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SYNTHESIS OF THE ENSEMBLES FROM SUCCINYLATED INTERLEUKIN-2 DERIVATIVES AND THEIR BIOLOGICAL ACTIVITY IN VITRO

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Purpose: *The biological activity (direct anticancer action in vitro) of combinatorial IL-2 succinylated derivatives was examined. Acylation of IL-2 was performed using succinic anhydride with various levels of acylation, with the formation of a complex assembly of many derivatives (self-assembled quasi-living structure).*
Methods: *In the study, we used recombinant interleukin-2 (IL-2) (Ronkoleukin, Russia) in oxidizing form and other reagents from Sigma-Aldrich and Fluka (USA). For acylation, recombinant IL-2 in the form of a matrix fluid with a protein concentration of 0.6 mg/ml was used. The IL-2 matrix solution was brought to a pH of 8.0 through the addition of a 0.01 % solution of sodium hydroxide. The synthesized ensemble of succinyl-IL-2 was analyzed using the capillary gel electrophoresis method in an Agilent-2100 bioanalyzer. The molecular masses of the synthesized ensemble were established in comparison to standard samples of low-molecular proteins with known molecular masses that were part of the bioanalyzer's collection. The additional negative charge of the modified IL-2 was determined by a FPLC (Fast Protein Liquid Chromatography) system from Pharmacia (Woerden, Netherlands). CTLL-2, a murine IL-2-dependent cell line, was obtained from Biokontrol (Kiev, Ukraine). These responding cells were used in proliferative assays comparing the activity of the IL-2 and succinylated IL-2 derivatives to the WHO International Standard. For statistical data analysis, the one-way ANOVA was used.*
Results: *After treatment with Suc-IL-2, BTL were studied in vitro on the CTLL-2 cell line, dose-dependently, in a BTL induction reaction. The highest level of biological activity in acylated IL-2 was observed in Suc-IL-2 with four modified lysine residues. In conclusion, succinylated IL-2 with four substituted lysines may be useful as a prospective anticancer agent.*
Conclusions: *Ten-fold increase in the biological activity of IL-2 was observed after partial succinylation. This phenomenon can be used in the further IL-2 drugs biotechnological development for increasing the concentration of the main active substance in medicinal form.*
Keywords: *Agilent-2100, bioactivity, succinylated interleukin-2, high-performance liquid chromatography, recombinant interleukin-2.*

Мета: *дослідити біологічну активність комбінаторних сукцинильованих похідних інтерлейкіну-2 (ІЛ-2). Ацилювання з різними ступенями ацилювання ІЛ-2 проводили з використанням бурштинового ангідриду, з утворенням складного ансамблю із багатьох похідних (квазі-жива структура, здатна до самоорганізації).*
Методи*: У дослідженні використовували рекомбінантний інтерлейкін-2 (ІЛ-2) (Ronkoleukin, Росія) в окисненій формі та інші реагенти Sigma-Aldrich і Fluka (США). Для ацилювання використовували рекомбінантний ІЛ-2 у вигляді матричної рідини з концентрацією білка 0,6 мг/мл. Матричний розчин ІЛ-2 був доведений до pH 8,0 шляхом додавання 0,01 % розчину гідроксиду натрію. Синтезований ансамбль сукциніл-ІЛ-2 аналізували за допомогою капілярного гель-електрофорезу на приладі Agilent- 2100 Bioanalyzer. Молекулярні маси синтезованого ансамблю аналізували у порівнянні зі стандартними зразками низькомолекулярних білків Bioanalyzer (Ladder). Додатковий негативний заряд модифікованого ІЛ-2 визначали за допомогою FPLC (швидкої рідинної хроматографії протеїнів) системи від Pharmacia (Woerden, Нідерланди). CTLL-2, мишача ІЛ-2-залежна клітинна лінія, була отримана з Biokontrol (Київ, Україна). Ці клітини були використані в аналізі рівню проліферації (реакція бластної трансформації або БТЛ) в порівнянні активності ІЛ-2 і сукцинильованих похідних ІЛ-2. Для статистичного аналізу даних був використаний дисперсійний аналіз.*
Результати*: клітинна лінія CTLL-2 була оброблена різними концентраціями похідних Suc-ІЛ-2. Найвищий рівень біологічної активності мав Suc-ІЛ-2 з чотирма модифікованих залишків лізину. Таким чином, сукцинильоване похідне ІЛ-2 з чотирма заміщеними залишками лізину може бути використане в розробці майбутніх імунотропних протиракових засобів.*
Висновки*: Десятикратне збільшення біологічної активності ІЛ-2 спостерігалося після часткового сукцинилювання його структури. Це явище можна використовувати в розробці нових біотехнологічних препаратів на основі похідних ІЛ-2 для зменшення концентрації основної активної речовини в лікарській формі при тій же біологічній активності та для розширення спектру активности інтерлейкіну-2*
Ключові слова*: Agilent-2100, біологічна активність, сукцинильований інтерлейкін-2, високоефективної рідинної хроматографії, рекомбінантний інтерлейкін-2*

1. Introduction

The biological activity (direct anticancer action in vitro) of combinatorial IL-2 succinylated derivatives was examined. Acylation of IL-2 was performed using succinic anhydride with various levels of acylation, with the formation of a complex assembly of many derivatives (self-assembled quasi-living structure).

2. Formulation of the problem in a general way, the relevance of the theme and its connection with important scientific and practical issues

Unlike antimicrobial drugs, there are almost no antiviral drugs on the pharmaceutical market. This is notwithstanding the fact that more than 90 % of animal pathology is connected with viruses. In many cases, antiviral drugs have many side effects that are very difficult to correct (e. g., ribavarin with hepatitis C). Viruses are able to very quickly adapt to drugs that have long been used in the population and to newly created drugs (for example, Acyclovir). We have addressed these problems through substituting the classical, precisely conservative structure of antiviral drugs for a dynamic, self-organizing system made of peptide molecules that are similar to but ultimately different from each other. The synthesized peptides have the properties of molecular robots; that is, they aggregate into a biologically active supramolecular complex when they have reached the target. A multi-surplus quantity of fragments is introduced into the organism.

3. Analysis of recent studies and publications in which a solution of the problem and which draws on the author

For example, recombinant interferons and interleukins have long been successfully applied in medicine and pharmaceutics [1, 2]. The effect of prolonging interferon action of using chemical modification through pegylation has long been known [3]. Earlier, it was shown that the partial chemical modification of an interferon results in an increase in its antiproliferative activity [4]. The basic parameters of quality of the succinylated protein analysis are molecular weight and a change in charges [5].

4. Allocation of unsolved parts of the general problem, which is dedicated to the article

IL-2 [6, 7] is a 133 amino acid protein that is used clinically for cancer immunotherapy [8, 9]. The initial clinical studies of IL-2 evaluated natural and recombinant preparations [10]. When these studies were initiated, there was not a uniform standard for calibrating the various IL-2 preparations. Each preparation was calibrated against an "in house" standard [11], and individual companies defined their own units of IL-2. The International Standard for IL-2 was established in 1988 [6]. This standard is available in lyophilized form, 100 IU/vial, to calibrate and standardize other various IL-2 preparations. An IU is defined as the amount of IL-2 that induces 50 % of the maximal proliferation of an established IL-2-dependent cell line.

5. Formulation of goals (tasks) of Article

The purpose of the study was to synthesize a series of IL-2 succinylated derivatives with different degrees of succinylation. The change in its structure correlates to the changes in molecular weight, charge, and ability to induce lymphocyte proliferation (dose-dependent).

6. Statement of the basic material of the study (methods and objects) with the justification of the results

In the study, we used recombinant interleukin-2 (IL-2) in oxidizing form, succinic anhydride (Fluka, USA), and other reagents from Sigma-Aldrich (USA).

The synthesis and purification of IL-2 derivatives with different degrees of succinylation were carried out in accordance with [4].

For acylation, recombinant IL-2 (Ronkoleukin, Russia) in the form of a matrix fluid with a protein concentration of 0.6 mg/ml was used. The IL-2 matrix solution was brought to a pH of 8.0 through the addition of a 0.01 % solution of sodium hydroxide. Succinic anhydride from Fluka was used as an acylating agent. Acylation was conducted at room temperature under aseptic conditions through adding a solution of succinic anhydride (SA) in dioxane in the corresponding ratio (Table 1). The solution was incubated for 30 minutes, diluted with water until a theoretical titer of 3 million IU in 1 ml was reached (in 1 ml of matrix solution with a protein concentration of 0.60 mg/ml, 180000000 IU of interferon); it was poured into ampoules and lyophilized in order to remove the dioxane from the drug.

The synthesized ensemble of succinyl-IL-2 was analyzed using the capillary gel electrophoresis method [12] in an Agilent-2100 bioanalyzer. The molecular masses of the synthesized ensemble were established in comparison to standard samples of low-molecular proteins with known molecular masses that were part of the bioanalyzer's collection.

The additional negative charge of the modified IL-2 was determined by a FPLC (Fast Protein Liquid Chromatography) system from Pharmacia (Woerden, Netherlands), using a mono-q anion exchange column from Pharmacia. Buffer A was a 0.02 M TrisHCl buffer (ph=7.4) and buffer B consisted of buffer A plus 1 M NaCl. Elution was carried out at a rate of 0.25 ml/min, using a gradient of 100 % A to 100 % B in 30 minutes. The samples of the substance to be investigated were dissolved in the amount of 1 mg/ml in buffer A, and 100 μl were injected into the FPLS system. The Millichrom-A02 chromatograph was also used as equipment for this study.

CTLL-2, a murine IL-2-dependent cell line, was obtained from Biocontrol (Kiev, Ukraine). These responding cells were used in proliferative assays comparing the activity of the IL-2 and succinylated IL-2 derivatives to the WHO International Standard. This standard is the WHO 1st International Standard for IL-2 (human) 86/504, obtained from the BRMP of the NCI[6]. The CTLL-2 cells were cultured at 8×10^3/well with dilutions of IL-2-derivatives or IL-2 standard for 20 h. All proliferative assays were cultured at 37 ℃ with 5 % CO_2. The Packard Filtermate 196 was used to harvest the cultures. The EC_{50}, the effective concentration necessary to induce 50 % of the maximal proliferation, was calculated using MS Excel and then converted to IAU/mg, international activity units per 1 mg of protein.

For statistical data analysis, the one-way ANOVA was used [13].

Structure [14] of the IL-2 dimer obtained by X-Ray analysis is presented in Fig. 1.

IL-2 is an acidic protein (with a molecular weight of 15300 Da), containing 7 lysines and 2 histidines. Seven lysines and one histidine are accessible for acylation. The second histidine is inside the molecule and is not accessible for acylation. In the structure, there are also 3 aspartic and 12 glutamic acids, which cause an excessive negative charge of –4 on the native molecule.

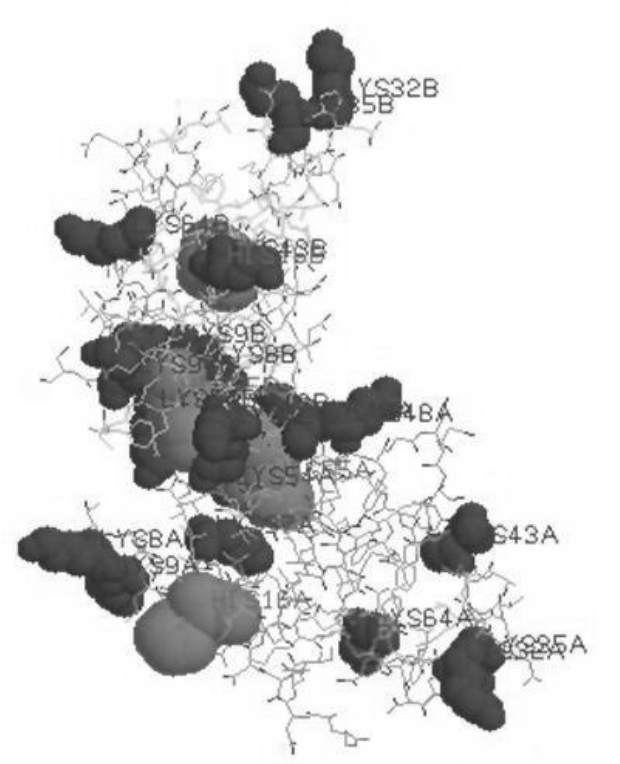

Fig. 1. IL-2 dimer structure obtained by means of x-ray analysis, PDB ID: 1M4C [14]

Thus, it is possible to synthesize eight succinylated derivatives of IL-2. All the derivatives are combinatorial IL-2 derivatives; that is, they are mixtures of IL-2 derivatives with various amino group substitution levels, from 0 to 8. For further studies, a protein monomer was used with a molecular weight of 15300 Da (determined by HPLC). The medicinal form also contains a lighter protein with a molecular weight of 13000 Da. Only the fraction with a molecular weight of 15300 Da was used in the studies.

The results are presented in Table 1 and Fig. 2 and 3.

Fig. 2 shows an electrophoretogram of the division of the initial IL-2 with the establishment of the components' molecular mass, obtained using an Agilent-2100 bioanalyzer. In capillary electrophoresis, a division occurs of the denatured and reconstituted form of the protein; division does not depend on the protein's tertiary structure. Accordingly, the initial IL-2 consists of two isomers with similar molecular masses; it is 97 % of the basic substance. The dual-band data is characteristic of all the medicinal forms of IL-2 and matrix solutions; thus the protein used for modification is in compliance with the requirements for pure interleukine-2.

As may be seen in Table 1, the difference in the molecular masses between the initial, unmodified IL-2 and the derivatives with one and two substituted groups is significantly insignificant, while the change to their charges is significant ($P \leq 0.05$). For derivatives with three to five substituted groups, the differences in both molecular masses and charges were significant and indicated that the acylation reaction had occurred and been completed. A similar picture is also seen for derivatives with six to eight substituted groups: the change in charge and molecular masses is significant. Accordingly, a conclusion may be drawn on the conclusion of the acylation reaction by the change to the molecular masses in all cases except with derivatives with one and two substituted groups. The insignificance of the changes in those two cases is caused by the insignificant changes in the molecular masses. At the same time, the change to the charges of the molecules in absolutely all of the derivatives are significant, which bears witness to the completion of the reaction.

The biological activity of IL-2 is represented in international activity units per 1 mg of protein (IAU/mg). Nine combinatory IL-2 derivatives were studied, of which one derivative was not initially modified (acylation index=0). The activity of each sample was verified seven times (Fig. 4).

Table 1

Reagent Ratio and Properties of Succinylated IL-2 Derivatives

Molar Ratio of Reagents, Succinic Anhydride: IL-2	Molecular Weight, Da		Molecular Charge	
	Calculated	Determined	Calculated	Determined
1:1	15402±100	15400±300	5±1	5±1**
2:1	15504±100	15500±310	6±1	5±1**
3:1	15606±100	15600±310**	7±1	8±2*
4:1	15708±100	15700±330**	8±1	8±2*
5:1	15810±100	15800±330**	9±1	10±2*
6:1	15912±100	15900±330*	10±1	10±2*
7:1	16014±100	16000±360*	11±1	11±2*
8:1	16116±100	16100±360*	12±1	11±2*
0:1 (control)	15300±100	15200±200	4±1	3±1

*Note: * – $P \leq 0.01$; ** – $P \leq 0.05$; n=6 (for molecular weight detection); n=7 (for charge detection)*

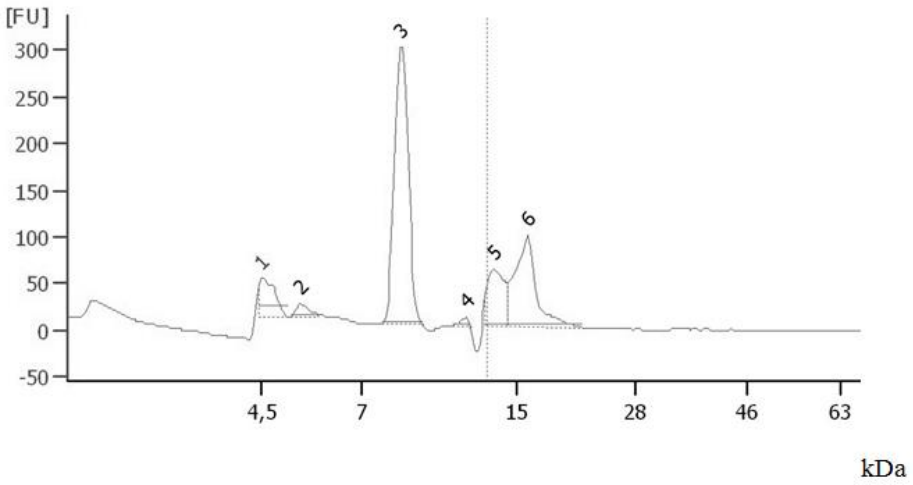

Fig. 2. Chromatogram of the separation of mixtures of succinylated IL-2 proteins into their components with the use of capillary gel electrophoresis in an Agilent-2100 biolanalyzer (Protein-80 chip) and the separation of the acylated ensemble with two substituted groups (X axis: molecular mass of the proteins in kDa)

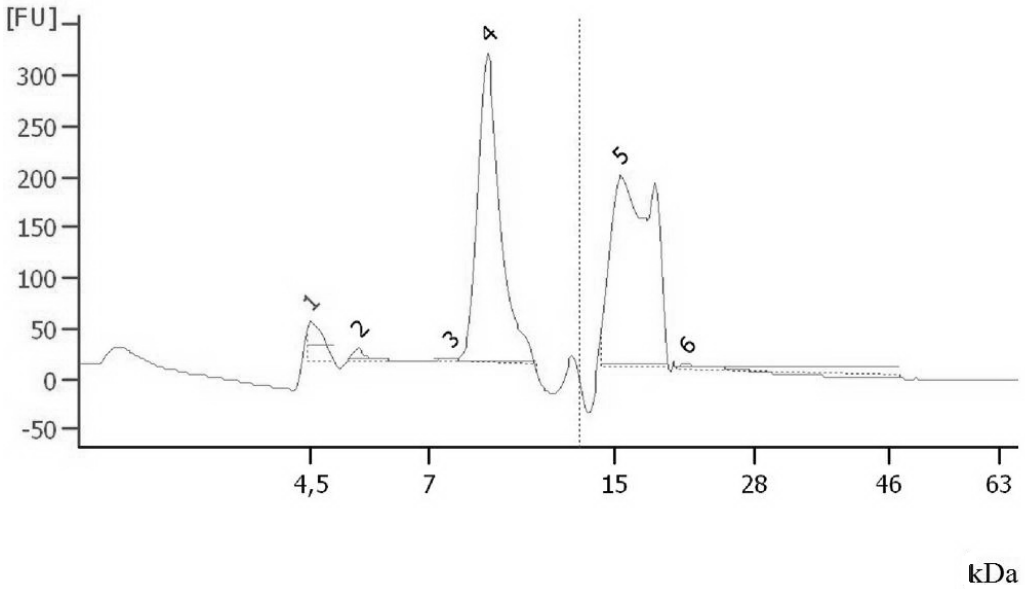

Fig. 3. Chromatogram of the separation of mixtures of succinylated IL-2 proteins into their components with the use of capillary gel electrophoresis in an Agilent-2100 biolanalyzer (Protein-80 chip) and the separation of the acylated ensemble with two substituted groups (X axis: molecular mass of the proteins in kDa)

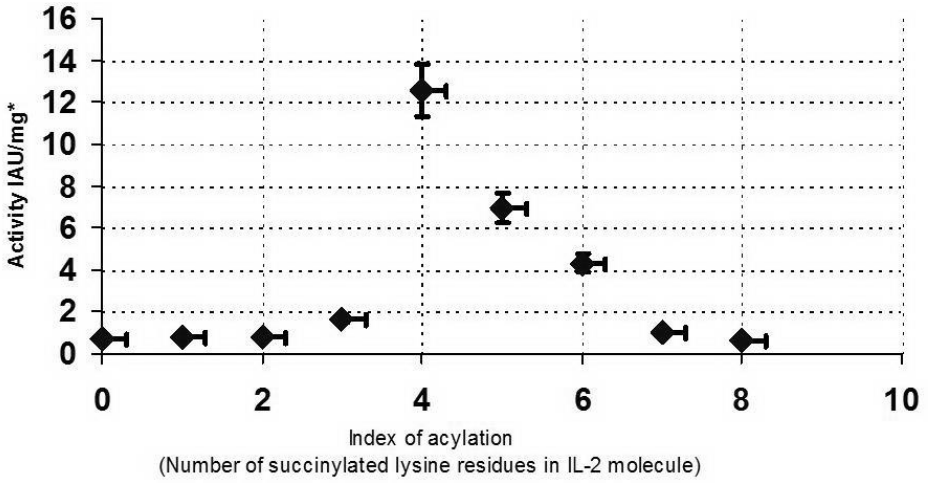

Fig. 4. Dependence of biological activity on the Suc-IL-2 acylation index:
* IAU/mg - international activity unit in 1 mg of protein, ($P \leq 0.01$)

A hypothesis on differences between the samples was checked using the one-way ANOVA method, in which the comparison group was the activity of the non-modified derivative. For all of the groups studied, the differences in the activity level from the non-modified derivative were statistically significant ($P \leq 0.01$).

The Suc-IL-2 derivative with 4 substituted lysines had the greatest activity. The activity of the IL-2 with 4 substituted lysines is more than ten times higher than the nonacylated derivative.

7. Findings from the research and prospects of further development of this area

IL-2 activity decreases gradually, not sharply, with an increase in the degree of molecular acylation. This fact indicates that high molecular acidity is not a necessary requirement for biological activity, and that even a completely substituted derivative displays biological activity similar to that of native IL-2.

In conclusion, a ten-fold increase in the biological activity of IL-2 was observed after its partial succinylation. This phenomenon can be used in the further biotechnological development of IL-2 drugs for increasing the concentration of the main active substance in medicinal form.

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АНАЛІЗ ФАРМАЦЕВТИЧНОГО РИНКУ НООТРОПНИХ ЗАСОБІВ В УКРАЇНІ

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Захворювання нервової системи посідають одне з провідних місць у структурі захворюваності та смертності в усьому світі. За даними ВООЗ, близько 30 % населення регулярно приймають нейротропні засоби. Для осіб літнього віку, чия питома частка в сучасному суспільстві неухильно зростає, цей показник складає близька 50 %. Проте слід відзначити і збільшення частоти захворюваності нервової системи і у осіб молодого віку. Дані факти свідчать про актуальність та затребуваність лікарських засобів даної групи, зокрема, ноотропних препаратів, які найчастіше застосовуються при лікуванні нервових захворювань.

Ціль. *Метою даної роботи було проведення аналітичного огляду фармацевтичного ринку ноотропних засобів в Україні.*

Методи. *Статистичні і маркетингові методи досліджень електронних і паперових джерел інформації. Об'єкт дослідження – інформація про зареєстровані в Україні ноотропні лікарські засоби.*

Результати. *Встановлено, що вітчизняні фармацевтичні препарати займають 57 % ринку ноотропних засобів. В Україні представлені 16 країн-виробників ноотропних препаратів. Дослідження ринку ноотропних препаратів показало, що вони представлені в різних лікарських формах (таблетки, капсули, сиропи, пілюлі, суспензії, розчини для ін'єкцій, розчини для інфузій, розчини для перорального застосування, порошок дозований у пакетах), серед яких переважають таблетки.*

Висновки. *Ноотропні препарати синтетичного походження переважають і займають 87 %, частка рослинних засобів – 13 %, які характеризуються одноманітністю складу і представлені лише препаратами гінкго білоба. Результати щодо співвідношення форм випуску засвідчують, що рослинні препарати ноотропної дії найбільше представленні у формі таблеток 67 %*

Ключові слова: *аналіз, фармацевтичний ринок, Україна, ноотропні препарати, рослинні засоби, номенклатура препаратів*