amyloid aggregation, due to the presence of a peripheral anionic site (PAS) in their structure[12,13]. These studies conducted to a resurgence of interest in cholinesterase enzyme as an important drug target in the therapy of Alzheimer's disease, and several researchers are involved in investigating concerning design and synthesis of new inhibitors[14,15].

Acetylcholinesterase is the main enzyme involved in the hydrolysis of acetylcholine. In addition, It is also responsible for cerebral blood flow modulation, β-amyloid aggregation, activation and expression of APP95 protein, τ(tau) protein phosphorylation and has an influence on inflammatory processes. It interacts with β-amyloid, leading to creation of stable complexes and formation of senile plaques[16]. Its role is not fully elucidated, but some studies proposed that it could enhance amyloid plaque formation.

In the 1970's and early 1980's, biochemical and neuropathological evidence emerged linking the degeneration of basal forebrain cholinergic neurons in Alzheimer's disease[17]. Relying on the cholinergic hypothesis, the rational therapeutic strategy to promote cholinergic neurotransmission is to increase the availability of acetylcholine in the synaptic cleft by inhibiting acetylcholinesterase, the enzyme that degrades acetylcholine[18]. Currently, many cholinesterase inhibitors are commercialized for the managemnt of mild-to-moderate dementia (donepezil, Rivastigmine and galanthamine). Moreover, memantine antagonist of NMDA (N-methyl-D-aspartate) receptor was approved for use in moderate to severe dementia[9].

Several novel acetylcholinesterase inhibitors have been described in recent years[19], and especially natural products and alkaloids pool, like deoxyvasicine dehydroevodiamine, continues to be a greater source for drug discovery of a new lead compounds20. Furthermore, they have been recently reported in literature many promising compounds as strong anticholinesterases possessing quinazoline nucleus. Decker et al, reported tri- and tetracyclic N-bridgehead cholinesterase inhibiting structures were synthesized based on quinazoline moiety[21] (**Fig. 1**). In the last two decades, it has to be emphasized that the huge majority of novel structures investigated are only moderately active, have failed in randomized controlled trials and rarely reach inhibitory activities under the micromolar range[22], which emphasizes the urgent need for nanomolar inhibitors, and how we can obtain them from lead structures with lower inhibitory activities.

Quinazoline modification achieved in this work was based on increasing the basicity of the core ring by introducing cyclohexyl group adjacent to the tertiary nitrogen atom of the lead quinazoline nu-

cleus. In addition, because several studies reported that the synthesis of spiro compounds increase the structural rigidity which could produce enhancement of the biological activity against some drug targets[23], we decided it interesting to synthesize novel spiro quinazoline derivative as a candidate inhibitor of the cholinesterase enzyme.

2. Materials and Methods

2.1 Reagents and chemicals

All reagents and solvents utilized were of high grade of purity, purchased from commercial suppliers and used without further purification. Deionized water (used in all solutions). N-Methylisatoic anhydride (4) (C9H7NO3), cyclohexanone [C6H12(=O)], lithium aluminium hydride (LiAlH4), tetrahydrofuran anhydrous (C4H8O), acetonitrile (CH3CN), GC grade methanol (CH3OH), dichloromethane (CH2Cl2), ethyl acetate (CH3COOC2H5), hexane (C6H14), p-toluenesulfonic acid monohydrate (CH3C6H-4SO3H.H2O), chlorotrimethylsilane (CH3)3SiCl, and anhydrous sodium sulfate (Na2SO4) were purchased from Sigma-Aldrich.

2.2. Thin layer chromatography (TLC)

TLC analysis were performed on commercial silica gel 60 Å on aluminum foils with fluorescent indicator 254 nm purchased from Sigma-Aldrich. The spots on TLC were further evidenced by UV-light detector (254 nm and 365 nm). The mobile phases exploited in TLC analysis composed of ethyl acetate/ hexane of ration 8:2, v/v; hexane/ethyl acetate of ration 7:3, v/v and hexane/ethyl acetate of ration 1:1, v/v they have been used in monitoring the preparation of 5, 6 and 7; respectively.

2.3. Chromatographic separation

The column dimension utilized (60 cm × 1 cm, or larger column based on the weight and the purity sample), manually packed with silica gel 60 Å (average particle size 63-200 μm, 60 g silica /1 g of product). Sample, dissolved in a minimum amount of the mobile phase used to pack the column, was loaded onto the top of the silica bed and the isocratic elution initiate utilizing ethyl acetate /hexane 8:2 (compound 5) and ethyl acetate /hexane 1:1 (compound 7) , v/v as mobile phases. The effluents obtained from the column were collected into test tubes with a fraction collector. Compounds were detected by UV-light detector. Fractions possessing the same purity were collected together, concentrated using a rotary evaporator. Further drying of the residue was achieved under vacuo to obtain the purified product as white solid (compound 5) or as brownish oil (compound 7).

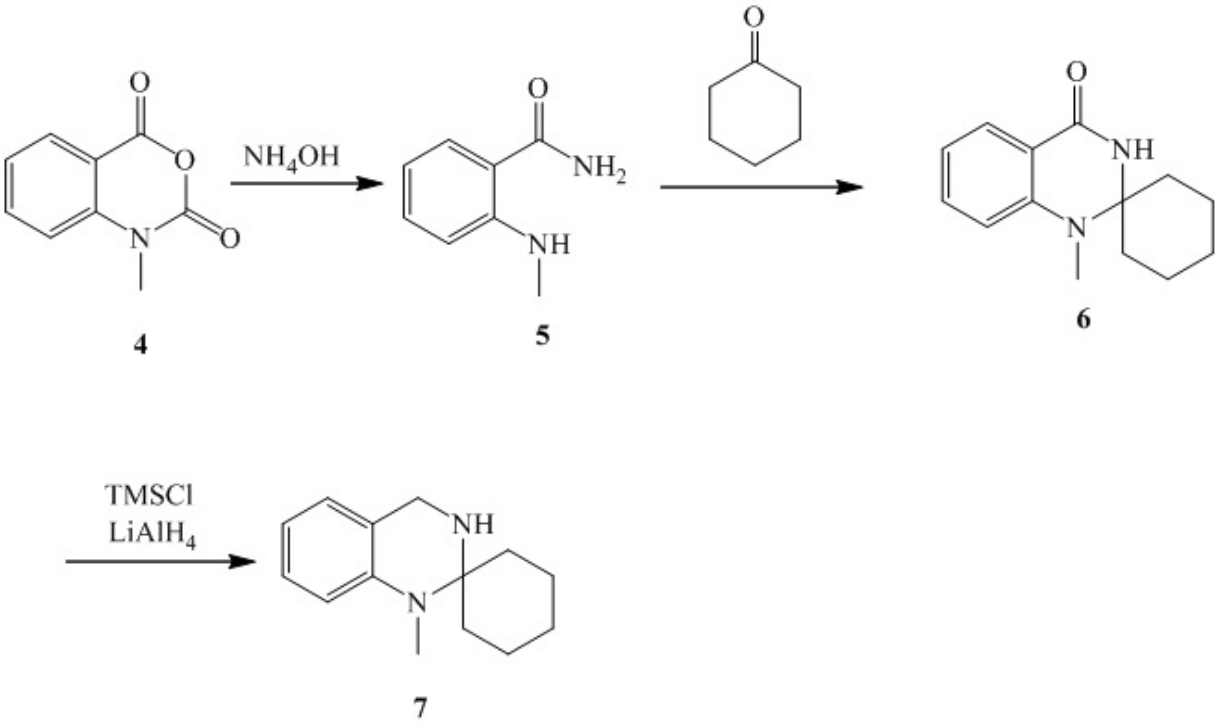
2.4. 1H and 13C NMR spectroscopy

1H and 13C NMR spectra were acquired at 500.13 and 125.75 MHz, respectively, using a Bruker Avance 500 spectrometer (Bruker, Karlsruhe, Germany). The Bruker Avance instrument was equipped with a 5 mm Broadband Observe (BBI) probe. Also, the spectrometer used was equipped with a Topspin Programming Package on a workstation running Windows Operating System.

Chemical shifts were recorded in ppm (δ) and were referenced to internal tetramethylsilane. Signal multiplicities in the 13C spectra were assigned accordingly to Distortionless Enhancement by Polarization Transfer (DEPT) experiments. Signals assignments were made on the basis of Correlated Spectroscopy (COSY).

2.5. Gas chromatography-mass spectrometry (GC-MS)

Characterization of products were achieved using directed-coupled GC/MS interface (GCMS-QP 2010 Plus), quadruple mass filter with electron impact (EI) ion source. Carrier gas is helium (purity 99.995%). GCMS solution software real-time analysis and postrun were used for data acquisition and reporting. The Perkin-Elmer column length 25 m, methyl 5% phenyl silicone, 0.32 mm inner diameter, 1.0 μm film thickness was used.



Synthesis of 1'-methyl-3',4'-dihydro-1'H-spiro[cyclohexane-1,2'-quinazoline] 7.

2.6. Thermogravimetric analysis (TGA)

The TGA-DTA curves were obtained using a Netzsch equipment, model STA 409 PG/PC, using 70 μL α-alumina open crucibles with samples of about 22.5 mg and a heating rate of 20.0 ºC min-1 in a N2 and a temperature range of 30.0-500.0 ºC.

2.7. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of the materials were recorded over the range of 400–4000 cm−1 on a Perkin Elmer (model Smart UAIR-two) with 4 cm-1 resolution.

3. Results and discussion

3.1. Synthesis

Synthesis of 2-(methylamino) benzamide 5 can be prepared by a widely described procedure utilizing an equimolar amount of an appropriate amine and N-methylisatoic anhydride. The mixture is heated under reflux over the formation of carbon dioxide. The reaction time was determined by detecting with GC-MS the formation of the desired product[24,25] (**Scheme 1**).

The synthesis of the spiro compound 6 was achieved using an inexpensive catalyst, p-toluenesulfonic acid (p-TsOH). The catalytic activity of this catalyst is not affected by the presence of solvents containing oxygen atom. Furthermore, in comparison with cyanuric acid and glacial acetic acid catalysts, p-TsOH shows more catalytic reactivity, reaction rate and yield[26].

Reduced structure of the spiro system compound 7 was prepared making the secondary amide more susceptible toward reduction by using chlorotrimethylsilane (TMSCl) followed by a large excess of lithium aluminium hydride (LAH). This procedure allowed to obtain the target product 7 in a good yield.

The structures of synthesized compounds 5, 6 and

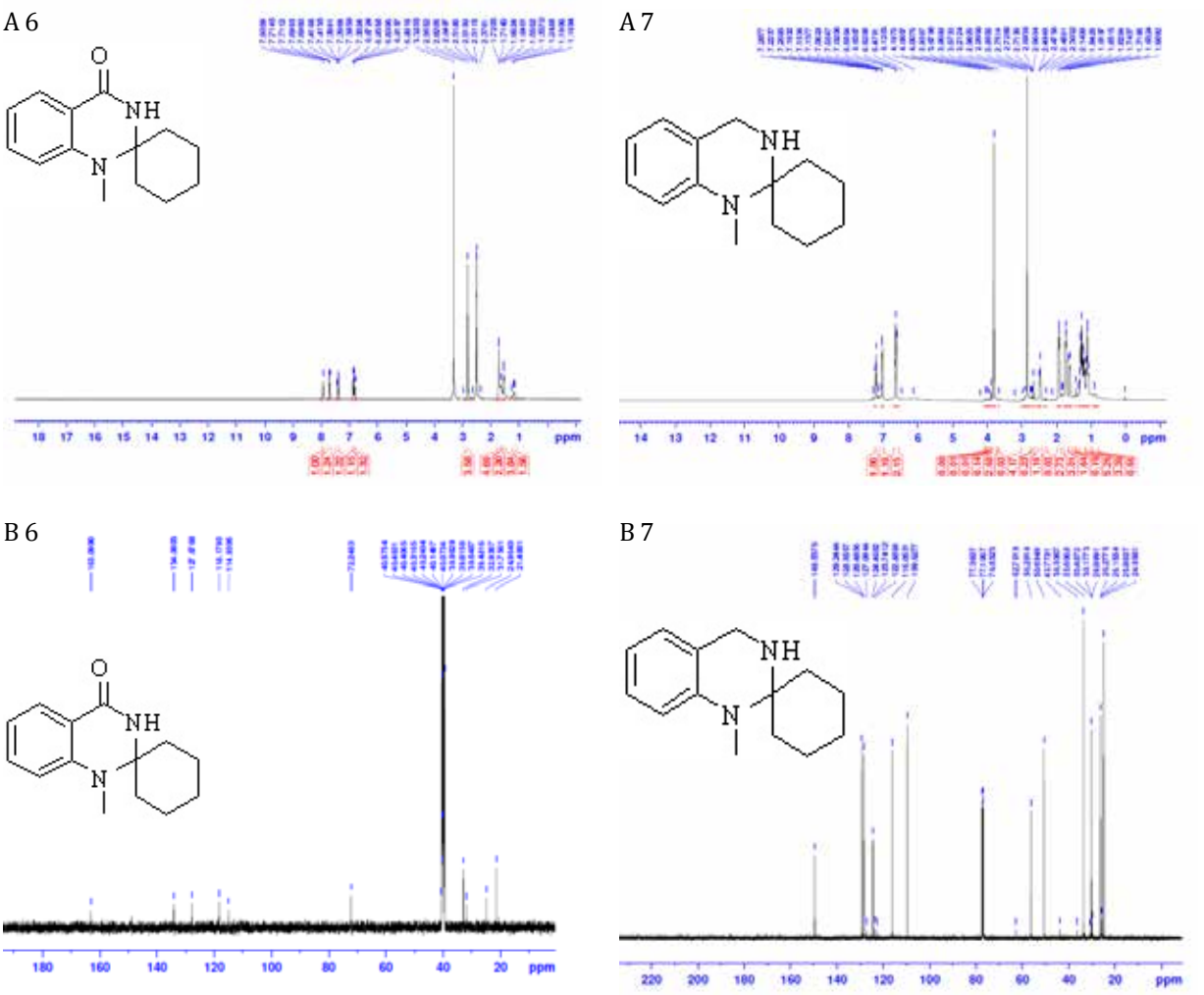


Fig. 2. *(A) 1H-NMR and (B) 13C-NMR spectrum of purified compounds 6 and 7.*

7 were confirmed after purification by GC-MS and 1H/13C NMR spectra (**Fig. 2 A and B**).

3.2. Synthesis of 2-(methylamino)benzamide (5)

N-methylisatoic anhydride (4) (10 g) was dissolve in 100 mL NH4OH solution (35%). The mixture was stirred at 40 C° for 1 hour untile the reaction was completed (TLC analysis) and was left to cool and then the solvent was removed in vacuo. The residue was purified by column chromatography (ethyl acetate/hexane, 8:2) affording the desired product as a white solid with a yield of 90%. Rf = 0.25 (ethyl acetate/hexane, 8 : 2). The product was characterized by GC-MS (EI) (C8H10N2O), m/z calcd: 150.08; found 150.25. 1H-NMR of 5 spectroscopy was in accordance with literature data[27].

1H-NMR (500 MHz, DMSO): δ = 2.77-2.78 (d, 3H, CH3) 3.32 (s, 1H, NH), 6.51-6.54 (t, 1H, J = 7.45, ArH), 6.61-6.63 (d, 1H, J = 8.3 Hz, ArH), 7.27-7.30 (dd, 1H, J = 7.5, 7.6 Hz, ArH), 7.58-7.60 (d, 1H, J = 7.8 Hz, ArH), 7.11-7.99 (br s, NH2).

3.3. Synthesis of 1'-methyl-1H'-spiro[cyclohexane-1,2'-quinazolin]-4'(3'H)-one (6)

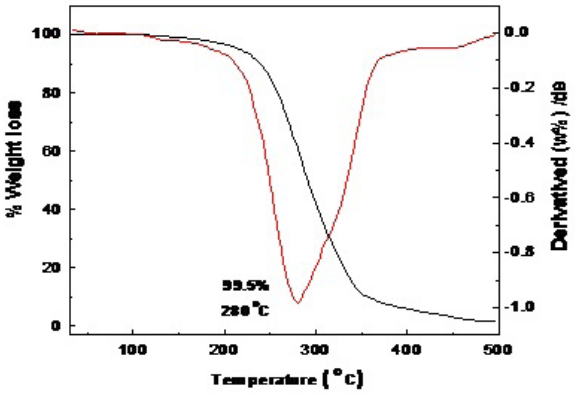


Fig. 3. *TGA analysis curves of the compound 7.*

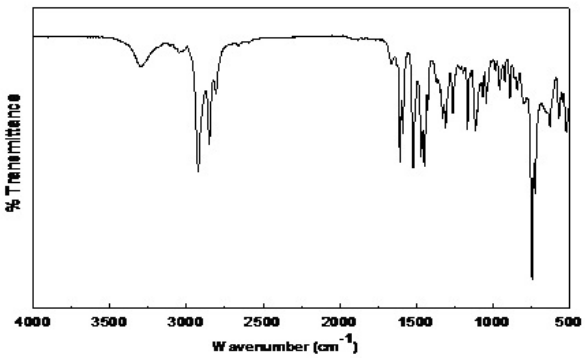


Fig. 4. *FTIR spectrum of compound 7 between 4000-500 cm-1.*

2-(methylamino)benzamide (5) (3 g, 13.88 mmol) was dissolved in CH3OH (30 mL). Subsequently, p-TsOH (0.3 g, 1.577 mmol) and cyclohexanone (5 mL, 48.24 mmol) were added. The mixture was stirred under reflux for 3 h. The solvent was removed in vacuo, and the resulting crude product was purified by crystallization from hot methanol, to afford the title compound as a white solid with a yield of 90%. Rf = 0.24 (n-hexane/ethyl acetate, 7:3).

1H-NMR (500 MHz, DMSO): δ = 1.53-1.72 (m, 10H, 2'-H2 - 6'-H2) 3.32 (s, 3H, CH3), 6.80-6.87 (m, 2H, ArH), 7.38-7.41 (m, 1H, ArH), 7.69-7.71 (dd, 1H, J = 7.6, 1.65 Hz, ArH), 7.93(s, 1H, NH, brd).

13C-NMR (125 Hz, CDCl3): δ = 21.48, 24.96 (cyclohex.), 31.75 (NCH3), 32.93 (cyclohex.), 72.24 (NCN), 114. 92 (arom.), 118.17 (arom.), 127.67 (arom.), 134.07 (arom.), 163.09 (CO).

MS m/z: calcd for C14H18N2O: 230.14; found: 230 .00

3.4. Synthesis of 1'-methyl-3',4'-dihydro-1'H-spiro[cyclohexane-1,2'-quinazoline] (7)

1'-methyl-1H'-spiro[cyclohexane-1,2'-quinazoline]-4(3'H)-one (6) (1.5 g, 6.5 mmol) was dissolved in 15 mL of anhydrous tetrahydrofuran. Subsequently, TMSCl (5.5 mL) was added and the reaction was stirred at room temperature for 15 minutes, and then lithium aluminium hydride (LAH) (4.28 g, 57.91 mmol) was added to the mixture of the reaction. After 1.5 hours the starting material was completely consumed and the reaction was quenched by the addition of 3.53 mL H2O and subsequently 3.53 mL of NaOH (15%) and finally with 10.7 mL of H2O. The reaction mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate (Na2SO4), filtered and evaporated in vacuo. The crude product was purified utilizing column chromatography (hexane/ethyl acetate, 1:1) to provide 7 in 70% yield (brownish oil). Rf = 0.2 (hexane/ethyl acetate, 1:1).

1H-NMR (500 MHz, CDCl3): δ = 1.07-1.94 (m, 10H, cyclohexyl), 2.47 (s, NH, brd), 2.85 (s, 3H, CH3), 3.84 (s, 2H, CH2), 6.62-6.65 (m, 2H, aromat.), 7.02-7.03 (d, 1H, J = 7.05 Hz, aromat.), 7.19-7.22 (dd, 1H, J = 7.65, 7.6 Hz, aromat.).

13C-NMR (125 Hz, CDCl3): δ = 24.95 (cyclohex.), 26.15 (cyclohex.), 30.17 (CH3), 33.60 (cyclohex.), 50.69 (CH2N), 56.28 (NCN), 109.62 (arom.), 116.05 (arom.), 124.46 (arom.), 128.48 (arom.), 129.24 (arom.), 149.53 (arom.).

MS m/z: calcd for C14H20N2: 216.16; found: 216.00

3.5. Thermogravimetric analysis (TGA)

TGA is a simple analytical technique used to study

Table 1. Assignment of FTIR absorption bands from 7[30-32].

Absorption peak (cm^{-1})	Interpretation
3296-3045	N-H stretching secondary amine
2924-2807	C-H stretching for benzene ring and cyclohexane
1663-1608	C=C benzene ring
1588-1521	N-H bending for secondary amine
1469	Aromatic ring stretching
1448	CH_2 scissors cyclohexane
1425-1327	CH_2 wag cyclohexane
1263	C-N stretching tertiary amine
1116	C-N stretching secondary amine
1066 and 921	CH_2 rock cyclohexane
1045	C-C stretching torsion cyclohexane
888	C-C stretching cyclohexane
744	Ortho substitution of benzene ring
518	C-C-C bending cyclohexane

the thermal stability of samples and their weight loss at different temperatures27. For compound 7 (**Fig. 3**), one thermal event was clearly observed. It occurred at 250–350 °C, which can be attributed to the decomposition and quick combustion of 7, with a 99.5% weight loss. This result indicates the stability of 7 up to 200 °C. Furthermore, the sample had a carbonaceous residual mass (average residual mass = 0,5%). The obtained result in our work was similar to literature[29].

3.6. FTIR spectroscopy

The FTIR spectrum for compound 7 is shown in Figure 4. The single weak band N-H stretches of secondary amines are appeared in the region 3300-3045cm-1 (**Table 1**). These bands are weaker and sharper than those of the alcohol O-H stretches which appear in the same region. Tertiary amines group in 7 do not show any band in this region since they do not have an N-H bond32. The FTIR spectrum of 7 showed three peaks at 2807, 2851 and 2924 cm−1 that might be attributed to an C-H vibration of the benzene ring and cyclohexane 31,32. The band between 1663 and 1608 cm−1 was due to benzene ring, whereas the band between 1588 and 152 cm−1 can attributed to N-H bond of secondary amine. The strong absorption band of 7 at 744 was due to ortho substitution of benzene ring30. Other absorption bands of 7 are shown in **Table 1**.

4. Conclusions

In the present study it was possible to obtain

spiro quinazolinone 6 in an excellent yield, using p-TsOH as a catalyst, which has proven to be the best reagent by comparing it with other chemical reagents.

Studying in depth the scientific literature concerning the structure-activity relationships of quinazoline derivatives, it has been syntheszied a novel spiro quinazoline 7 in a good yield, utilizing TMSCl as activating agnet and subsequently LAH as a powerful reducing agent.

The physical and chemical properties of compound 7 were identified utilizing FTIR and TGA. Compound 7 offers a new quinazoline based template for further biological evaluation against Alzheimer disease. □

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Therapeutic potential of N-terminal fragments of galanin in cardiovascular diseases

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SUMMARY

Under normal conditions, pleiotropic neuropeptide galanin (Gal) is involved in central cardiovascular regulation but its influence in heart disease remains unclear. The present study was designed to evaluate effects of N-terminal Gal fragments on rat models of myocardial ischemia/reperfusion (I/R) injury and heart failure (HF). Natural and modified Gal fragments (2-11) and (2-15) were synthesized by the automatic solid phase method using Fmoc technology and purified by preparative HPLC. Their chemical structure was identified by 1H-NMR spectroscopy and MALDI-TOF mass spectrometry. They were examined in acute myocardial infarction and doxorubicin-induced HF. The optimal doses of the peptides significantly reduced the infarction area and decreased the activity of necrosis markers in blood plasma at the end of reperfusion compared with the control. The chimeric ligand of Gal receptors [βAla14, His15]-galanin (2-15) (G5) exhibited the most beneficial effect. Administration of the peptide G5 improved LV systolic dysfunction, reduced activation of lipid peroxidation and decreased the plasma activity of creatine kinase-MB in doxorubicin-treated animals. This peptide attenuated the disturbances of myocardial energy metabolism induced by doxorubicin cardiotoxicity. The results suggest that pharmacological ligands of the GalR1-3 receptors can be a rational basis for the development of drugs in the field of cardiovascular diseases.

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1. Introduction

Prevention and treatment of cardiovascular diseases is one of the key tasks of modern molecular and cellular cardiology. In this regard, the development of new cardioprotective compounds that can reduce damage to the heart during various pathological influences is topical. Galanin, a 29 (30 in human) amino acid residues neuropeptide, is involved in a large range of different vital functions, including regulation of the cardiovas-

cular system[1]. There is experimental evidence that constitute the basis of targeting galanin receptors for the treatment of various human pathological conditions, including Alzheimer's disease, mood disorders, anxiety, metabolic diseases, pain and solid tumors[2]. Data on the cardiovascular effects of galanin and its bioactive N-terminal fragments are quite few. It is known that intracisternal injections of these peptides may affect arterial pressure and heart rate[3]. In addition, galanin is able to act directly on the cardiomyocytes since GalR1-3 receptor subtypes are expressed in the heart[4]. In isolated guinea pig papillary muscle galanin exerted a positive inotropic action under hypoxic conditions and prolonged effective refractory period[5] probably due to activation of inwardly rectifying K^+ channels[6]. Contents of galanin mRNA in cardiac sympathetic neurons and heart tissue are increased after myocardial ischemia/reperfusion (I/R) injury in rats[7]. The increased galanin content in the damaged left ventricle of the heart is consistent with studies showing that the peptide is transported to regenerating nerve endings after axon damage to promote neurogenesis[8, 9]. These observations suggest that agonists of galanin receptors GalR1-3 may be useful pharmacological agents in cardioprotection.

We have recently shown that N-terminal fragment of galanin (2-11) (G1) can reduce experimental myocardial I/R injury[10]. This peptide increased cell viability, inhibited apoptosis and the formation of excessive reactive oxygen species (ROS) in mitochondria in response to hypoxia-reoxygenation in cultured rat cardiomyoblast H9c2 cells. Postischemic infusion of galanin fragment (2-11) improved functional recovery, reduced cell membrane damage in perfused rat heart and enhanced restoration of myocardial metabolic state during reperfusion. These findings demonstrated a direct action of G1 on the heart damaged by ischemia and reperfusion. Moreover, intravenous administration of the peptide G1 limited myocardial infarct size and decreased activity of necrosis markers in rats in vivo. The cardioprotective efficacy of G1 is likely related to its antioxidant properties and binding to the GalR2 receptor, since this ligand has a poor affinity for the receptor subtypes GalR1 and GalR3[1, 11]. GalR2 activation triggers different signaling pathways that stimulate the mechanisms of cell survival and the regulation of metabolic and ion homeostasis[12]. Despite these beneficial effects, poor solubility in water and a short half-life in blood circulation due to hydrolysis by peptidases complicate the study of this peptide as an alleged therapeutic tool.

The objective of the present work was to overcome these problems by design and synthesis of novel ligands of galanin receptors. As a basis, we used the galanin fragment (2-15) (G2) which has a preferential binding towards GalR2 receptor with no appreciable activation of GalR1 and GalR3 receptors[13], and a better solubility in water compared to the peptide G1. Preliminary experiments confirmed ability of G2 to attenuate heart damage caused by ischemia and reperfusion[14]. In this study, we examined ability of natural and synthesized peptide ligands of galanin receptors to limit acute myocardial infarction and attenuate doxorubicin-induced cardiotoxicity in rats.

2. Materials and Methods

Galanin peptides. Peptides were synthesized by the automatic solid phase method using Fmoc technology and purified by preparative HPLC. Their chemical structure was identified by ^{1}H-NMR spectroscopy (a Bruker WM-500 spectrometer, Germany) and MALDI-TOF mass spectrometry (Bruker Daltonics, Germany).

Reagents. Fmoc-protected amino acids derivatives were purchased from Novabiochem and Bachem (Switzerland). Chemicals for peptide synthesis were from Fluka (Switzerland**).** Enzymes and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO USA). Solutions were prepared using deionized water (Millipore Corp. Bedford, MA, USA).

Animals. Male Wistar rats (300 - 350 g) were used in these experiments. They were housed in cages under standard conditions and a 12 h light-dark cycle. A commercially available pellet diet (Aller Petfood, St. Petersburg, Russia) and tap water were provided ad libitum. The experimental protocol was performed according to the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (No. 123 of 18 March 1986).

Acute myocardial infarction. Rats were anesthetized with 20% urethane (120 mg/kg body wt. i.p.) and artificially ventilated with a KTR-5 animal respirator (Hugo Sachs Electronik GmbH) with a volume of 2–3 ml at a rate of 70–75 breaths/min. Preparation of animals was performed as described earlier[10]. The mean arterial pressure, HR and standard lead II ECG were recorded throughout the experiment using a LabVIEW 7.1 data acquisition system (National Instruments, USA). After stabilization of hemodynamic parameters (steady state), the left anterior descending (LAD) coronary artery was occluded for 40 min to simulate regional ischemia; the duration of subsequent reperfusion was 1 h. In control, saline (0.5 ml) was administrated by i.v. bolus injection at the onset of reperfusion. In the experimental series, peptides G1-G5 were administrated by i.v. bolus injection at the onset of reperfusion at doses of 0.25, 0.50, 1.0, 2.0 or 3.0 mg/kg. At the end of reperfusion, LAD coronary artery was reoccluded and 2 ml of 2% Evans Blue (Sigma, USA) solution was injected through the jugular vein to distinguish the myocardial non-ischemic area from the area at risk (AAR). The heart was then excised and the left ventricle (LV) was frozen. A frozen LV was transversely cut into 1.5 mm thick slices which were incubated in 0.1 M potassium phosphate buffer pH 7.40, containing 1% 2,3,5-triphenyl-tetrazolium chloride for 10 min at 37^0C. The slices were fixed in 10% formalin for 5 min. They were placed between two transparent glasses and captured on both sides using a scanner at 600 d.p.i. resolution; the saved images were analyzed by computerized planimetry using Imagecal software. The slices were weighed for determination of LV weight. The AAR was expressed as a percentage of LV weight, myocardial infarction (MI) was expressed as a percentage of the AAR in each group.

Doxorubicin-induced heart failure. Rats were randomized into 4 groups: control group (C) received saline weekly (1.0 ml/kg for eight weeks, subcutaneously); doxorubicin group (D) received doxorubicin (2.0 mg/kg/week for the first four weeks, intraperitoneally) and then saline (1.0 ml/kg/week for the next four weeks, subcutaneously); the doxorubicin+peptide G5 group (D+G) received doxorubicin and saline as stated above and simultaneously peptide G5 (50 nmol/kg/day for eight weeks, subcutaneously); peptide G5 group (G) received peptide G5 (50 nmol/kg/day for eight weeks, subcutaneously). Before treatment and at the end of the study, the animals were weighed and cardiac function was evaluated by echocardiography, concentration of thiobarbituric acid reactive substances (TBARS) and activity of creatine kinase-MB (CK-MB) were determined in blood plasma. At the end of experiments, the hearts were used to determine energy metabolites and mitochondrial respiration in permeabilized fibers of the left ventricle (LV).

Echocardiographic study. Animals were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (1 mg/kg). Echocardiography was performed using a Philips Envisor C ultrasound system with a 10-MHz electronic transducer. Images were obtained from the left parasternal short-axis views of the LV at the level of papillary muscles. LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD) were measured from M-mode images. LV fractional shortening (LVFS) was calculated as (LVEDD-LVESD)/LVEDD × 100%. LV ejection fraction (LVEF) was calculated using the Teichholz method.

Determination of necrosis markers, TBARS and metabolites. Plasma CK-MB and LDH activity was determined using standard kits from BioSystems S.A. (Barcelona, Spain). Cayman's TBARS Kit was used for colorimetric measurement of lipid peroxidation products in plasma. The hearts frozen in liquid nitrogen were quickly homogenized in cooled 6% $HClO_4$ (10 ml/g) using an Ultra-Turrax T-25 homogenizer (IKA-Labortechnik, Staufen, Germany), and the homogenates were centrifuged at 2800×g for 10 min at 4^0C. The supernatants were neutralized with 5 M K_2CO_3 to pH 7.40, and the extracts were centrifuged after cooling to remove $KClO_4$ precipitate. Tissue dry weights were determined by weighing a portion of the pellets after extraction with 6% $HClO_4$ and drying overnight at 110^0C. Concentrations of ATP, ADP, AMP, phosphocreatine (PCr), creatine (Cr) and lactate in neutralized tissue extracts were determined by enzymatic methods[15].

Mitochondrial respiration in permeabilized LV fibers. Saponin-treated LV fibers were prepared accord-

Table 1. Physicochemical characteristics of galanin peptides

Peptide	Sequence	MW, g/mol	MALDI-TOF, m/z	HPLC R_t, min	Yield*, %
G1	H-W-T-L-N-S-A-G-Y-L-L-NH_2	1136.3	1136.77	16.53	23.9
G2	H-W-T-L-N-S-A-G-Y-L-L-G-P-H-A-OH	1499.67	1499.72	15.34	45.4
G3	H-W-T-L-N-S-A-G-Y-L-L-G-P-H-A-**NH_2**	1498.68	1498.64	14.85	27.4
G4	H-W-T-L-N-S-A-G-Y-L-**Nle**-G-P-H-**R**-OH	1584.88	1584.66	14.25	21.2
G5	H-W-T-L-N-S-A-G-Y-L-L-G-P-**βAla-H**-OH	1499.67	1499.76	14.66	46.3

*The substitutions are shown in bold. R_t - retention time. * The yields are given relatively to the first amino acid, which is attached to the polymer.*

ing the method described in[16]. Mitochondrial respiratory parameters were determined using an Oxygraph plus system (Hansatech Instr., UK) and expressed in nmol O_2/min/mg dry weight. The energy substrates were 10 mM glutamate +5 mM malate. State 3 was induced by 2 mM ADP. Dry weight of the muscle samples was determined after drying for 48 h at 80^0C.

Statistical analysis. Data are presented as means ± SEM. All statistical analyses were performed using SigmaPlot version 12 (Systat Software Inc, San Jose, CA). $P<0.05$ was defined as significant.

3. Results and Discussion

Design strategy and characteristics of galanin peptides. To increase the resistance of novel ligands to the action of exopeptidases, standard molecular design techniques were used: amidation of the C-terminal amino acid and incorporation of unnatural amino acid residues into the sequence[17]. Modification of the N-terminal Trp residue was not carried out, as it was previously shown that changes in the N-terminal part of galanin lead to a loss of affinity for the GalR1-3[19]. Considering the necessity of testing the synthetized agonists on models of myocardial I/R injury and heart failure *in vivo*, significant attention was paid to increase solubility of peptides. We have synthesized amide G3, which has a greater resistance to degradation by carboxypeptidases, and two novel analogues of the galanin fragment (2-15), G4 and G5. The peptide G4 was obtained by replacing Leu[11] with Nle and substituting Ala[14] for a basic amino acid Arg to enhance proteolytic stability and solubility in water. A highly water-soluble chimeric peptide G5 was synthesized as a conjugate of galanin (2-13) with the dipeptide carnosine. Amino acid sequences of galanin peptides are given in **Table 1**. Modification of natural galanin fragments G1 and G2 resulted in a significant increase in solubility of the pharmacological ligands G3-5 in water. This parameter increased in the following sequence G1 (0.25 mg/ml) < G2 (4 mg/ml) < G3 (10 mg/ml) < G4 ≈ G5 (>20 mg/ml) and tightly correlated with HPLC retention time.

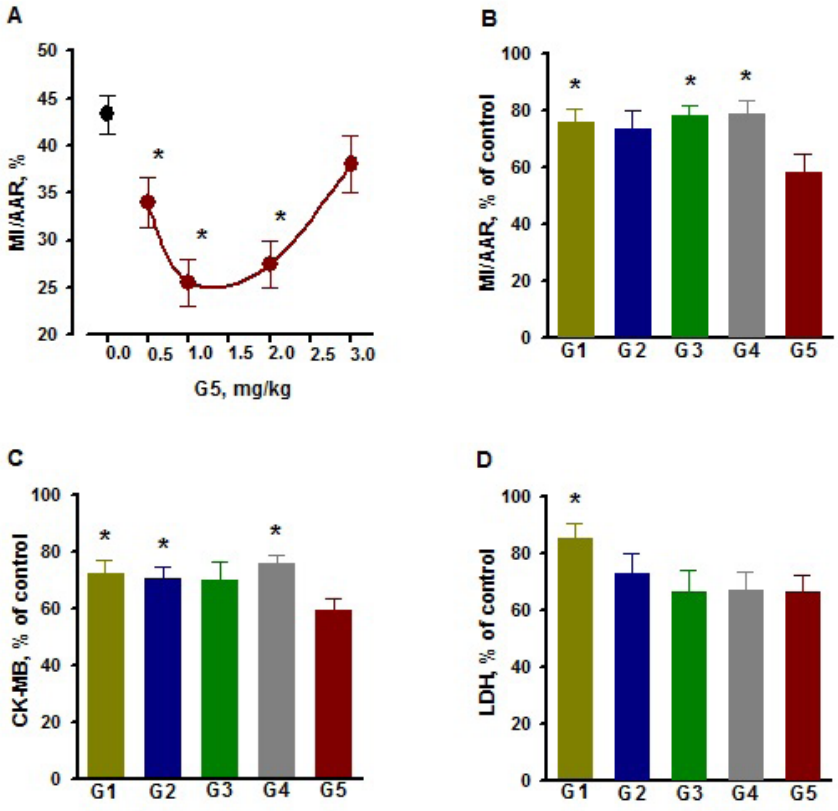

Figure 1. *Effects of intravenous administration of peptides G1-5 on myocardial infarct size and activity of necrosis markers in blood plasma in rats. Data are the means ± SEM from 10 experiments.*
*(A) Dose-dependent effect of G5 bolus injection after occlusion of LAD coronary artery on myocardial infarct size (MI/AAR, %) at the end of reperfusion. Black circle corresponds to myocardial infarction in control (43.3±2.0%). *P<0.05 vs. control. (B) Reduction of myocardial infarction with the optimal doses of peptides G1-5. Data are expressed as a percentage of the value in control. * P<0.05 vs. G5. (C) Effects of the optimal doses of peptides G1-5 on the plasma activity of CK-MB expressed as a percentage of the value in control. * P<0.05 vs G5. (D) Effects of the optimal doses of peptides G1-5 on the plasma activity of LDH expressed as a percentage of the value in control. * P<0.05 vs G5.*

Effects of intravenous G1-5 administration on myocardial I/R injury. The percentage ratios of AAR/LV did not differ significantly between the studied groups and averaged 41.0±1.6%. This suggested that the ligation of LAD coronary artery was performed equally in all animals. At the end of reperfusion, myocardial infarct size (MI/AAR, %), was 43.3±2.0% in control. Peptides G1-5 exhibited a dose-dependent effect on limiting infarct size in the dose range from 0.5 to 3.0 mg/kg. An example of such dependence for intravenous G5 injection is shown in **Fig. 1A**. The optimal dose was 2.0 mg/kg for peptide G1 and 1.0 mg/kg for peptides G2-5. The most effective of the peptides was G5. It reduced infarct size by 40% compared with the control (P<0.005) and to a significantly greater extent than the natural galanin fragment (2-11) G1 and the modified analogues of galanin (2-15) G3 and G4 (**Fig. 1B**). Effects of the optimal doses of peptides G1-5 on the plasma activity of CK-MB and on the plasma activity of LDH are shown in **Fig.3C** and **3D**.

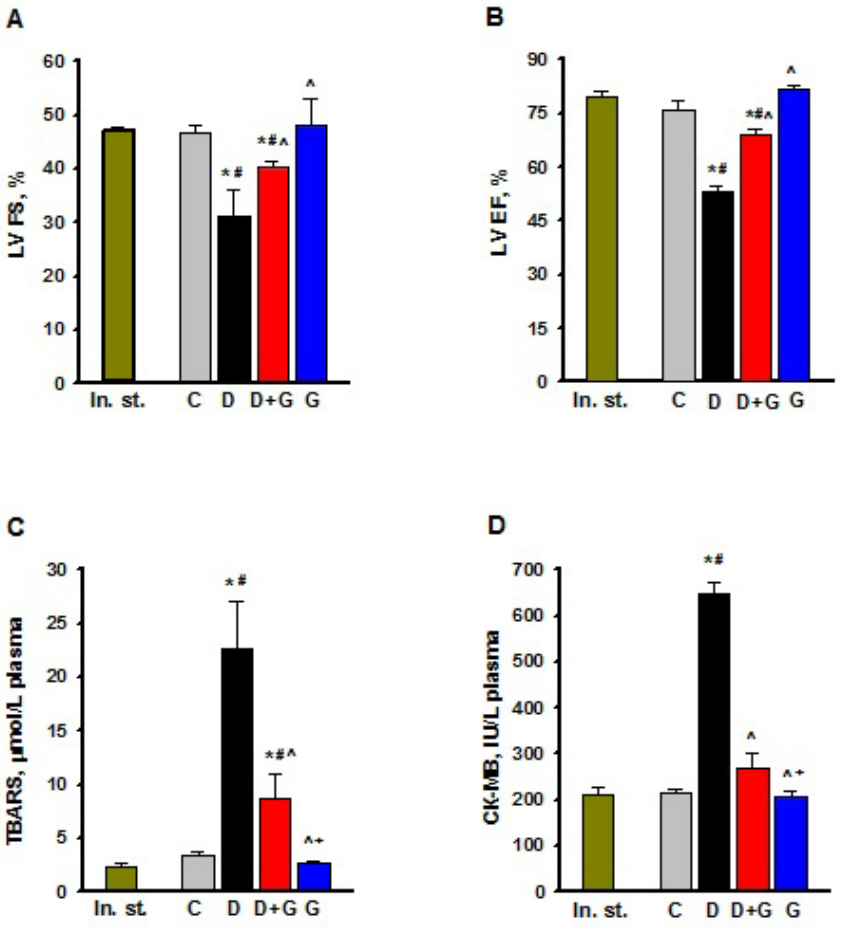

Figure 2. *Changes in LV fractional short¬ening (A), LV ejection fraction (B), the plasma concentration of thiobarbituric acid reactive substances (C) and plasma activity of creatine kinase –MB (D) in the control and treated groups during the study. Values are expressed as means +SEM. In. st.: initial state before treatment. The values for C, D, D+G and G groups correspond to the 8-week study. P<0.05 vs: * initial state, # :C, ^:D, +: D+G.*
(A, B) In. st., n=48. For groups C, D, D+G and G n=12.
(C, D) In. st., n=24. For groups C, D, D+G and G n=6.

In the control animals, the activity of CK-MB and LDH increased to 2347.1±78.7 and 1402.7±107.9 IU/l plasma, respectively, by the end of reperfusion compared to the values in the steady state (277.5±27.0 and 72.5±10 IU/l plasma, respectively).The activity of both enzymes was lower after administration of the optimal doses of peptides G1-5 (P<0.05-0.01). **Fig. 2A** shows that the activity of CK-MB expressed as a percentage of the value in control was significantly lower in rats treated with G5 than in rats treated with peptides G1, G3 or G4. Administration of peptide G5 significantly reduced plasma activity of LDH compared with this parameter in animals treated with peptide G1 (**Fig. 2B**).

The results obtained indicate that peptide G5, the chimeric ligand for galanin receptors, exhibited the most beneficial effect in this model of myocardial I/R injury in vivo. It is important that treatment with this ligand did not affect the hemodynamic parame-

Table 2. Effects of doxorubicin and peptide G5 on animal mass, echocardiography variables and heart rate in experimental groups.

	Initial state	After 8 weeks of observation			
		Control	Doxorubicin	Doxorubicin + G5	G5
Body mass, g	338±5	425±12*	354±6$^{\#}$	398±5$^{*\#\wedge}$	423±6$^{*\wedge+}$
LVEDD, mm	6.4±0.1	6.6±0.2	7.2±0.1$^{*\#}$	7.0±0.1*	6.6±0.2$^{\wedge}$
LVESD, mm	3.3±0.1	3.9±0.3	4.9±0.1$^{*\#}$	4.2±0.1$^{*\wedge}$	3.3±0.2$^{\wedge+}$
HR, bpm	450±8	432±19	440±4	434±8	445±10

*Values are expressed as means +SEM for groups of 12 animals each. LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; HR, heart rate. P<0.05 vs: * initial state, # control, ^ D, + D+G.*

ters, heart rate and mean arterial pressure. Based on these findings, we evaluated the effect of G5 on doxorubicin-induced cardiotoxicity in rats. This part of research was focused on the parameters of LV cardiac function and oxidative stress, and the effect of peptide on myocardial energy state.

Effects of peptide G5 on LV dysfunction, cardiotoxicity and oxidative stress caused by DOX. In our model, doxorubicin cardiotoxicity was accompanied by development of heart failure by the end of the eighth week, as evidenced by a significant decrease in EF and FS (**Fig. 3A**) and an increase in the LV cavity (**Table 2**). The cardioprotective effect of peptide G5 against systolic dysfunction was manifested in a decrease in LVESD and LVEDD and a significant increase in LVFS and LVEF compared with these parameters in D group. Treatment of animals with G5 alone for 8 weeks did not affect the echocardiography variables compared with control.

We have assessed the effect of peptide G5 on one of the most important markers of doxorubicin toxicity, body mass loss[19]. The control group demonstrated a progressive increase in body mass during the observation period (**Table 2**). After 8 weeks of the study, the mean body mass in D group was the same as at the beginning of the study and was 20% lower than in the animals of the control group. Co-administration of G5 and doxorubicin increased body mass during the study. By the end of 8 weeks, body mass in D+G group was significantly higher than in D group. Throughout the observation period, the increase in body mass of animals treated with the peptide G5 was the same as in control animals.

Effect of peptide G5 on plasma concentrations of TBARS, low molecular weight end products of oxidative damage is shown in Fig. 3B. There were no significant differences in plasma TBARS levels between the groups before treatment. After 8 weeks of the study, plasma TBARS concentration did not change in rats of the control group compared to this value. On the contrary, this index increased 7-fold in rats of D group thus indicating activation of lipid peroxidation. Plasma TBARS concentration was 2.6 times lower in D+G group than in the D group but remained higher than in the control group. As expected, treatment of animals with peptide G for 8 weeks did not affect plasma TBARS level in comparison with the values in the initial state and in the control group.

There were no significant differences in plasma CK-MB activity between the groups before treatment

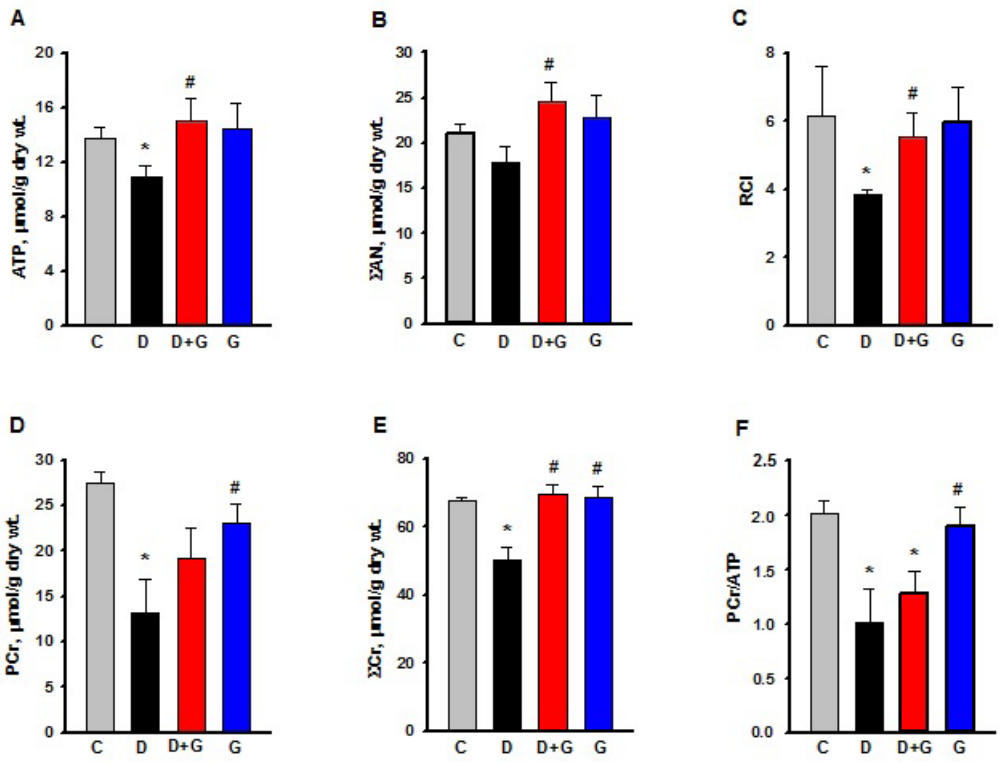

Figure 3. *Myocardial energy state in the control and treated groups after 8 weeks of the study. Values are expressed as means +SEM for groups of 6 animals each. Myocardial content of metabolites: (A) ATP; (B) ΣAN=ATP+ADP+AMP; (D) PCr: phosphocreatine; (E) ΣCr= phosphocreatine+creatine. (C) RCI: respiratory control index. (F) PCr/ATP: myocardial PCr/ATP ratio. P<0.05 vs: # :C, ^ :D.??? symbol ^ is not in the figures*

(**Fig. 3D**). After 8 weeks of the study, plasma CK-MB activity did not change in rats of the control group compared to this value. Treatment with doxorubicin produced severe cardiotoxicity, which was manifested in a three-fold increase in CK-MB activity compared with the control. Plasma CK-MB activity was 2.5 times lower in D+G group than in D group (268.9±30.8 and 646.2±28.4 IU/l, respectively, P<0.001) and did not differ from this index in the control group. These data suggest that peptide G5 significantly reduced the myocardial tissue injury caused by doxorubicin. Treatment of rats with peptide G5 for 8 weeks did not affect plasma CK-MB activity in comparison with the initial state and the value in the control group.

Effects of peptide G5 on myocardial energy metabolism. We examined cardiometabolic effect of peptide G in DOX treated rats (**Fig. 3A-F**). In D group, myocardial ATP was significantly reduced on average by 20% in comparison with this value in the control group. This resulted in a clear trend to a decrease in the total adenine nucleotide pool (SAN) in the hearts of doxorubicin-treated rats since ADP and AMP levels in groups C and D did not differ significantly. Myocardial PCr and the total creatine (SCr=PCr+Cr) were 50 and 75%, respectively, of the values in the control group. The hearts of doxorubicin-treated animals showed a twice lower myocardial PCr/ATP ratio, thus reflecting the development of LV dysfunction. Respiratory control index (RCI) in permeabilized LV fibers, which characterizes oxidative phosphorylation, was reduced by 40% compared to control (although this difference was not statistically significant). These data suggested impaired mitochondrial function, compromised cardi-

ac energy homeostasis and loss of sarcolemma integrity in animals that received doxorubicin.

Coadministration of doxorubicin with peptide G5 attenuated the disturbances of myocardial energy metabolism. At the end of the experiments, group D+G showed a trend towards an increase in myocardial ATP and PCr combined with a significant increase in both SAN and SCr pools. Noteworthy, in the hearts of animals of group D+G, myocardial contents of ATP, PCr, SAN and SCr as well as the adenylate energy charge (AEC=ATP+0.5ADP/SAN) did not differ from the values in the control. In parallel, there was a slight increase in myocardial PCr/ATP ratio and a significant augmentation of RCI (by 25 and 43%, respectively) compared to those in the group D. Administration of peptide G for 8 weeks did not affect indices of myocardial energy metabolism and mitochondrial respiratory parameters of permeabilized fibers compared to the control group. Thus, peptide G5 attenuated doxorubicin induced cardiac dysfunction in rats by suppressing oxidative stress and increasing the functional activity of mitochondria, which led to an improvement in the metabolic state of the heart.

Mechanisms of action of peptide G5. We have found that the overall protective effect of the modified galanin fragment G5 was the highest among the studied peptides on the model of acute myocardial infarction. In addition, this compound has demonstrated the properties of a pharmacological agent capable of attenuating the cardiotoxic effect of doxorubicin. Noteworthy, administration of peptide G5 to healthy animals was not accompanied by potentially detrimental effects or changes in metabolic and functional parameters of the heart.

The potency of G5 as the ligand for galanin receptor subtypes GalR1-3 and molecular mechanisms of peptide G5-related cardioprotection have yet to be investigated. It is known that the N-terminal fragments of galanin are of critical importance for its biological activity including cardiovascular regulation, and the first 15 amino acid residues are conserved in most species, while the C-terminal region (residues 17-29) varies among species and lacks receptor affinity[11]. Docking of galanin into the GalR1 receptor revealed that galanin (1–13) interacts with this receptor subtype via binding of Gly^1, Trp^2, Asn^5 and Tyr^9 to the receptor[20]. The deletion of the Gly1 residue results in loss of affinity for GalR1[21]. The Trp^2 and Tyr^9 residues, together with Asn^5, Gly^8, Leu^{10} and Gly^{12}, were identified as the critical pharmacophores for selective galanin (1-16) binding to the GalR2 receptor[22]. Galanin (1-16) displays a low affinity and efficacy for GalR3 receptor[13]. Thus, most likely, the peptide G5 synthesized by conjugation of galanin (2-13) with the dipeptide carnosine acts as a chimeric ligand having a preferential binding towards GalR2. GalR2 receptor signaling through several classes of G-proteins stimulates multiple intracellular pathways that may play a role in metabolic and functional myocardial protection. The most significant of them enhance cell survival by suppressing caspase-3 and caspase-9 activity, mediate the release of Ca^{2+} into the cytoplasm from intracellular stores and opening Ca^{2+}-dependent channels, cause GLUT4 translocation from intracellular membrane compartments to plasma membranes to enhance glucose uptake[12, 22]. It is possible that peptide G5, like the natural galanin fragments (2-11) and (2-15), has antioxidant properties. Since myocardial I/R injury and treatment with doxorubicin reduce the activity of catalase, glutathione peroxidase and superoxide dismutase[23, 24], a direct scavenging of ROS or replenishing depleted antioxidant system may be one of the effective mechanisms of cardioprotection. To date, to delineate the contribution of galaninergic system in protective effects of peptide G5 is difficult due to a lack of ligands with receptor subtype specificity, the complexity of intracellular signaling cascades, switching between different conformational states of G protein-coupled receptors in vivo, the existence of GalR1-3 heteroreceptor complexes and potential antioxidant properties of G5. Despite this uncertainty, galanin receptor subtypes can be considered as putative drug targets in various cardiac pathology.

4. Conclusions

We synthesized two natural N-terminal fragments of galanin, G1 and G2, and three pharmacological peptide ligands, G3, G4 and G5, which may have a high affinity for the receptor subtype GalR2. The optimal doses of

peptides G1-5 significantly reduced myocardial infarct size and decreased the plasma activity of CK-MB and LDH in at the end of reperfusion in rats in vivo. The chimeric agonist of galanin receptors, WTLNSAGYLLGPβAH (G5), most effectively reduces irreversible damage to cardiomyocytes and slightly affects the hemodynamic parameters when administered intravenously. We believe that this compound can be used to correct myocardial I/R injury with a greater advantage than the other GalR1-3 receptor ligands studied in this work. We examined effects of peptide G5 on doxorubicin-induced cardiotoxicity in rats. Co-administration of G5 with doxorubicin significantly decreased the formation of TBARS, the products of oxidative stress, in plasma and prevented an increase in plasma CK-MB activity. These effects were combined with enhanced preservation of myocardial high-energy phosphates and mitochondrial respiration. Peptide G5 significantly improved LV function and caused weight gain in doxorubicin-treated animals. These findings demonstrate cardiometabolic efficacy of peptide G5 as a pharmacological agent that attenuates toxic effects of doxorubicin. Thus, the potential clinical application of a new ligand of the GalR1-3 receptors G5 may include various pathologies of the heart. However, further research is needed on its mechanisms of action and pharmacological properties. □

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Conflict of Interest
The authors declare that they have no conflict of interest.

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Personalized and Precision Medicine (PPM) as National and International Models of Healthcare Services of the Newest Generation: A Green Light Towards the Innovations to Secure Biopharma and Translational Resources

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SUMMARY

Higher education is key to economic and social development. Despite significant invest-ments in education, science and innovations in recent years, Russia, unfortu-nately, continues to noticeably lag behind the world.

The completion of several fundamental international projects focused on understanding the functioning of human body at all levels has caused the appearance of omics technologies. It is marked the creation of new directions in the development of biotechnology and biopharmaceutical industry and medical practice. The rapid emergence of novel high-tech drugs, the needs of potential employers and the demands of the labour-market have clearly demonstrated that the introduction of new approaches in the pharmaceutical

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and biotechnology practice requires a global reforms not only in the field of biopharmaceutical production, but also in the areas of healthcare and education. These reforms should solve problems of training universal specialists who have both fundamental scientific education and professional competences. Such reforms significantly require

Introduction

Translational Research is a term used to describe a complex process aimed to build on basic scientific research to create new therapies, medical procedures and diagnostics[1]. It is obvious that basic researchers are the key bricks within the frame of the translational research initiatives. It is critical that such scientists are well acquainted with the what is known and not known about the biological processes of human development (organogenesis) and of human pathogenesis arising from microbial infection, environmental toxins, and natural errors in gene functioning. The challenging issue is that the vast majority of graduate students and even these scientists are not so trained.

Speaking of medical students, they are acquainted with these concepts, basic anatomy, some microanatomy, and even basic molecular biology. However, their understanding of fundamental biological processes is limited. They are generally instructed in current interpretations of cellular and molecular mechanisms but are not acquainted with the gross uncertainties that extrapolation and assumptions untested. For instance, the concept of "stem cell" in human development and in pathogeneses as in the development of cancers or atherosclerotic plaque are not yet understood even in the world's leading centers of basic and medical research.

The work of a medical researcher is a way to understand fundamental biological processes and mechanisms of disease pathogenesis, and it has been critical to preventing, diagnosing, and treating diseases and conditions that afflict millions of people. However, despite a tremendous impact, for instance, of human genome project on our understanding of the pathogenesis, such as cancer and autoimmune diseases, and an invention of different techniques (approaches) such as single cell sequencing or proteomic profiling, the current educational system is not sufficient at preparing a next-generation specialist, which is able to use all the advances have been made[2]. For example, an implementation of next-generation sequencing (NGS) into clinical practice requires a “Big data” approach based on high integrity between clinical informatics, bioinformatics, and fundamental studies as well. A major challenge in the clinical setting is the need to support a dynamic workflow associated with the constant growth of the laboratory's NGS test menu and expanding specimen volume[3]. To perform such kind of a mission it is crucial to educate specialists or thinkers, which will know medical, biological and informatics aspects of the problem and will know how to use their knowledge in solving this problem as well. And that is just the tip of the iceberg. Human genetic databases are corrupted by false results. NGS studies have built-in error rates of approximately 0.3 to 3.0% per base pair, which does not favor an improved understanding of diseases and the implementation of advanced therapeutics. In addition, the concept of Personalized & Precision Medicine (PPM) aimed to create a cutting-edge treatment, diagnostic and preventive tools for a wide range of diseases also requires high integrity between fundamental research, industry, and clinic[4, 5].

The lack of translation is the challenging problem in the various fields of medicine such as creating of brain-computer interfaces or investigations of drug resistance and cancer[6,7,8]. We are not saying that the problem is mainly due to the obsolete education system, for example, the lack of clinical trans-

lation in cancer research can be explained by the fact that animal models is not a precise reflection of a human organism[8]. Conversion of research findings into meaningful human applications, mostly as novel remedies of human diseases, needs progress of appropriate animal models. Research methodologies to test new drugs in preclinical phases often demanded animal models that not only replicate human disease in etiological mechanisms and pathobiology but also biomarkers for early diagnosis, prog-nosis, and toxicity prediction. Whereas the transgenic and knockout proce-dures have developed guidance of rodents and other species to get greater un-derstandings of human disease pathogenesis, but still generating perfect animal models of most human disease is not available[8].

Notably, clinical trials themselves have some considerable limitations, and hence the results of these studies could be misunderstood[9]. The point is: in order to provide an effective "bench to bed" workflow there is a huge need for specialists, which are capable of performing a wide range of tasks. Nowadays, however, due to a tremendous amount of available information, it is feasible to create a specialist who knows how to interconnect different areas of research and how to adapt to constantly changing conditions, whereas to create a specialist knowing how to do everything on his own is not. An education of these specialists is the pivotal objective for the new education system, and the creation of this system is at the top of the agenda for this paper.

Drug discovery is extremely both time- and money-consuming process. The basic translational pipeline here consists of at least eight units, namely, target to hit, hit to lead, lead optimization, preclinical trials, three stages of clinical trials, and finally, submission to launch[10]. The whole process lasts as long as a decade and a half and requires interdisciplinary-educated staff not only familiar with fundamental research but also with different techniques and approaches used in the drug discovery. A number of potential solutions to improve R&D productivity and increase clinical translation of drug candidates have been offered by Paul et al[10]. Some of these solutions propose a total transformation of the current single company-owned R&D enterprise to one that is highly networked, partnered and leveraged (Fully Integrated Pharmaceutical Network or FIPNet)[10]. Authors also stated that in order to improve drug development it is vital to provide a cashflow from the high expensive phase II and III trials to less expensive preclinical and 1-st phase clinical trials, thereby increasing the number of drug candidates to select the most promising ones. These candidates, in turn, would have a higher chance to be approved[10]. Obviously, both of the ideas aforementioned require a strong collaboration between research, stakeholders, and government. And we suggest that the education is the starting point to deploy such a network. Once developed, this new education system should kill two birds with one stone, namely, should prompt the collaboration between fundamental research and industry, yet also should allow to use an approach close to simulation-based medical education that has been proved to be highly effective in different areas of medical education[11-15]. The main difference of the approach proposed is the use of it in the settings of drug discovery.

Mastery learning is another approach, which could be used in the modern education system. Despite its development as early as in 1963, it has a lot of progressive features, such as clear learning objectives, deliberate skills practice, and complete mastery of the discipline selected[16]. However, it also has some considerable limitations especially meaningful in a case of drug development. One of these limitations is the unlimited time to reach the mastery. The time factor is one of the most important ones during the translation process. To reduce the negative influence of unlimited time of mastering one could involve only talented students in such a program, which expected time to acquire a new skill or master a new subject is relatively low. However, does it fit in with a standard mastery learning paradigm?

The problem of great concern is the designing of a mechanism that could detect, educate, and implicate highly motivated students and young scientists in order to meet the needs from industry and healthcare system. It is clear that somehow we should tightly

interconnect different areas of research, saving student-oriented education principles. Some attempts are already ongoing: universities are experimenting with new programs and courses to teach innovation. Within the life sciences, there is particularly strong traction in the area of biomedical technology innovation, in which a number of interesting new training initiatives are being developed and deployed. However, how this experiments will affect the healthcare system remains to be determined. We suggest that the system itself requires not just new courses and programs, but a total rearrangement at all. Practically, in most universities, preclinical students often see academic and medical professionalism as two separate paradigms characterized by dissimilar elements. It is very critical to measure medical students' understandings of the relationships between these two constructs. We suggest that the system itself requires not just new courses and programs, but a total rearrangement at all.

Fundamental aspects of this educational reform.

At the present stage, the task of national importance is the development of the concept and strategy of radical changes in Healthcare Service and the creation of new medical education model. The purpose of employment of the knowledge is to predict and prevent diseases, increase the life expectancy, strengthen and preserve human health, as well as the identification and monitoring patients with underlying risk for the development of a particular pathology.

At present, a leading strategy for the development of health care system is the creation of new directions in medicine using modern achievements of basic sciences and high technology, as well as the development of the concept and strategy for radical transformations in the field of health care and medical education. The strategy for innovative development of Russia is based on implementation of intellectual potential of the country and sets high requirements to the level and quality of professional education. In this respect, an innovative model for the development of the health care system provides for close cooperation between the health care system, medical science, government and business community, planning of scientific medical research depending on the needs of health care service, active introduction of scientific results into medical practice, as well as targeted training of specialists capable to ensure implementation of scientific achievements[17].

The implications in PPM include interpretation and clinical use of novel and personalized information including genetic testing; anticipation of results and treatment; ongoing chronic monitoring; and support for patient decision-making. Health care service lies in managing human body reserves while its main purpose is to detect hidden abnormalities in a body and to take measures in order to eliminate them and prevent occurrence of decease. A key reason for changing the health care system became an active use in the practice of a hospital physician of advances in omics, allowing to penetrate inside biostructures and create therein conditions for visualization of lesions, previously concealed from the eyes of a clinician.

At the heart of the developed concept of PPM and the model proposed for practical use there are postulates which promote change in the culture and the mindset of society as a whole. In the first place it is the awareness of individuals that they are responsible for their own health and the health of their children, an active participation of population in preventive measures designed for promotion of individual and public health. In this connection, one of the major organizational tasks of the Russian Ministry of Healthcare is to carry out restructuring of the existent health care system to ensure implementation of preventive, diagnosis, remedial and rehabilitation measures designed to reduce morbidity and death rate of population, ensure maternal and infant health care and promote healthy lifestyle.

Implementation of the PPM model will lead to the replacement of the existing "doctor-patient" relationship model by the "doctor-consultant-healthy person" model.

In this regard, it is obvious that the society needs a new scientific and technical school for the formation

of specialists of a new generation, using non-traditional methods and a technological arsenal based on the achievements of system biology and translational medicine.

The most important factor for successful implementation of the reforms is staffing. Specialists in the field of PPM should be experts not only in interdisciplinary areas of activity, but also in the fundamental areas that provide a clue for generations of essentially new medicinal drugs and diagnostic technologies as well as for personalization of protocols of diagnostics and prevention[18].

Correspondingly, preparation of specialist capable of building the interdisciplinary healthcare system of the future should be built on the novel principles taking into account the following:

• common architectonics of pre-university, university and post-graduate education;

• features of introducing a secondary school-university PAIR into international educational environment;

• the role of additional or further education and bioinformational technologies as the basis of such environment;

• principles of design in the structure of additional education and design of business, research and engineering plays;

• a rigid necessity of a multiple-level testing and dialog in the learner-teacher pair with due account of personality characteristics of the two when forming vocational self-sufficiency and professional potential of a specialist as a personality of the future;

• the value of innovation risks of the educational process and possibility to monitor such risks in case of emergency situations.

This educational model should contain:

• educational-methodological kernel;

• key platforms of basic knowledge and qualities, and

• a system of group and individual vectors demonstrating priority directions and substantive potentials of intensiveness and quality of knowledge development[19].

In this sense, to have to design initiatives to secure targeted steps in reforming of education:

• to harmonize accreditation standards across the continuum of Hi-Tech education;

• to catalyze educational innovations in pre-university, undergraduate, graduate, and continuing Hi-Tech education;

• to empower more interdisciplinary curricular arrangement;

• to inform the public about improvement initiatives in Biopharma and Biomedicine-related education

For training of specialists it is required to restructure programs of pre-university (high-school), undergraduate, graduate and postdoctoral medical training as well as to develop fundamentally new generation interdisciplinary programs, focused on training and retraining of specialists in the areas related to PPM. In implementing the principle of continuity of an ongoing education a model of multi-stage training of a specialist is being built, which is characterized by a phase-by-phase process of individual development going over, while information is learned, from one level of an ongoing training to another. To implement the reform it is required to introduce in the medical universities special training programs, comprising 12-15 disciplines of different level and content and requiring at least 8-12 years of general and 4-5 years of target education to train a specialist with the required set of competencies. The latter incorporating technical, psychological and legal aspects of establishing a pre-hospital diagnosis and development, if necessary, of a personalized prevention protocol, using an appropriate evidence base and procedures for informing the "healthy" individuals at risk and their relatives. In such a manner, at the 1st level of education (pre-university) school pupils will be made familiar with the modern model of PPM without in-depth study of any particular aspects. Special significance on the pre-university level is the selection of talented young specialists and involvement them into creative activities. At the 2nd (university) level, students will be offered in-depth study of fundamental and applied aspects of PPM. The core of the third (post-university) level will be interdisciplinary aspects of PPM, targeted to resident physicians and postgraduates[19-21].

The new development model for branches of the biopharmaceutical industry and TraMed will cover almost all global markets. Accordingly, countries and individual companies which adopt this model, will have a significant advantage through synergy and an already established demand arising from the patient and the medical community simultaneously. The long time developmental forecast pertaining to the biopharmaceutical industry's aforementioned market–which has been supplied with resources by drug design and TraMed–will be determined by:

1. The "binding" of technologies in the spheres of: fundamental biology, nanotechnology, system engineering, with stockpiles of medical experience;

2. An increased connection between the instrument making industry, biopharmaceuticals and the nanotechnology segment of the biopharmaceutical industry;

3.The miniaturization of monitoring facilities, which allow the minilab to be connected to real patients, and is designed to accommodate biosensors or biorobot injector units: including the formation of networks and private clouds (cloudy technologies);

4. Explosive technological growth addressing preventative/prophylactic tasks within personalised protocols (increasing the share of MPs which aim to stimulate adaptive/coping mechanisms in the body and the strengthening of genomic health), and the development of personal health management programs;

5. Predicting changes to competitive models: from in-house development to integrating the efforts of various companies (synergy);

6. The strengthened role of emerging markets whose influence is dictated by the rapid growth of university technoparks (e.g South Korea, the Philippines, Singapore, Iran and other countries dynamically characterised by personnel possessing the potential to be extremely highly qualified).

Integration of science and education is important to increase the quality of preparation of specialists. As an example: within the framework of higher educational institution structural integration there may be integrated university departments and research institutes with similar interests to create scientific and educational complexes having a common academic council and administration system. By doing this, university lecturers do scientific work in research institutes, and researchers give lessons in university department, and the academic council coordinates all this complex work, approves educational plans of departments and research plans in research institutes. Some of the Russian universities have set up Education-Science-Innovation Complexes (ESIC) or BioClusters in terms of long-term cooperation to secure the quality of education[20].

One of the tasks of higher professional education is the participation of students and teachers in basic research with the aim of mastering modern methods of searching for, processing and using scientific information, the ability to concentrate the work of its intellect on an unconventional solution of theoretical and experimental problems.

An important part of the new educational model is the allocation of specific methods and approaches to training for different age levels. Of particular interest is training of high-school students in high technologies on the basis of leading research institutes and universities with a strong and mod-ern research capacity. Another important component of the new educational model is its focus on the practical skills and the ability to apply knowledge. To ensure efficient interface between educational institutions and production it is proposed to introduce educational cluster "school-university-production". An active cooperation of educational institutions and the production will create favorable conditions for enhancement of their investment attractiveness and competitiveness of graduates on the labor market, and it will help to ensure speedy adaptation of students to the conditions of an enterprise and it will prevent occurrence of shortfall in human resources. Early postgraduate work assignment at enterprises will significantly improve the planning of training of specialists in conjunction with the ability to more effectively specialize students in the profile of specific companies.

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A number of universities has already organized ESIC for the purpose of increasing the quality of edu-cation and strengthening the liaison with the production. The specifics of ESIC consists in that thanks to the cooperation of scientific research, educational and production capacities, there is ensured a new quality of education, development of research and commercialization of the results of scientific and technological joint performance.

Creation of ESIC on the basis of a structural subdivision of a higher educational institution/university (a department – laboratory of a research institute – pilot-scale production of university) without establishment or attraction of a legal entity permits to avoid problems associated with the interaction of two and more legal entities, especially when they have different forms of in-corporation. In this case it becomes is easier to solve questions associated with intellectual property and organize a joint educational, scientific and innovation processes. Setting up of ESIC on the basis of a university and a large industri-al enterprise permits developing long-term cooperation and prepare duly trained personnel for the needs of biopharmaceutical industry.

In addition to the creation of a "secondary school-university" cluster with the aim to increase the quality of education, a particular place in prepara-tion of bioengineers is occupied by the "secondary school-university/college-production" cluster. The cluster pattern of this system will help prepare a competent employee for regional labor market and achieve maximum result in minimum time limit. Exactly the cluster approach predetermines mutually beneficial relationship, uninterruptedness and cooperation. It is evident that active joint functioning of educational establishments with production facilities will create favorable conditions for increasing their investment attractiveness and competitiveness of graduates at the regional labor markets.

The demonstrated "good practices" uniting educational and innovation activities in universities show that with the established ties with revived or newly organized private enterprises, the scientific and educational cooperation starts its active growth. In short, the state, market and production, universities and research institutes should promote expansion of biopharmaceutical sector of economics, contribute to effective national industrial policy, and stimulate innovations of small businesses, thus facilitating the progress of society as a whole.

Some specifics of the model.

Currently, a new educational program is being developed on the basis of the Sechenov First Moscow State Medical University, aimed at training doctors and specialists in the field of biopharmaceutical industry. Within the program it is planned to train specialists for medical, pediatric and bioengineering faculties. The courses of the program are divided into three categories - basic, elective and specialized. At the first stage of pre-university training, general aspects of human physiology and anatomy, the foundations of molecular and cell biology will be considered, and also students will learn the basics of PPM. The program of higher education will be divided into three stages. The first includes the first two courses, in which students will study the fundamental foundations of PPM (Omics, Genetic Engineering, Genomic Editing and Gene Therapy, Immunology, Biomarkers, Bioinformatics, Targeting, Technologies for Working with Proteins and Genes, Biobanks). Further, at the stage of the three-year university education will be the study of diagnostic, preventive and therapeutic diagnostic platforms of target categories of PPPM, among them pharmacogenomics, oncology, pulmonology, pediatrics and others. At the next one-year training stage, students will study clinical and preclinical models with pre-

dictive-diagnostic and preventive-preventive orientation, risks, their evaluation and the formation of diagnostic protocols. At the postgraduate stage students will study preclinical and clinical trials using the biobank base, a program for managing one's own health, including family planning, the stage of genomic scanning and clinical evaluation, clinical bioinformatics, as well as interdisciplinary aspects, including bioethics, the basis of public-private partnerships in modeling personalized and preventive medicine and questions of sociology.

In the training programs will include the tasks of training the students and planting skills in the following fields:

• understanding of the key molecular mechanisms of disease devel-opment and designing models of pathophysiologic mechanisms of the latter with preliminary selection of potential pPTT;

• identification of basic structural-functional shifts in the physiologi-cal architectonics of cell biomolecules causing generation of cell pathology, pathology of intercellular interactions and, as a result, the overall pattern of clinical symptomatics;

• screening of biomarkers necessary for use in predictive diagnostics, prognostication and monitoring of diseases at the preclinical and clinical stages of disease;

• understanding of the principles of modern diagnostics, analysis and interpretation of laboratory data permitting to perform identification of key cellular shifts when various pathologies are formed;

• use of molecular targets with the aim of prophylactics and preven-tion of disease at the clinical stage or typical pathological process at preclin-ical stage.

A graduate of specialized courses should know the following:

• theoretical and methodological basis of the fundamental medicine corresponding to the contemporary level of world knowledge in the field of systems biology and fundamental biomedicine;

• principles of designing of a model of pathological process with identification of biomarkers and selection of targets necessary for effective control of pathological process;

• pool of modern technologies used to improve the effectiveness of the analytical cascade including with the use of interdisciplinary approaches and modernization of the integrated infrastructure of biopharmindustry;

An important part of program is the creation and development of fundamentally new technological platforms with elements the commercialization of the results of basic research and following introduction of them into clinical practice. For example, the development of innovative methods system of screening and monitoring will allow estimating the reserves of health, allocate among the asymptomatic contingent in the process of preventive examinations of patients and persons from risk groups with preclinical stages, and create objective prerequisites for personalized therapy. And the creation of an information system for personalized medicine prescribes the development of a new model of the patient and people at risk with using biomarkers, preclinical and predictive diagnostics technologies, and the development of new methods for targeting and motivating healthy lifestyles and active longevity. The key to implementing PPM in clinical practice is information technologies, including machine learning and artificial intelligence.

Such a faculty's lectures and practical courses will touch upon all aspects of next generation MPs and PDTs modelling and processing (at different stages and levels of the creation process), as well as elements involving the forecasting of their respective market segments. Being involved with a student's development is of particular importance, as they should be familiarised with the principles and methods of validating the final product, the procedures for market calculations, and the prospective applications of a product in clinical practice. The specialist of the future will also have the ability to pursue follow-up research to enhance the product itself and widen the scope of its application (repurposing), and this will all be achieved with the aid of cutting edge equipment.

Special attention will be delegated to school-based (Pre-University) courses including: Next-Generation Sequencing (NGS) and related technologies;

mass-spectrometry and proteomics; immunoassay platforms and techniques; bioinformatics-related network technologies.

A program is being created for the purpose of training specialist bioengineers in the fields of genetic-engineering and genome editing technologies, biotechnology, clinical pharmacology, biomedicine and biomedical application of nanotechnology.. This program should be a mandatory component of every pharmaceutical designer's and bioengineer's training in specialized Russian higher education establishments which possess the relevant human resources and bioengineering infrastructure to accommodate it.

Thereafter the curriculum for training of physicians at the premises of University will be developed, the aim of which is to acquire general professional and specific professional competencies to provide on-going medical assistance to each applicant - patient or person at risk, (regardless age, sex and character of disease); for the implementation of patient care, taking into account family history, the society in which he lives, its culture, ensuring respect for the principle of personality's individuality.

The programme focuses on familiarising bachelors, masters and Ph.D. students with scientific research, applied production as well as managerial and pedagogical activities. These assets are then implemented in the creation of a cluster (unifying I.M Sechenov First MSMU with a number of specialist higher education establishments) which provides:

1. Unity between scientific, educational/pedagogical, production, and monitoring/oversight processes;

2. The continuation of the educational process within flexible international platforms, and the interrelation of these platforms and programmes within a single cluster;

3. Innovative focus on the academic and applied sections of the educational process before the transition into practices involving advanced technologies;

4. A program involving constant consumer marketing and calculations of potential, actual and projected market segments, which function as a practice model for the training of biopharmaceutical industry's future entrepreneurs/executives.

To secure the positive outcome of the reforms we are planning to take several steps.

1. Globally, to focus on the importance of/new generation disciplines whilst better incorporating omics-technologies and bioinformatics into the Curricula so that students understand the underlying science and its application to Targeted Medicines and Predictive Diagnostics tools.

2. To provide at regular schools fellowships in Fundamental Medicine and Pharmacy-related Biomedical Areas, which would help to establish the field as a subspecialty.

3. To actively engage at regular schools in developing new standards of health care to integrate new targeted therapeutics, personalized and predictive tests, and quality standards for testing.

Possible problems in the implementation.

World practice has shown that as soon as a country enters a phase of sustainable economic development, there is an increase in the social welfare of people and an increase in the life expectancy of the population, then at the same time an increase in the death rate of the population from cancer and cardiovascular diseases is observed. The priority struggle against socially significant ills of modern civilization is an important step, but it is not decisive in increasing the life expectancy of the population of the country. In the civilized world, there was a steady idea of how to fundamentally reverse the negative trend of growth of socially significant diseases without financial bleeding of the country's budget. More and more economically developed countries are converting their health care in line with the concept of PPM.

Changing the paradigm of health care actually entails reformatting the system for training specialists, reorienting research centers to solving health problems and creating new breakthrough technologies, and qualitatively modernizing the domestic bio-pharmaceutical industry and related industries in the Russian Federation. It is obvious that without

interactive regulation and restriction of "egoistic" requests of departments, participants of this global project, any financial investments only in health care and education will be ineffective.

The implementation of the project to modernize health care in its scientific, technical and social significance is akin to a nuclear project of the USSR. Its result was not only the emergence of the country's "atomic shield", but also the creation of new knowledge-intensive branches of the national economy, which ensured economic progress and improved well-being of citizens. The PPM project is aimed at preserving and improving the quality of health of those who are protected by the "atomic shield" of the country. Taking into account the modern structure of the Russian economy, as well as the role of the state in regulating financial flows in the implementation of projects of such scale, it is necessary to give it a special status with the involvement of all possible sources of financing for its implementation.

If we consider the modernization of education as an element of the project with modern scientific and technical achievements, then we have a chance to transform the educational system taking into account breakthrough precision technological platforms. At the same time, in the very system of today's education, there are yesterday's mechanisms that inhibit its mobility and ability to reform.

Despite an ample need to Implementing new educational system into practice, there are some considerable limitations, which could hamper all the process.

First of all, how should we evaluate the impact of the reform on national health care system, quality of life, and even the employment of biopharma specialists? And, in the case of failure, what actions should be performed to prevent additional aggravations in the industry? The main issue here is that we don't have an approach to a transparent analysis of such the data. For instance, a social return on investment - based approach seems to be a promising one due to the fact that it includes the information on the amounts of resources used by a program, in addition to program activities, and represent program value to society as a whole rather than a specific stakeholder group. Unfortunately, this approach is not devoid of flaws, such as raw methods in use and the possibility of inclusion only "appropriate" social groups in the analysis[21]. Additionally, several years (or even decades) should pass to enrich the data of reform outcome, and thus allowing to analyze the impact of this reform.

The second issue is the cost of the reform in a broad sense. When speaking of a total rearrangement of the educational system, it is of great importance to determine the source of financing. It seems to be obvious that both the government and the industry are interested in a new education system. However, are these sides interested enough to provide an immense amount of investments required to reach this goal, taking into account that return on investment is not expected in upcoming years? Moreover, to make the reform real it is crucial to implicate well-qualified staff, which demand a salary at least higher than average. Increased administrative expenses, expenses on reform implication, wages for workers, and on additional factors, such as new equipment, could eventually increase the cost of undergraduate and graduate education.

And finally, the educational reform is multifaceted, time-consuming process, which could be viewed as a process with its own translation pathway. Taking into account that calls for reform of graduate medical education started as early as in 1940[18], and nothing has changed dramatically ever since (in terms of the education system), the major issue is to prevent the reform from getting stuck in the translation.

Conclusion

Our model for accelerated development of continuous vocational educa-tion in the sphere of biopharmaceutics and biopharmaceutical industries is based on the combinatorial approaches (competence, module, personality-activity, program-design and problem-oriented) to the elucidation of innova-tive processes of modernization of the existing system. Correspondingly, the unit to build up the content of educational programs and sites is the task of pedagogics oriented for the innovation context in

education development, and it allows each hearer to organically combine individual and group work with the aim to enrich oneself with the experience of the colleagues, and also to use own professional experience.

The use of the accelerated model for development of continuous vocational education has required a new type of organization of the educational process. The place of formalized methods and means of education focused on the transfer and learning of information is now occupied by innovation, inter-active, problem- and-practice-oriented, and research-and -design methods. All that ensures solving of pedagogical problems on the basis of constructive dialog, exchange of opinions, role and positional interaction, practical solution of educational tasks, and use of information technologies.

The afore stated reform of bio-pharmaceutical education, when implemented, will provide the ability to attain and maintain a professional standard of training for specialists in Russian universities, which in turn, will bring them up to world standards and promote academic, professional and inter-regional mobility. It will also enable the creation of an open system of university education, which will ensure that specialists are well enough trained to work in a constantly changing environment. □

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Forskolin potentiates the effects of GSKJ4 in human acute myeloid leukemia cells through Protein Kinase A pathway

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SUMMARY

Forskolin is a natural cAMP elevating agent that is emerging as one of the most promising molecules for its potential use in cancer therapy. The epigenetic marks play a relevant role in several cancer types, including in leukemia. In particular, GSKJ4 has been recently demonstrated to act as a potent proliferation inhibitor in many tumor cell lines. Due to the failure of the single agent therapy, recently the chemotherapy combination has received more attention in order to increase the therapeutic index and to reduce the side effects. In this scenario, naturally occurring molecules represent the ideal candidates to investigate cooperative responses with conventional and new antineoplastic drugs. Here, we investigate the capability of forskolin to potentiate the effects of GSKJ4 in human leukemia U937 cells. We provide evidence that forskolin markedly sensitizes GSKJ4-induced antiproliferative effects through apoptosis induction (caspase-3 activation and PARP cleavage). In addition, we demonstrate that this phenomenon is mediated by cAMP elevation and Protein Kinase A (PKA) involvement, as indicated by the use of PKA inhibitor (KT-5720). In conclusion, our findings provide initial evidence about the efficacy of forskolin/GSKJ4 combination in leukemia cells and suggest a new possible therapeutic approach for AML treatment.

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Introduction

Extracted from the roots of the Coleus Forskohlii, forskolin is a derived diterpenoid that was discovered in India in 1970s[1]. This plant has been used in Hindu Ayurvedic preparations as an herbal medicine to treat various pathological disorders such as hypertension, congestive heart failure and many others[1-2]. Nota-

bly, all potential pharmacological activities related to Coleus Forskohlii are connected to the forskolin and to its capability to be a potent, rapid and reversible stimulator of adenylate cyclase activity, resulting in the increase of intracellular cAMP[3-4]. At this regard, it has been shown that forskolin rises cAMP formation in mammalian cells, influencing various physiological and not physiological functions and processes, such as blood and intraocular pressure, platelets aggregation and lipolysis[5-6]. The clinical use of forskolin is now a reality for the treatment of different pathological conditions[7-8]. Moreover, it has also been shown that the cAMP elevating agent forskolin has numerous relevant anticancer effects in many types of cancer cells. The induction of mesenchymal-to-epithelial transition, the inhibition of proliferation, motility/migration and the enhancement of the sensitivity to conventional antineoplastic drugs represent the main responses induced by forskolin in cancer cell lines[8-17]. In this respect, we demonstrated that in MDA-MB-231 triple negative breast cancer cells, forskolin inhibits basal and leptin-induced proliferation and migration preventing ERK1/2 and STAT3 phosphorylation in response to leptin. In addition, we also found that the inhibition by forskolin of leptin-mediated cell migration is accompanied by a strong decrease of β3 integrin subunit and FAK protein levels[18]. Concerning the ability of forskolin to sensitive antineoplastic drugs, we demonstrated that forskolin strongly enhances gemcitabine-induced antiproliferative effects in pancreatic cancer and improves sensitivity to doxorubicin of triple negative breast cancer cells[17, 19].

More recently, several epigenetic compounds, which target specific enzymes involved in the determination of the chromatin status, have been developed and proposed to have beneficial effects in cancer care, including leukemia[20-21]. Among all the compounds, the histone deacetylase inhibitors (HDACi), such as MS275 (entinostat) and SAHA (vorinostat), represent one of the best-studied and well-characterized classes of inhibitors designed in this field. Although the HDACi are in advanced phases of clinical trials, the widespread inhibition of the plethora of HDAC-containing complexes represents their major drawback[22-23]. The methylation status of the four histone lysine residues (K4, K9, K27, K36) stands for another critical epigenetic mark for many biological functions, and methyltransferases and demethylases are the enzymes by which this process is regulated[24-25]. Notably, the tri-methylation on lysine 27 of histone H3 (H3K27me3) controls the gene expression patterns acting as a transcriptional repressor and it has been described to have a relevant role in several cancer types[26]. GSKJ4 is a novel, selective inhibitor of the jumonji family of histone demethylases JMJD3 and UTX, which catalyzes H3K27me3 in to H3K27me2/1. Furthermore, it has been shown that GSKJ4 acts as a potent small molecule inhibitor of the proliferation in many cancer cells, including glioma, breast, ovarian, lung cancer and leukemia.[28-32]. In addition, in diffuse large B-cell lymphoma GSKJ4 is directly toxic as a single agent and even more in combination with various clinically approved drugs[33]. Sadly, due to the failure of the single agent in cancer therapy, the chemotherapy combination has received more attention in order to increase the therapeutic index of each individual drug and to reduce the side effects simultaneously. In this scenario, naturally occurring molecules represent the ideal candidates to investigate cooperative responses with conventional and new antineoplastic drugs. In view of the above, no evidence has been reported in literature concerning the possible effect of forskolin in leukemia, while few data are available regarding GSKJ4 and its possible partners to serve for the combination therapy in the same cancer types[34]. In particular, it has been described that GSKJ4 has cytotoxic effects in acute lymphoblastic leukemia and that co-treatment with All Trans Retinoic Acid (ATRA) significantly increases cell death respect to ATRA or GSKJ4 alone in PML-RARα-positive leukemic cells[34]. For these reasons, the present study has been designed to investigate the possible effects of forskolin on the chemo-sensitivity of acute myeloid leukemia cells to GSKJ4 and the underlying molecular mechanisms.

2. Materials and Methods

2.1. Antibodies and chemical reagents

Chemical Reagents: Propidium Iodide (PI) (Sigma Life Science, Milan, Italy), bovine serum albu-

min (BSA) (Microtech, Naples, Italy), 8-Br-cAMP, cAMP analogue 8-pCPT-2'-O-Me-cAMP, forskolin, 3-isobutyl-1-methylxanthine (IBMX), Protein Kinase A inhibitors KT5720 and H-89 were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA), GSKJ4 (SIGMA-ALDRICH #SML0701) Primary antibodies used for immunoblotting: Anti-tubulin antibody (CP06, Oncogene-Calbiochem, La Jolla, CA), anti-ERK (#9102), anti-p-CREB (Ser133, #9198) and anti-CREB (#9197) (Cell Signaling Technology, Danvers, MA, USA) Anti-H3K27me2 (Diagenode, Cat. No. C15410046) and Anti-H3K27me3 (Diagenode, Cat. No. C15410069). All other antibodies were obtained from Santa Cruz Biotechnology (San Diego, CA, USA). Secondary horseradish peroxidase (HRP) conjugated antibodies used for immunoblotting: goat anti-rabbit (GtxRb-003-DHRPX) and goat anti-mouse (GtxMu-003-EHRPX.0.05) (Immunoreagents Inc., Raleigh, NC, USA). Solutions and buffers were prepared with ultra-high-quality water.

2.2. Cell culture and treatments

U937 and NB4 cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI-1640 culture medium containing phenol red (Sigma Life Science), supplemented with L-Glutamine (2mM), 10% of Fetal Bovine Serum (FBS) (Hyclone), penicillin (100 mg/ml), streptomycin (100mg/ml), amphotericin B (250mg/ml), (Sigma, UK). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Forskolin and GSKJ4 were dissolved in DMSO and added to culture medium in order to obtain the final concentration required. Negative control cells were treated with an equal volume of DMSO (< 0.1% v/v).

2.3. Cell proliferation and cell cycle analysis

Colorimetric exclusion: $2x10^5$ cells/mL cells were seeded and treated with different times and concentrations as indicated in results section. Thereafter, cells were diluted 1:1 in Trypan Blue (Sigma Life Science) and counted with an optical microscope in order to discriminate dead cells (stained in blue) from living cells (not stained). Flow cytometry analysis was performed to evaluate cell cycle, using a FACSCalibur flow cytometer (Becton Dickinson). The cells were harvested, spun-down at 1200 rpm for 5 minutes and resuspended in 500 µL of a hypotonic solution containing 1X PBS, Sodium Citrate 0.1%, 0.1% NP-40, RNAase A and 50 mg/mL Propidium Iodide (PI). After 30 minutes of incubation, at room temperature and protected from light, samples were acquired. ModiFIT Cell Cycle Analysis software was used to define the percentage of sub-G1, G1, S and G2/M regions. All experiments were performed at least in triplicate.

2.4. Cell death assay by propidium iodide uptake and Flow Cytometry analysis

Cell death was measured as previously described[35]. Changing of plasma membrane permeability is the essential condition to allow Propidium Iodide (PI) to bind DNA. Due to mechanisms of death (apoptosis, necrosis, autophagy etc.) plasma membranes generally become permeable. Comparing PI uptake, in different cells populations or in the same one, is used as a method to discriminate dead cells from live cells. Briefly, cells were plated ($2x10^5$ cells/mL) and treated for different times and concentrations. After that cells were recovered and incubated with PI-FACS buffer containing 0,4 µg/mL of PI in 1X PBS and analyzed by flow cytometry.

2.5. Preparation of total cell lysates

Cell extracts were prepared as follows. 3 to 5 volumes of RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 µg/ml aprotinin, 1 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride, PMSF) were added to recovered cells. After 1 hour of incubation, samples were spun-down at 14000 rpm in a table top centrifuge for 15 min at 4° C. Supernatant (SDS total extract) was recovered in order to determine proteins concentration (using Brad-

ford Method) and diluted in Leammli Buffer, boiled and stored as samples for immunoblotting analysis.

2.6. Protein histone extraction

Cells were harvested and washed with 1X PBS and lysed in Triton extraction buffer (TEB: PBS containing 0.5% Triton X-100 (v/v), 2 mM PMSF, 0.02% (w/v) NaN3) at a cellular density of 10^7cells/ mL for 10 minutes on ice, with gentle stirring. After centrifugation (2000 rpm at 4° C for 10 min), the supernatant was removed, the pellet was washed in TEB and centrifuged as before. The pellet was suspended in 0.2 M HCl overnight at 4° C on rolling table. Next, the samples were centrifuged at 2000 rpm for 10 minutes at 4° C, the supernatant was removed and proteins content was determined using the Bradford assay (Bio-Rad CA, USA).

2.7. Immunodetection of proteins

20-40 μg of proteins were employed for immunoblotting. Extracts were separated by SDS-PAGE and transferred on nitrocellulose membrane (Sigma-Aldrich, St. Louis, MO, USA) using Mini Trans-Blot (Bio Rad Laboratories, Hercules, CA, USA). Nitrocellulose Membranes were blocked in no-fat milk 5% w/v and incubate at 4° C overnight with specific primary antibodies. Nitrocellulose Membranes were washed three times per 5 minutes with TBS Tween-20 (TermoFisher Scientific, Waltham, MA, USA) and incubate at room temperature for 1 hour with goat anti-rabbit or anti-mouse antibodies conjugated with horseradish peroxidase, used as a detection system (ECL) according to the manufacturer's instructions (Amersham Biosciences, UK).

2.8. Statistical analysis

Data were presented as the mean ± s.d. of biological replicates. Differences in mean between different groups were calculated using analysis of variance (ANOVA) plus Student's t-test. P values of less than 0.05 were recognized as significant.

3.Results and Discussion

3.1. Results

In order to evaluate the possible combination effects of forskolin/GSKJ4 in leukemia, we utilized U937 cell line as a representative model of human leukemia cells [36-37]. With regard to *in-vitro* experiments we treated U937 cells with 10 μM of forskolin consistent with previous findings and studies performed *in-vitro* on cancer cell lines, in which the range 1 to 100 μM represents the concentration used[37-39]. GSKJ4 shows antiproliferative effects in many cancer cells associated with low/no toxicity in normal cells[28-29, 33-34]. Taking into account the above research we decided to use 10 μM as a final concentration[40-41].

3.1.1. Forskolin enhances GSKJ4-induced antiproliferative effects in U937 leukemia cells

Initially, we explored whether GSKJ4 and forskolin could have a possible action in U937 cells. To achieve our aims, we treated U937 cells with 1 and 10 μM of GSKJ4, in the presence or absence of 10 μM forskolin, for 24 and 48 hours. The effects of these two agents have been evaluated through cell counting and cell death assays, as described in materials and methods section. **Figure 1A** shows that GSKJ4 inhibits U937 proliferation index in dose- and time-dependent manner. In details, 10 μM GSKJ4 induces a decrease of 15% at 24 hours and 35% at 48 hours in terms of cell growth, whereas 1 μM GSKJ4 has an antiproliferative effect only after 48 hours of treatment (15%). At the same time, the presence of forskolin clearly enhances the antiproliferative effects of GSKJ4 in all the conditions tested. Notably, 1 μM of GSKJ4 plus 10 μM of forskolin improves the cell growth inhibition from 15% to 35% after 48 hours of treatment. As expected, the 10 μM GSKJ4/forskolin combination results in a growth inhibition of more than 40% at 24 hours, respect to GSKJ4 alone (15%). Moreover, according to previous findings, forskolin does not affect U937 viability [35-36]. In order to confirm and extend these data, we also evaluated the effects of GSKJ4 and forskolin, alone

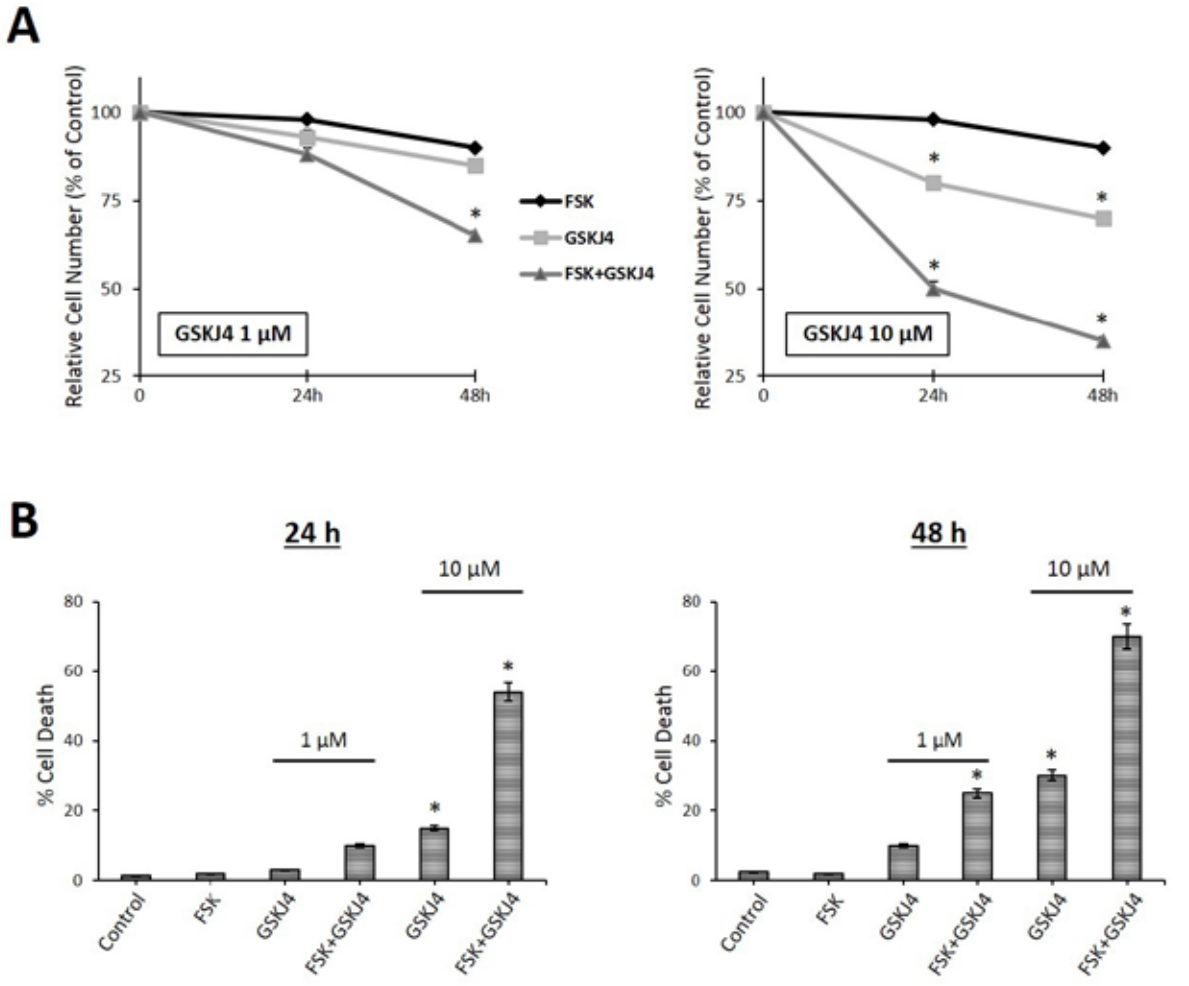

Figure 1. *Effects of forskolin on the sensitivity of U937 cells to GSKJ4. U937 cells were treated or not with 1 and 10 μM of GSKJ4, alone or in combination with 10 μM forskolin for 24 and 48 hours. Subsequently, the cell number was recorded (panel A) and PI cell death assay was performed (panel B). Data represent the average of four independent experiments. The means and S.D. are shown. *, P < 0.05, compared to control untreated cells.*

or in combination, in other leukemia models. Comparable data were obtained in NB4 leukemia cell line (data not shown). **Figure 1B** shows the quantification of cell death obtained in response to different experimental settings indicated in. According to the above cell counting data, comparing single agent treatment with GSKJ4/forskolin co-treatment, the combination clearly enhances the percentage of cell death at 48 hours, whereas this evidence is pure only in presence of 10 μM of GSKJ4 at 24 hours.

Altogether, the above data strongly suggest that forskolin increases the sensitivity of U937 cells to GSKJ4.

3.1.2. The induction of apoptosis is the means by which forskolin enhances the sensitivity of U937 to GSKJ4.

To further investigate the mechanism(s) by which for-

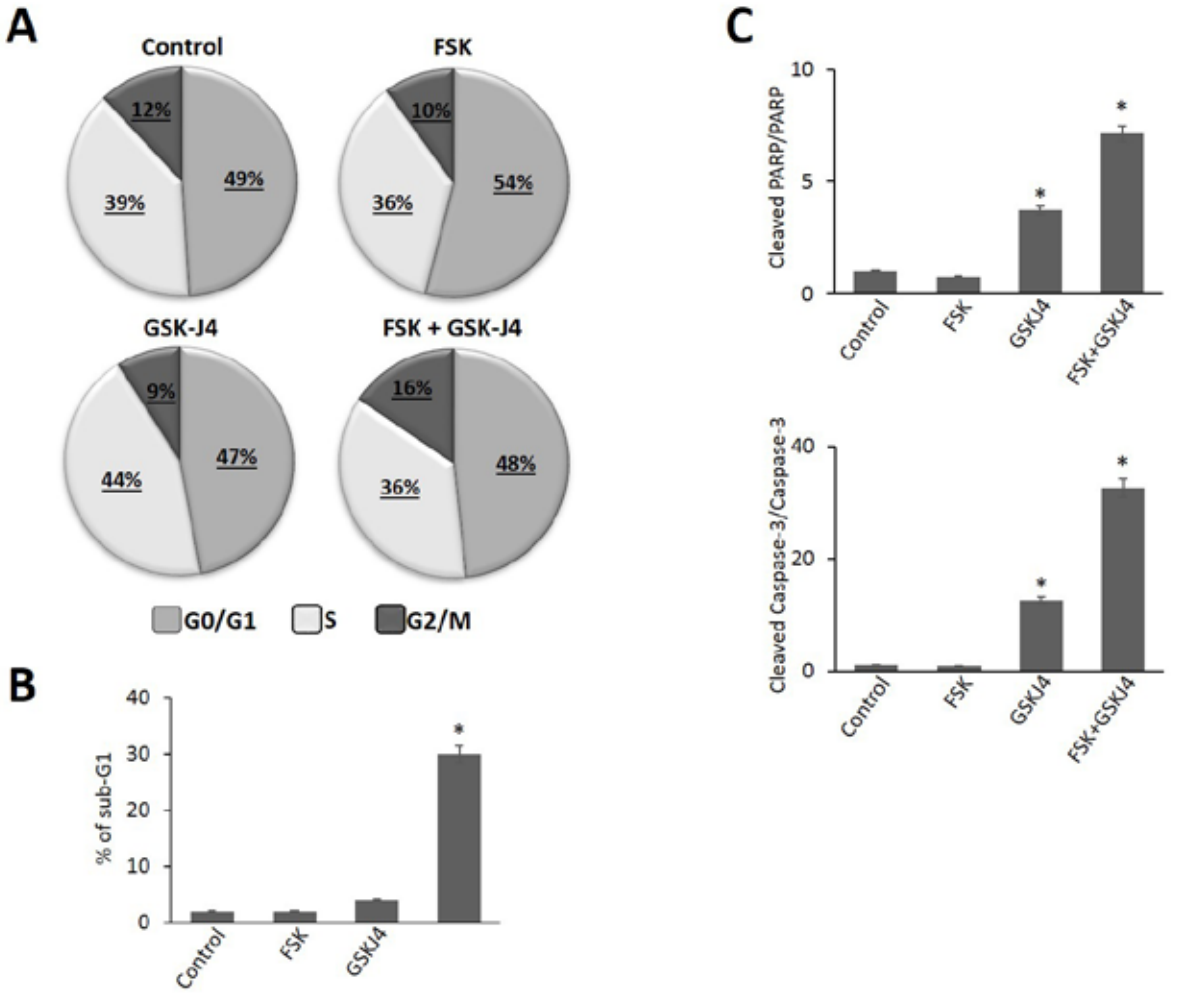

Figure 2. *Effects of forskolin, GSKJ4 and forskolin/GSKJ4 combination on cell cycle distribution, subG1 phase and apoptotic proteins in U937. Treatments with 10 μM forskolin, 10 μM GSKJ4, forskolin/GSKJ4 combination were carried out for 24 hours. Subsequently, sub-G1 and (Panel A and B) were evaluated by Flow Cytometry. In panel A, quantitative data from four independent experiments indicating the percentage of G1, S, and G2/M cells. In panel B is indicated the relative sub-G1 percentage. Panel C shows Cleaved Caspase-3/Caspase-3 Ratio and Cleaved PARP/PARP Ratio as outcome of the analysis of the relative protein levels assessed by western blotting. The means and S.D. are shown. *, P < 0.05, compared to control untreated cells.*

skolin improves the sensitivity of U937 to GSKJ4, cells were treated, alone or in combination, with 10 μM of GSKJ4 and 10 μM of forskolin for 24 hours and undergo to cell cycle investigation assessed by flow cytometric analysis of propidium iodide-stained cells (**Fig 2A and 2B**). The determination of the sub-G1 population, characteristic of cells having undergone DNA fragmentation, was also evaluated. **Figure 2A** shows no significative variations in cell cycle distribution in response to GSKJ4 and forskolin when used alone or in combination. Analyzing the percentage of proportion of cells with hypodiploid DNA content, we observed no

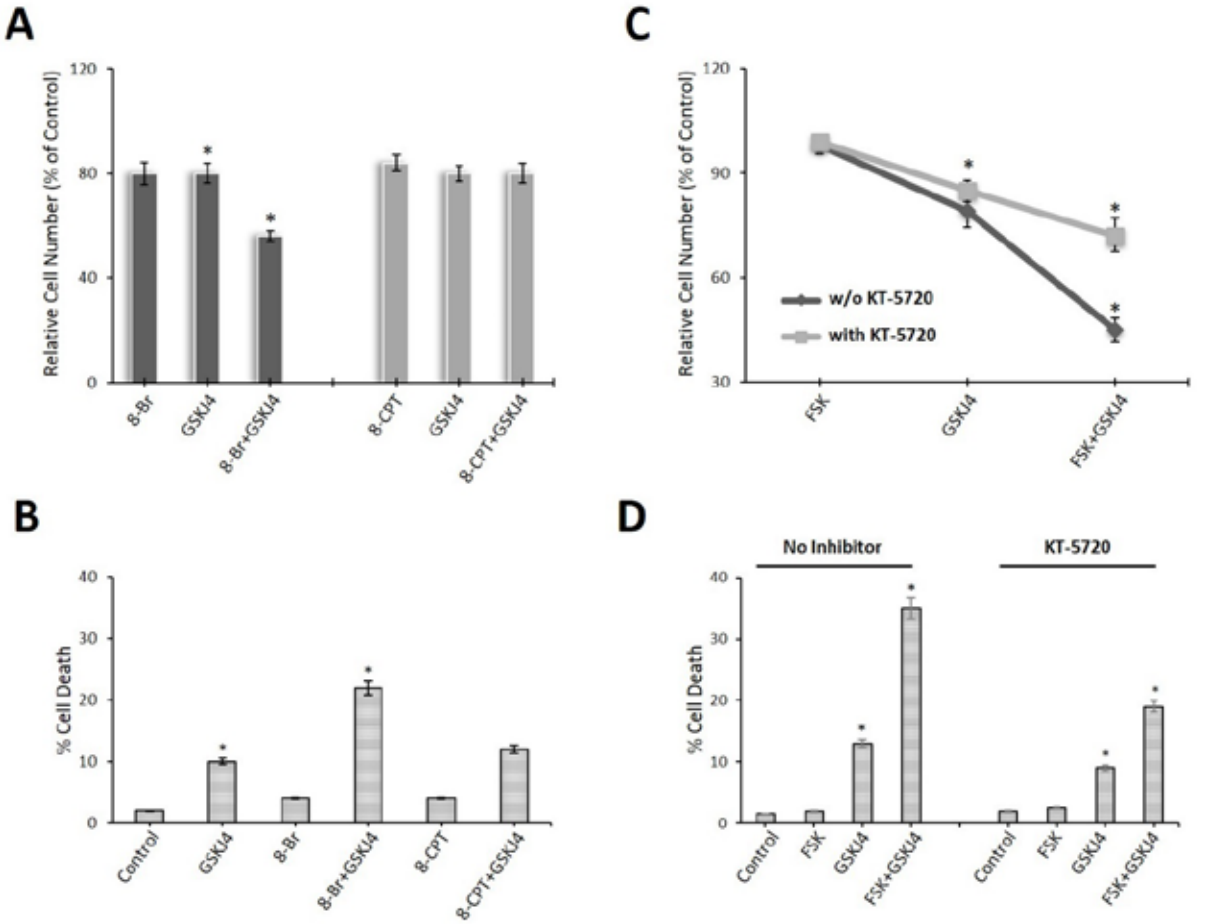

Figure 3. *Effects of 8-Br-cAMP and 8-pCPT-2'-O-Me-cAMP on GSKJ4-induced cytotoxicity. Evaluation of PKA inhibitor KT5720 on the proliferation of U937 cells in response to forskolin, GSKJ4, and forskolin/GSKJ4 combination. Panels A and B: U937 cells were treated or not for 24 hours with GSKJ4 10 μM in absence or presence of 25 μM 8-pCPT-Me-cAMP and 25 μM 8-Br-cAMP. Panels C and D: U937 cells were treated or not for 24 hours with 10 μM GSKJ4, 10 μM forskolin and forskolin/GSKJ4 combination in absence or presence of 10 μM PKA inhibitor KT5720. Subsequently, cell number was recorded (panels A and C) and PI cell death assay was performed (panels B and D). Data represent the average of three independent experiments. The means and S.D. are shown. *, P < 0.05, compared to control untreated cells.*

sub-G1 population in response to forskolin, whereas a little percentage (5%) of sub-G1 is evident upon treatment with GSKJ4 demethylase inhibitor. Interestingly, a huge amount of sub-G1 cells was recognized after forskolin/GSKJ4 co-treatment (**Fig 2B**). As well described in literature, generally, the presence of sub-G1 can be regarded as a biochemical hallmark of apoptosis[42-43]. To prove that the increase of sub-G1 is associated with an increase of the apoptotic process, we assessed the levels of the proteins relevantly involved in apoptosis. To this purpose, U937 cells were exposed for 24 hours to forskolin, GSKJ4 and forskolin/GSKJ4 combination. Thereafter, cell extracts were analyzed by western blotting to evaluate the protein levels of caspase 3 and PARP. **Figure 3C**, according to sub-G1 appearance, displays an increase of cleaved caspase-3/caspase-3

ratio in response to GSKJ4 and forskolin/GSKJ4 combination, as a consequence of the activation of the terminal caspase-3 and a contemporary decrease of the uncleaved isoform. Even PARP pattern proceeds in the same way of caspase-3, suggesting the increase of its activity.

Taken together, these data indicate that the induction of apoptosis is the means by which forskolin enhances the sensitivity of U937 to GSKJ4.

3.1.3. Forskolin potentiates the effects of GSKJ4 in U937 cells through cAMP/PKA involvement.

It is known that forskolin is a cAMP elevating compound as a consequence of its direct interaction with adenylate cyclase enzyme. However, other cellular activities can be affected by forskolin[44-45]. In order to understand whether the phenomena forskolin-mediated in presence of GSKJ4 could be attributed to the cAMP increase, we evaluated the effects of others cAMP elevating agents on the GSKJ4-induced cytotoxicity. To investigate our hypothesis, we treated for 24 hours U937 cells with GSKJ4 10 μM in the absence or presence of 8-Br-cAMP, a common analogue of cAMP, and 8-pCPT-2'-O-Me-cAMP, a cAMP analogue that specifically activates Epac and not PKA[46-47]. As shown in **Figure 3A**, 8-Br-cAMP, which is expected to activate both PKA and Epac, potentiated the GSKJ4-induced cytotoxicity, while, in contrast, the Epac activator 8-pCPT-2'-O-Me-cAMP had only a minimal impact on GSKJ4 effect. Analog data have been obtained analyzing the percentage of death cells in response to our treatments (**Fig 3B**). Overall, the above data indicate that forskolin potentiates the sensitivity of U937 cells to GSKJ4 via cAMP elevation and that, very likely, PKA might be involved in.

To further explore the role of PKA in the forskolin-mediated enhancement in response to GSKJ4 antiproliferative effects in U937 cells, we used a cell-permeable, selective and potent PKA inhibitor (KT5720)[48-49]. We exposed U937 cells to forskolin, GSKJ4 and forskolin/GSKJ4 combination, in presence or not of KT5720 (**Fig 3C**). We found that in presence of KT5720 inhibitor, forskolin was not able to enhance the GSKJ4-induced cytotoxicity (**Fig 3C and D**). Similar results were obtained using H-89, another PKA inhibitor (data not shown).

Overall, these data suggest that cAMP mediates forskolin-induced enhancement in U937 leukemia cell line to GSKJ4 and that PKA pathway is strongly involved in.

3.2. Discussion

According to the previous findings, we confirm the evidence about GSKJ4 as a small molecule inhibitor of the proliferation in myeloid leukemia cells[50]. Moreover, the mechanisms by which GSKJ4 works as an anticancer drug remain unclear. A possible explanation is that the up-regulation of the global level of H3K27me3, as a direct consequence of GSKJ4 treatment, might silence transcription factors involved in leukemia, such as CREB and other oncogenes[51-52]. For this reason, understanding how GSKJ4 inhibits leukemia cell proliferation represents an important issue that needs to be addressed. In addition, although previous studies have demonstrated that cyclic AMP elevation in leukemia cells confers drug resistance and protects cells against DNA damaging agents-induced apoptosis, through PKA-mediated mechanism, here we describe that cAMP elevating agent forskolin causes cell death induction when used in combination with GSKJ4 inhibitor in U937 leukemia cells, even via PKA[37, 53]. Depending on the cell type and nature of death-inducing signal, controversial effects of cAMP concerning cell death and potentiation of chemotherapeutic drugs have been reported in literature[17, 19, 37, 53-54]. However, molecular mechanisms underlying the increase of sensitivity induced by cAMP/PKA/forskolin to GSKJ4 in U937 are initially discuss in this present article and speaking of which, further and more exhaustive studies are needed. At this regard, we cannot finally be ruled out other cAMP dependent mechanisms.

4. Conclusion

In conclusion, our findings provide initial evidence of anticancer activity induced by forskolin/GSKJ4 combination in vitro in leukemia cells and underline the potential use of forskolin and GSKJ4 in the development of innovative and effective therapeutic approaches for AML treatment. □

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ΕΚΔΗΛΩΣΕΙΣ - MEETINGS

• 15-17 MAY 2019, AMSTERDAM, NETHERLANDS

2nd EUROPEAN CHEMISTRY CONFERENCE , ECC-2019
http://europeanchemistry.madridge.com

• 10-13 JUNE 2019, KRAKOW, POLAND

EFMC-ACSMEDI: Medicinal Chemistry Frontiers 2019

• 16-20 JUNE 2019 MILAN, ITALY

48TH International Symposium on High-Performance Liquid Phase Separations and Related Techniques
https://www.hplc2019-milan.org/

• 23-27 JUNE 2019, IOANNINA, GREECE

CMTPI-2019: 10th International Symposium on Computational Methods in Toxicology
and Pharmacology Integrating Internet Resources http://cmtpi.net/

• 27-30 JUNE 2019, PRAGUE, CZECH REPUBLIC.

11th Joint Meeting on Medicinal Chemistry 2019, https://www.jmmc2019.cz/

• 1-5 SEPTEMBER 2019, ATHENS, GREECE

EFMC-ASMC'19 - EFMC International Symposium on Advances in Synthetic and Medicinal Chemistry
http://www.rsc.org/events/detail/35235/efmc-asmc19-efmc-international-symposium-on-advancesin-synthetic-and-medicinal-chemistry

• 08-11 SEPTEMBER 2019, ISTANBUL, TURKEY

12th AFMC / AIMECS 2019
Asian Federation for Medicinal Chemistry (AFMC) 12th International Symposium
“New Avenues for Design and Development of Translational Medicine”
http://www.aimecs2019.org/

• 8-11 SEPTEMBER 2019, PESCARA, ITALY

Recent Developments in Pharmaceutical Analysis, RDPA2019
https://rdpa2019.wixsite.com/rdpa2019

• 22- 25 SEPTEMBER 2019, IOANNINA, GREECE

IMA-2019: 11th International Conference on Instrumental Methods of Analysis
http://www.speciation.net/Events/IMA2019-11th-International-Conference-on-Instrumental-Methods-of-Analysis-;~/2018/12/11/8941.html

• 18-19 NOVEMBER 2019, PARIS, FRANCE

31st International Conference and Expo on Nanosciences and Nanotechnology
https://nanotechnology.conferenceseries.com/