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(71) Demandeur/Applicant:
ZAKRYTOE AKTSIONERNOE OBSCHESTVO "RUSSKIE BIOTECHNOLOGII", RU

(72) Inventeurs/Inventors:
SEVERIN, EVGENIY SERGEEVICH, RU;
SEVERIN, SERGEY EVGENYEVICH, RU;
LUTSENKO, SERGEY VIKTOROVICH, RU

(74) Agent: OGILVY RENAULT

(54) Titre : POLYPEPTIDE, SON CONJUGUE AVEC DOXORUBICINE ET COMPOSITION PHARMACEUTIQUE SUR CETTE BASE

(54) Title: POLYPEPTIDE, THE CONJUGATE THEREOF CONTAINING DOXORUBICINE AND A PHARMACEUTICAL COMPOSITION BASED THEREON

(57) Abrégé/Abstract:
The inventive polypeptide is embodied in the form of a similar fragment of the epidermal growth factor of aminoacid ranging from 21 to 31 and capable of efficiently binding with the receptor of the epidermal growth factor as a vector for the directed supply of anticancer agents to tumour cells. The inventive conjugate of said polypeptide contains doxorubicine and has a selective action on cancer tumours and can substantially reduce the resistance of the tumour cells with respect to the anticancer agents, the conjugated parts of said agents being binded with the aid of a chemical bond which is unstable in relation to acid hydrolysis. The inventive pharmaceutical composition comprises an efficient quantity of conjugate and a carrier which is fit for intravenous injection. Said invention can be used for medicine for treating cancer patients.

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ABSTRACT

The inventive polypeptide is embodied in the form of a similar fragment of the epidermal growth factor of aminoacid ranging from 21 to 31 and capable of efficiently binding with the receptor of the epidermal growth factor as a vector for the directed supply of anticancer agent to tumour cells. The inventive conjugate of said polypeptide contains doxorubicine and has a selective action on cancer tumours and can substantially reduce the resistance of tumour cells with respect to the anticancer agents, the conjugated parts of said agents being binded with the aid of a chemical bond which is unstable in relation to acid hydrolysis. The inventive pharmaceutical composition comprises an efficient quantity of conjugate and a carrier which is fit for intravenous injection. Said invention can be used for medicine for treating cancer patients.

POLYPEPTIDE, ITS CONJUGATE WITH DOXORUBICIN AND CONJUGATE-BASED PHARMACEUTICAL COMPOSITION

FIELD OF ENGINEERING

The invention is related to the chemistry of natural compounds, pharmacology and medicine and can be used in medicine for the treatment of cancer patients.

TECHNICAL ANCESTORS

The primary and acquired resistance of cancer cells to and low selectivity of anticancer drugs are among major factors which limit the efficacy of anticancer chemotherapy. Selectivity of these drugs can be increased via their targeted delivery directly to cancer cells by using various protein vectors as carrier. In this case, drugs penetrate in the targeted cells through a mechanism of receptor-mediated endocytosis of a conjugate in which a vector protein is covalently linked with a drug. Selectivity of the above conjugates is achieved through either the presence of specific receptors in the walls of cancer cells which are "recognizable" by the vector protein or antibody or due to a much higher level of receptors of the vector protein in the walls of cancer as compared to non-transformed cells. A presumed efficacy of the epidermal growth factor (EGF) as vector molecule is derived from its high stability, availability as a recombinant protein and a higher level of EGF-receptors in the walls of cancer cells as compared to healthy cells.

Numerous studies and patents are known which describe different conjugates of the EGF with doxorubicin (DR) and other anticancer drugs, methods of their manufacturing and pharmaceutical compositions on their basis [U.S. Patents Nos. 5824805, 5349066, 5087616, 5393737, 5505931, 5622929, 5122368, 5776458 etc.].

Doxorubicin is a known anticancer antibiotic drug which, although widely used in clinical practice, has a number of practical drawbacks such as high cardiotoxicity and induction of quick development of resistance of cancer cells following repeated intravenous injections of the drug. The use of DR in a conjugated form could result in a considerable improvement of its therapeutic qualities.

Short-chain EGF peptide fragments are of particular interest as vector molecules to be used for the targeted delivery of DR and other anticancer drugs to cancer cells. As the method of their manufacture is relatively simple, economical and does not require using any starting materials of biological origin, conjugates derived from them are easier to standardize.

Mixtures (pharmaceutical compositions) of DR (and other anticancer drugs) with different EGF peptide fragments are known to be used in the chemotherapy of cancer [U.S. Patent No.4863902]. The authors explain an enhanced anticancer efficacy of the patented drugs by a higher sensitivity of cancer cells to DR which is due to a proliferative effect produced by the EGF and its active fragments. However, as the link between DR and EGF and its fragments in the conjugate is not covalent, the targeted delivery of the drug to cancer cells cannot be ensured, leading to an increase of the drug nonspecific toxicity.

Also known to be used in the treatment of cancer is a biological active cyclic polypeptide having an amino acid sequence similar to that of an EGF fragment from 32 to 48 amino acid [U.S. Patent No.5183805]. The patent says that the peptide fragment can be conjugated with chemical agents such as DR, but neither conjugates nor methods of their manufacture nor their biological qualities are claimed in the patent.

Also known are a polypetide which is an EGF fragment from 21 to 31 amino acid, of the following formula:

$$H_2N\text{-MYIEALDKYAC-COOH} \qquad (I),$$

capable of effectively binding to EGF-receptors and causing cellular proliferation, and its conjugate with DR in which the DR-to-polypeptide ratio is 1:1 and the conjugated moieties are linked together with an acidlabile azomethin linkage as a result of using glutaric aldehyde as cross-linking agent. [*Russian Oncological Journal*, No.3, 2001, pp.33-37, The Enhancement of Anticancer Activity of Doxorubicin by its Targeted Delivery to Cancer Cells via Protein Vectors]. As pharmaceutical compositions, any compositions may be indicated, described in U.S. Patents Nos.5349066, 5087616, 5122368, which contain DR-EGF conjugates at effective concentrations and a pharmaceutical carrier suitable for intravenous administration.

DISCLOSURE OF THE INVENTION

The goal of the invention is to widen a choice of agents for the purpose described and to enhance their efficacy.

The goal is accomplished by that a new synthetic polypeptide similar to a fragment of the epidermal growth factor from 21 to 31 amino acid is proposed, which is capable of effectively binding to receptors of the epidermal growth factor, causing cellular proliferation and acting as vector for the targeted delivery of anticancer drugs to cancer cells. The polypeptide has the following formula:

H_2N-MYIEALDSYAC-COOH (II)

Unlike polypeptide (I), the proposed polypeptide has an amino acid Lys in the active center of the receptor-binding fragment of human EGF replaced with Ser which is one of the amino acids forming a binding site in murine EGF. It was established accidentally that replacement of Lys with Ser precludes undesirable conjugation at Lysine ε-amino groups in the active center of the receptor-binding fragment which, in turn, prevents the inhibition of the binding of the proposed polypetide to an EGF-receptor.

Although the efficacy of the proposed polypeptide is demonstrated in a form of a conjugate with a known antibiotic doxorubicin, other anticancer antibiotics such as vincristine, cisplatin, daunomycin, methotrexate and other anticancer agents such as toxins (such as diphtheria or ricin), cyclophosphamide and other medications, also can be used.

Also, a conjugate of the above polypeptide with doxorubicin is proposed, in which doxorubicin is covalently linked with polypeptide through an acidlabile azomethin linkage and a polypeptide MYIEALDSYAC is used as polypeptide. The conjugate acts selectively on tumors and may substantially reduce resistance of cancer cells to anticancer drugs.

Glutaric aldehyde or any other bifunctional agent can be used as cross-linking agent for linking polypeptide and doxorubicin at amino groups. As a result, a conjugate with an acidlabile linkage is obtained.

Also, a pharmaceutical composition is proposed, which shows cytotoxic activity and contains the proposed conjugate of a receptor-binding fragment of the epidermal growth factor with doxorubicin at an effective concentration (0.05-0.1%) and carriers suitable for intravenous administration.

A physiologic salt solution, phosphate saline solution or analgetic preparations can be used as carrier. The composition may additionally contain microbicidal, antiviral and antiparasitic medicines and other medications used for the treatment of cancer patients, in the amounts of no more than 50% of the total weight of the composition.

BRIEF DESCRIPTION OF FIGURES

Fig. 1 presents data to compare proliferative effects of the proposed polypeptide and known polypetide (I) on murine fibroplat cell line NIH 3T3.

Fig. 2 presents data demonstrating a delay in the onset of tumors and their growth retardation as a result of administration of DR-containing compositions, in a murine implanted tumors model.

DETAILED DESCRIPTION OF THE INVENTION

Below are given examples which illustrate the invention and prove the possibility of using the proposed receptor-binding fragment of the epidermal growth factor, conjugate and conjugate-based composition for the purpose described.

Example 1. Manufacture of polypeptide.

The polypeptide was obtained by the method of solid-state peptide chemistry using a Fmoc strategy of synthesis. Wash-resin was chosen as polymer carrier. HOBt and N,N-diisopropyl carbodiimide were used to activate carboxyl groups of Fmoc-amino acids by conversion to hydroxybenzotriazolic ester at each step of peptide condensation. Fmoc-amino acids, HOBt and N,N-diisopropyl carbodiimide were taken in 10-fold molar excesses relative to amino groups of the polymer. The course of the chemical reaction was controlled by discoloration of the bromophenol blue which was added in the reactor as solution in dimethyl sulfoxide (DMSO) in the ratio 1/1000 (mol) relative to amino groups. The reaction proceeded for 1.5-2.0 hours. After the condensation was over, the resin was washed 4 times in dimethyl formamide (DMF) and then processed by a 50% solution of piperidine in DMF. The polypeptide was removed from the polymer carrier using a mixture of trifluoroacetic acid - 95%, water - 2.5% and 1,2-ethanedithiol - 2.5%. After the resin had been filtered, the peptide was precipitated from the solution by diethyl ether. The precipitate was separated by centrifugation, dried in vacuum and analyzed by reversed-phase HPLC on a column Phenomenex Luna C18 250*3. Gradient elution of the polypeptide from the column was carried out with a system of the following solvents: acetonitrile-0.1% trifluoroacetic acid/water. Semipreparative reversed-phase HPLC was conducted under the same conditions on a column Nucleosil C18 250*8. The purity of the polypeptide obtained with chromatographic separation was > 96%.

The obtained polypeptide was characterized by the method of mass spectrometry (molecular ion - 1265). The structure of the polypeptide is given in Table 1.

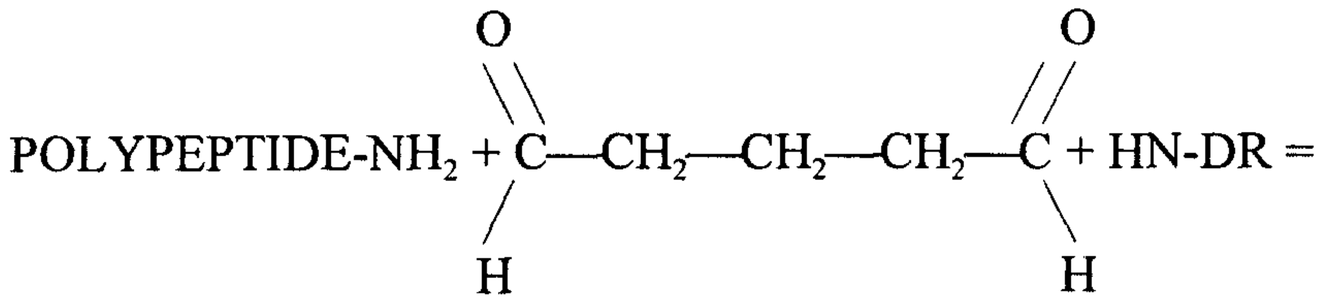
Table 1. Structures of the known and proposed polypeptides

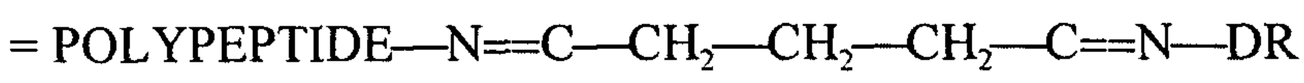
Amino acid sequence of the prototype polypeptide (EGF fragment)
NH_2-Met-Tyr-Ile-Glu-Ala-Leu-Asp-Lys-Tyr-Ala-Cys-COOH | (ε-NH_2)
Amino acid sequence of the proposed polypeptide
NH_2-Met-Tyr-Ile-Glu-Ala-Leu-Asp-Ser-Tyr-Ala-Cys-COOH

Example 2. Manufacture of a conjugate of the polypeptide with DR.

The polypeptide was dissolved in 5ml of 0.05M phosphate buffer (pH 7) (concentration 1 mg/ml) and doxorubicin preliminary dissolved in 5 ml of distilled water (pH 5.5) (concentration 1.2 mg/ml) was added. The synthesis was run by scheme 1:

Scheme 1





Glutaric aldehyde (GA) in 0.05M phosphate buffer (pH 7.2) (concentration 0.5 mg/ml) was added drop-by-drop to the reaction mixture. The volume of the added GA was 3 ml. After that, the mixture was incubated at 50°C for 1 hour. The obtained conjugate was separated from starting materials using a column PD-10 Sephadex G-25 (Pharmacia, Sweden). The conjugate was analyzed by reversed-phase HPLC on a column Nucleosil C18 250*4.6. Gradient elution of the polypeptide from the column was carried out with a system of the following solvents: acetonitrile-0.1% trifluoroacetic acid/water.

Concentration of the polypeptide in the conjugate was determined by spectrophotometry (at λ=280 nm). Concentration of DR in the conjugate was determined by spectrophotometry at λ=495 nm). The molar polypeptide-to-DR ratio in the conjugate was 1:1.

To conclude, a method is developed of chemical conjugation of the proposed polypeptide and doxorubicin with the use of glutaric aldehyde as cross-linking agent. A polypeptide-DR conjugate is obtained and characterized, in which the ratio of the conjugated moieties is 1:1.

Example 3. Demonstration of biological activity of the polypeptide.

Proliferative activity of the polypeptide was tested on line NIH 3T3 murine fibroplat cells. The rate of DNA biosynthesis was judged from the amount of ^{3}H-timidine incorporated in the acid-soluble cell fraction.

Line NIH 3T3 murine fibroplat cells were cultivated in plastic flasks in the RPMI medium (Sigma), which contained 10% of fetal bovine serum (Sigma), 100 U/ml of penicillin and 100 μg/ml of streptomycin, in a wet environment of 5% CO_2 at 37°C. Proliferation of synchronized line NIH 3T3 fibroplats was induced by adding to the cellular suspension the proposed polypeptide at different concentrations (0.1-1000 ng/ml). The fibroplasts were cultivated in 96-well plates in the complete medium, 200-800 cells in each well containing 200 μl of the medium. ^{3}H-timidine (1 μCi/well, 40 μCi/mol) was added 2 hours prior to the end of the incubation period. Radioactivity was measured by a scintillation counter (Rackbeta, LKB) with the use of a toluene-based scintillation fluid ZhS-8. The rate of DNA biosynthesis was expressed as the stimulation index (stimulated-to-control cell ratio) calculated by the formula: I = N/No x 100%, where I is the stimulation index, N is the number of EGF-stimulated cells, and No is the number of control cells.

Fig. 1 presents data to compare proliferative effects of the proposed polypeptide and known polypetide I (EGF fragment) on murine fibroplat cell line NIH 3T3. The data show that the proposed polypeptide has a more pronounced dose-dependent proliferative effect on this line cells than the known polypeptide, which may be indicative of a higher affinity of the proposed polypeptide to receptors. As the expression of EGF-receptors in the walls of cancer cells is several orders of magnitude higher than that in non-transformed cells, the above finding supports a possibility of using the proposed polypeptide as a more effective vector molecule for the targeted delivery of anticancer drugs to cancer cells.

Example 4. Efficacy of the proposed polypetide-DR conjugate against different cancer cell lines, evaluated by the rate of survival.

Efficacy of the conjugate was studied on the following cell lines: human breast carcinoma cell line MCF-7^{wt} and resistant human breast carcinoma cell line MCF-7^{AdrR}; human ovary carcinoma cell line SKOV3 and resistant human ovary carcinoma cell line SKVLB; and murine melanoma cell line B16.

Study protocol was common for all test runs. The cells cultivated in plastic flasks (Costar) in the RPMI medium (Sigma), which contained 10% of fetal bovine serum (Sigma), 100 U/ml of penicillin and 100 μg/ml of streptomycin, in a wet environment of 5% CO_2 at 37°C, were inoculated in 96-well plates (Costar), 10000 in each well, and studied drugs were then added at different concentrations. After that, the cells were incubated for 72 hours. Two-four hours prior to the end of the incubation period, 50 μl (1mg/ml) of the MTT solution (3-[4,5-dimethylthiazole-2yl]-2,5-diphenyltetrazole bromide, Sigma) in the culture medium were added in each well. After the development of coloration, the medium was removed, formazan crystals were dissolved in 150 μl of dimethyl sulfoxide, and the absorbance was measured by spectrophotometry at 540 nm. Survival rates of the cells exposed to free and conjugated DR were determined in per cent relative to the survival rate of controls taken as 100%.

Survival rates of the cells exposed to free doxorubicin and doxorubicin conjugated with either proposed polypeptide or EGF fragment are given in Table 2.

Table 2. Survival rates of the cells exposed to free or conjugated doxorubicin

Cellular line	Cytotoxicity (IC_{50})		
	DR	EGF fragment-DR conjugate	Polypeptide-DR conjugate
MCF-7^{wt}	58.6 nM	2.5 nM	1.3 nM
MCF-7^{AdrR}	3.7 μM	0.3 μM	0.15 μM
SKOV3	209 nM	35 nM	22 nM
SKVLB ^{AdrR}	2.01 μM	0.2 μM	0.07 μM
B16	35 nM	1.5 nM	1.1 nM

It follows from the data presented that the proposed conjugate of polypeptide with doxorubicin has a much more pronounced toxic effect on human cancer cell lines MCF-7^{wt} and SKOV3, which are doxorubicin-sensitive, and murine melanoma cell line B16 than free DR or its conjugate with EGF fragment (prototype). Also, the proposed conjugate of polypeptide with doxorubicin shows a high cytotoxic activity against cell lines MCF-7^{AdrR} and SKVLB which are resistant to anthracycline antibiotics.

To conclude, an anticancer drug (doxorubicin) conjugated with the proposed polypeptide is shown to have a pronounced toxic effect on cancer cells, both sensitive and resistant to doxorubicin, which fact proves the efficacy of the proposed polypeptide as vector molecule for the targeted delivery of anticancer drugs in vitro.

Example 5. Pharmaceutical compositions for injections are obtained by dissolving the conjugate in a physiological salt solution or phosphate saline solution with pH about 7.4. Concentration of the conjugate in the solution is 0.05-0.1%.

Example 6. Cytotoxic activity of a composition of the polypeptide-DR conjugate with a physiological solution (described in Example 5) demonstrated in a murine implanted tumor model.

To demonstrate cytotoxic activity of the polypeptide-DR conjugate against solid tumors, pharmaceutical compositions described in Example 5 were administered intravenously to mice with subcutaneously implanted line B16 cancer cells. The results obtained are given in Figs. 1 and 2. The medicines were administered at a dose of 0.2 mg/kg

of doxorubicin once every four days, three injections in total, starting from day 3 after the cancer implantation.

It follows from Fig. 1 that, unlike free DR, its conjugate caused a considerable delay in the onset of tumors and slowed their growth. The tumor growth inhibition rate was 58%.

It follows from Table 3 that the proposed conjugate has a therapeutic effect when given intravenously, which could be seen from an increase in the average duration of life (ADL) of laboratory animals. Unlike free doxorubicin, administration of its conjugate allowed a 46% increase in ADL to achieve.

Table 3. Increase in the average duration of life of animals treated intravenously with DR-containing compositions

Animal group	**ADL, days**	**Increase in ADL, %**
Controls	31.4±4.8	-
DR	23.6±4.0	- 24.8
Conjugate	46.6±5.7	48.5

An increase in the average duration of life and higher tumor growth inhibition rates suggest a therapeutic efficacy of the targeted delivery of an anticancer agent in the proposed composition in vivo.

A conjugate of the proposed polypeptide with an anthracycline antibiotic doxorubicin (polypeptide-DR) is shown (Examples 4, 6) to exert a therapeutic action on solid tumors when given intravenously.

AMINO ACID SEQUENCE OF THE PROPOSED POLYPEPTIDE

Met-Tyr-Ile-Glu-Ala-Leu-Asp-Ser-Tyr-Ala-Cys
1 5 10

CLAIMS

1. A biological active polypeptide of the formula MYEIALDSYAC, capable of binding to receptors of the epidermal growth factor, causing cellular proliferation, and acting as vector molecule for the targeted delivery of anticancer drugs to cancer cells.
2. A conjugate of the polypetide with doxorubicin, in which doxorubicin is covalently linked with polypeptide through an acidlabile azomethin linkage and a polypeptide of the formula MYIEALDSYAC is used as polypeptide.
3. A conjugate of the polypetide with doxorubicin per Claim 2, in which doxorubicin is covalently linked with polypeptide as a result of using glutaric aldehyde as cross-linking agent.
4. A pharmaceutical composition having an anticancer effect, which contains doxorubicin conjugated with a vector molecule at effective concentration and a pharmaceutical carrier suitable for intravenous administration, distinguished by that it contains doxorubicin conjugated with a polypeptide of the formula MYIEALDSYAC as doxorubicin conjugate.

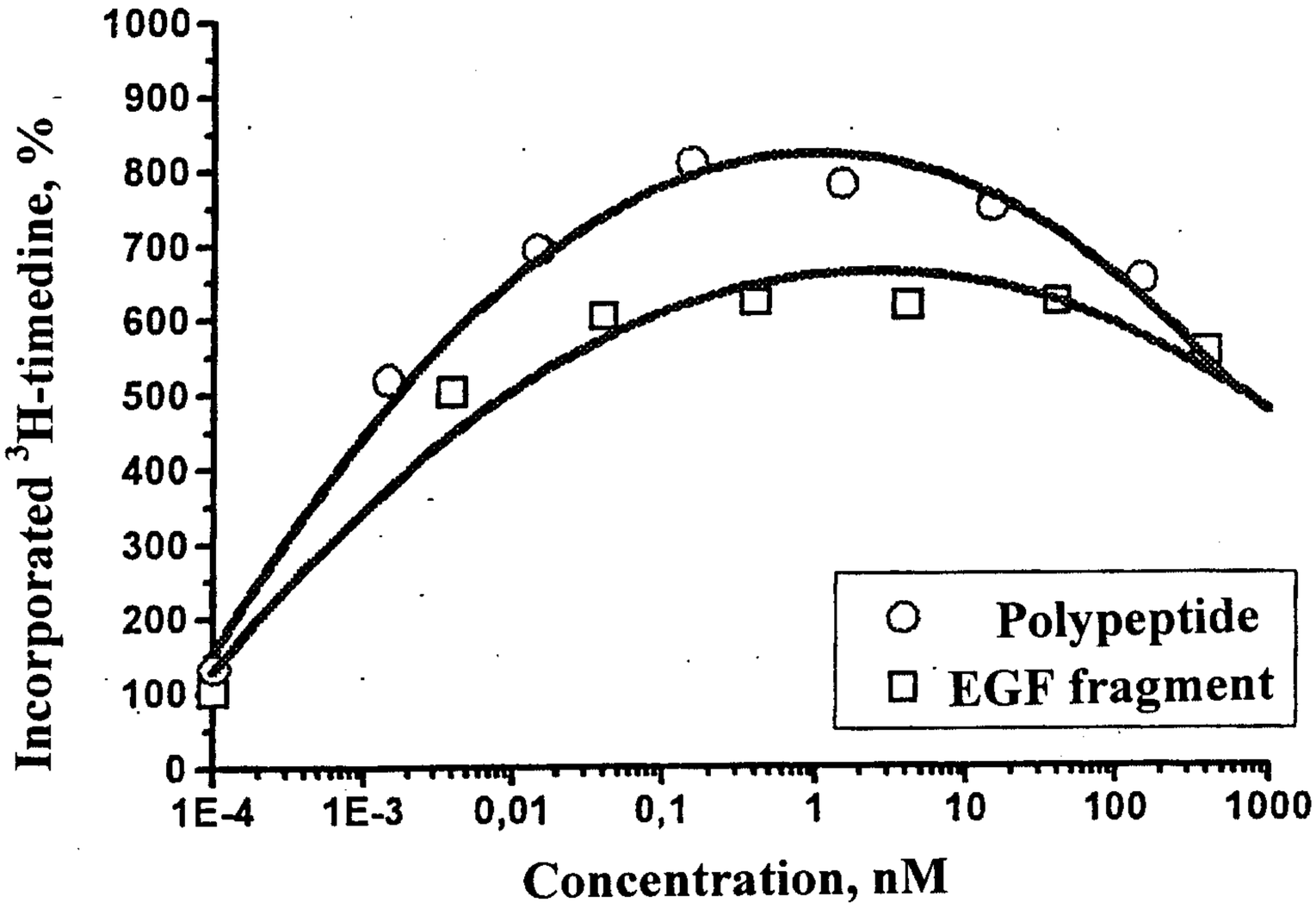

Incorporated ^{3}H-timedine, %
1000
900
800
700
600
500
400
300
200
100
0
Polypeptide
EGF fragment
1E-4
1E-3
0,01
0,1
1
10
100
1000
Concentration, nM

Fig. 1.

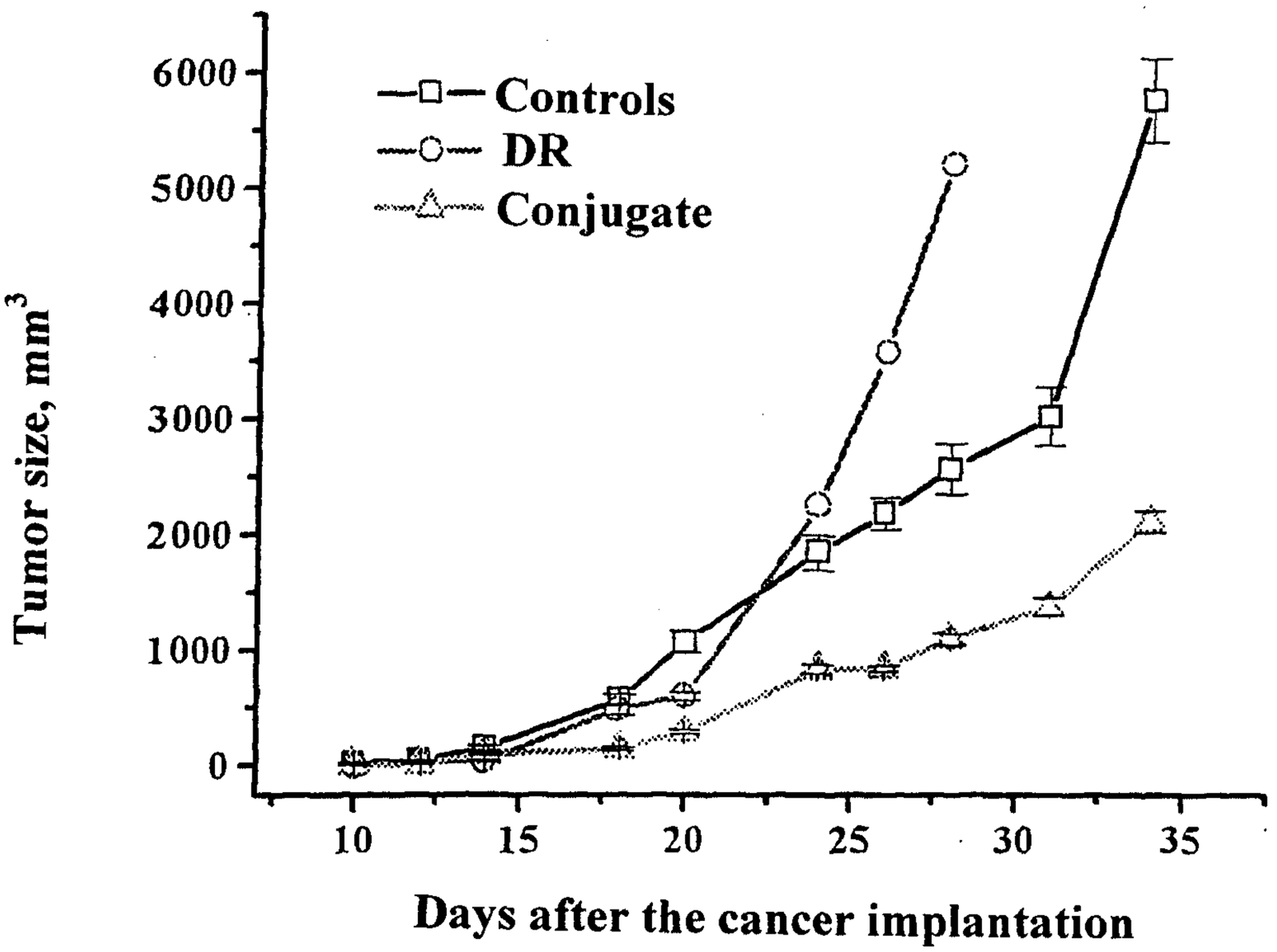

Controls
DR
Conjugate
Tumor size, mm3
0
1000
2000
3000
4000
5000
6000
10
15
20
25
30
35
Days after the cancer implantation

Fig. 2.