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SUMMARY
of dissertation for the Academic and Scientific Degree Ph.D.

Thema: INVESTIGATION OF THE PHARMACODINAMIC EFFECTS OF ERUFOSINE ON MALIGNANT AND NORMAL HAEMATOPOIETIC CELLS

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The dissertation is written on 373 pages and includes 49 tables, 70 figures and 16 applications. The bibliography includes 273 articles, 5 in Bulgarian language and 268 in English language.

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The PhD-student Maya Zaharieva is a doctorant in the Department of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Pharmacy, Medical University of Sofia.

The promotion of the dissertation will take place on November 29, 2007 at 1:30 pm, in the conference hall of the Institute of Plant Physiology at the Bulgarian Academy of Sciences, Acad. G. Bonchev ", bl. 21, 2nd floor, at an open session of the Specialized Scientific Counsil for Physiology, Pathophysiology and Pharmacology of the Superior Certifying Commission.

The dissertation is available in the library of the Institute of Neurobiology, Bulgarian Academy of Sciences, "Acad. G. Bontchev" Str. 23, 2^{-nd} floor.

Abbreviations

ASO, ASO	Antisense oligonucleotide
AΦX	Alkylphosphocholines
Abl	Abelson tyrosine kinase
Bad	Proapoptotic protein related to the mitochondrial way for apoptosis induction
Cdk	Cyclin dependent kinases
CFUs	Colony Forming Units,
CML	Chronic myeloid leukemia
hpRNA	hairpin RNA
LTBMCCs	Long Term Bone Marrow Cell Cultures
NSO	Nonsense-oligonucleotide
PCR	polymerase chain reaction
PKB	Protein kinase B
PTEN	Phosphatase and tensin homolog on chromosome ten
qPCR	quantitative PCR
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
RNAi	RNA-interference
WT	wild type cells

Introduction

Treatment of malignant tumors follows therapeutic schemas with various cytostatic drugs, which differ in pharmacodynamics and pharmacocynetics. Often their therapeutic effect is accompanied by serious side effects on various organs and systems. Most of the cytostatics have a pronounced suppressive effect on the hematopoietic system, with leukopoiesis being most severely affected. The leukopenic effect is desirable in hemoblastosis, but the use of aggressive cytostatic combinations or long-term polychemotherapy in the treatment of neoplasms can cause severe drug-induced leukopenia and granulocytopenia, conditions that require costly and not always effective treatment with recombinant growth factors and systematic haemotransfusions. Drug-induced bone marrow aplasia in many cases has progressive development and poor prognosis due to the presence of anemia, hemorrhage and the risk of secondary infections. In this regard, the study of cytostatic compounds with low hematological toxicity is particularly relevant and important for therapeutic practice. Alkylphosphocholines combine the presence of a cytotoxic effect on malignant transformed cells with stimulation of hematopoietic progenitor cells.

Today, there is an increasing emphasis on the thesis that malignant diseases can be long-lasting and well controlled with modern pharmacological agents. In this regard, the development of new drugs that are modulators of pathological signal transduction, as well as the development of new therapeutic drug regimens, will allow to achieve good pharmacological control and reduce or eliminate the myelosuppressive effect of conventional cytostatics.

AIM AND OBJECTIVES

I. Aim

The aim of the present study was to determine the cytostatic effect of the alkylphosphocholine erufosine on a panel of tumor cell lines when used alone and in combination; to elucidate the mechanisms of selective cytotoxicity, its effect on normal hematopoietic cells and on various signaling biomolecules.

II. Objectives

1. 1. To determine the cytotoxic and antiproliferative effect of erufosine on a spectrum of leukemic and tumor cell lines by comparative analysis of the IC_{50}-values.
2. To create long-term cultures from normal mouse bone marrow on which to study the protective effect of erufosine against hematopoietic cells in single and combined use.
3. To determine the effect of combinations between erufosine and classical cytostatics on a panel of leukemic cell lines.
4. To determine the effect of erufosine in combination with antimetabolites or anthracycline antibiotics on the distribution of cells in the different phases of the cell cycle by flow cytometric analysis.
5. 5. To determine the influence of erufosine on the reorganization of lipid rafts in the cell membrane and their components with respect to the signaling molecules BCR-ABL, ABL and PKB/AKT.
6. To investigate the effect of erufosine on proteins of signal transduction pathways, including the oncoprotein BCR-ABL, cell cycle regulation and apoptosis - Rb, PKB / AKT, PTEN, p27, Bad, BCL-xL, caspase 8 and caspase 9.
7. To determine the cytotoxic effect of erufosine on leukemic cell line models with suppressed retinoblastoma protein Rb.

MATERIALS AND METHODS*

1. Drugs

Erufosine, Imatinib, Epirubicine, Bendamustine, Cytosine arabinoside (Ara C), Pemetrexede и Gemcitabine.

2. Cell lines

2.1. Malignant cell lines

K-562, BV-173, SKW-3, LAMA-84, AR-230, HD-MY-Z, HuT 78, KM-H2, MDA-MB, SAOS-2, 293T HEK

2.1. Primary cell lultures

Mouse haematopoietic cells, isolated from mice ICR and C57/Bl6

3. Cell culturing

3.1.Culturing of human cell lines.
3.2.Cultivation sof long term bone marrow cell cultures

4. Assay for in vitro evaluation of cell vitality and proliferation

4.1.MTT-test
4.2.Combined application of two and more drugs
4.3.CFU-assay

5. Flowcytometry for cell cycle analysis

6. Molecular biology methods for gene expression analysis

6.1.Cryopreservation of cell samples for DNA and RNA analysis
6.2.Isolation and prification of total RNA from eukaryotic cells
6.3.Lysis of eukaryotic cells for protein isolation
6.4.Isolation of proteins from lipid rafts
6.5.Determination of protein concetrations using the Pierce BSA Protein Assay
6.6.Protein separation in PAAG
6.7.RT-PCR
6.8.PCR
6.8.1. Conventional PCR
6.8.2. Dwtermination of positive clones with the virus vector pLL 3.7
6.8.3. Quantitative PCR
6.9.DNA separation by electrophoresis
6.10. Immunoblot

7. Recombinant DNA technology

7.1.DNA annealing
7.2.Preparation of dsDNA fragments for cloning plasmid vectors
7.3.Clonin gof dsDNA into the vector pSUPER - ligation
7.4.Selection of positive clones with the pSUPER vector in electrocompetent *E. coli* (DH 10β)
7.5.Cloning of H1-shRNA cassette from the pSUPER vector into the virus vector pLentiLox 3.7

8. Purification of nucleic acids

9. Restriction analysis of plasmids in agarose gel

10. DNA sequencing

11. Transduction of nucleic acid fragments into cells in vitro

11.1. Transfection of nucleic cells into prokaryotic cells
11.2. Transfection of nucleic cells into eukaryotic cells
11.2.1. Unstable transfectio with cationi arriers
11.2.2. Transduction with lentiviruses – stable transfection

11.2.3. Evaluation of the transfectoin effectivness

12. Preparation of samples using cytospin

13. Statistics

RESULTS AND DISCUSSION

The main results are published in the following two articles:

Erufosine

A Membrane Targeting Antineoplastic Agent with Signal Transduction Modulating Effects

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ABSTRACT: **The ether lipid analog erufosine (erucylphospho-N,N,N,-trimethylpropylammonium, ErPC3) has high activity against leukemic cells without affecting the normal hematopoiesis. It belongs to the group of alkylphosphocholines (APC) that are inhibitors of protein kinase C and phospholipase C. However, the mechanism of action of erufosine remains rather unclear. We focused on combination effects with the tyrosine kinase inhibitor imatinib mesylate (gleevec, former STI-571 or CGP-57148) against two chronic myeloid leukemia (CML)-derived cell lines (K-562 and BV-173). The influence of erufosine on proteins involved in the phosphatidylinositol-3-phosphate pathway and on expression of the retinoblastoma protein Rb was studied, the latter being a key component for cell cycle entry and progression in mammalian cells. The consecutive treatment of K-562 and BV-173 cells with erufosine (2.5, 5, 15, 30 μM) and imatinib mesylate (0.05, 0.1 μM) led to synergism as measured by the MTT-dye reduction assay and this is reason to hypothesize that such combinations could be beneficial for relapsed patients with drug-resistant disease. Whole cell lysates from K-562 and BV-173 were investigated for the expression of Rb, PKB/Akt, pAkt, and p27 by Western blot. Erufosine caused decreases of pAkt and CML fusion protein p210 (BCR-ABL) protein expression, but induced the Rb protein expression in K-562 cells. A parallel increase in p27 level was observed after 24 and 48 h treatment. These alterations in signal transduction could be an explanation for the drug interaction found. Furthermore, Rb is a substrate of caspases and is cleaved during apoptosis as already evidenced for BV-173 cells. Our experimental findings suggest that erufosine acts through induction of changes in protein signaling and especially through Rb induction. This unique mode of action makes it an attractive partner**

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Research Paper

The Expression Level of the Tumor Suppressor Retinoblastoma Protein (Rb) Influences the Antileukemic Efficacy of Erucylphospho-N,N,N-Trimethylpropylammonium (ErPC3)

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KEY WORDS

alkylphosphocholines, erucylphospho-N,N,N-trimethylpropylammonium (erufosine), Rb, antisense, cytotoxic efficacy, chronic myeloid leukemia (CML)

ABBREVIATIONS

CML	chronic myeloid leukemia
AML	acute myeloid leukemia
ALL	acute lymphoid leukemia
APC	alkylphosphocholine
ErPC3	erucylphospho-N,N,N-trimethyl-propylammonium
ASO	antisense oligonucleotide
NSO	nonsense oligonucleotide
FITC	fluorescein isothiocyanate
PBS	phosphate buffered saline
TBS	tris buffered saline
SDS	sodium dodecyl sulfate
DTT	dithiothreitol
FCS	fetal calf serum

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See page 934.

ABSTRACT

The alkylphosphocholine erucylphospho-N,N,N-trimethylpropylammonium (ErPC$_3$) is a promising new drug for treating various types of cancer. Its mechanism of action is not yet fully understood but is related to the Rb tumor suppressor protein. In the present study, we investigated the role of decreased Rb expression levels for the antileukemic efficacy of ErPC$_3$ in BV-173 and K-562 CML-derived cell lines. We used antisense technique to knock down Rb levels in the two cell lines in addition to ErPC$_3$ treatment. Cells with reduced Rb expression showed a diminished sensitivity to ErPC$_3$ exposure, as determined by MTT (BV-173 and K-562) and clonogenicity assays (K-562 only), if concentrations below the IC$_{50}$ were used. The feasibility of Rb knockdown varied between BV-173 and K-562 cells, with the former being distinctly more sensitive than the latter. We conclude that sufficient Rb levels are important for the cytotoxic and anticlonogenic effects of ErPC$_3$ at levels below the IC$_{50}$, but that higher concentrations of ErPC$_3$ are less dependent on Rb status.

INTRODUCTION

Alkylphosphocholines (APCs) are a class of antineoplastic compounds that are derived from ether lipids.[1] APCs exhibit significant cytotoxic activity towards a vast number of leukemic cell lines, such as the promyelocytic AML-derived HL-60 cells, the ALL-derived Reh cells, the T-cell lines Jurkat and MOLT-4, and the Burkitt lymphoma cells Raji and Ramos.[2] APCs are also effective against CML-derived leukemic cells, but in that case the efficacy of APCs depends on the level of the BCR-ABL fusion oncoprotein.[3] The BV-173 and CML-T1 cell lines, expressing relatively low levels of BCR-ABL, are more sensitive to the action of APCs, as compared to K-562, LAMA-84 and AR-230 which express higher levels of BCR-ABL.[4] It is noteworthy that APCs are not myelotoxic and even stimulate normal haematopoiesis in the bone marrow, in contrast to conventional cytostatics.[2,5] Their exact mechanism of action is not fully understood but it is known that they modulate different signal transduction pathways through interaction with membrane components.[2,5,6-12] For example, they inhibit the phosphoinositide pathway,[2,6,12] lead to dephosphorylation of Akt and, interestingly, dephosphorylation and induction of Rb.[9] Rb is a nuclear protein that controls the cell's entry and transition through a new cycle of cell division.[13,14] Rb also plays a key role in the processes of differentiation[13,15,16] and apoptosis.[13,15,17-19] The activity of Rb is regulated by phosphorylation with the phosphorylated form being inactive and the dephosphorylated form active, i.e., only the dephosphorylated and hypophosphorylated forms of Rb bind to E2F transcription factors and block them from activating the transcription of genes crucial for DNA replication and mitosis.[13] The pathways controlling the activity of Rb start from membrane proteins.[13,14] Recently, we have described that erucylphospho-N,N,N-trimethylpropylammonium (ErPC$_3$, Erufosine) can elicit changes in Rb protein status and these changes include hypophosphorylation, increased expression and fragmentation that are typical for cell cycle arrest and apoptosis.[9] In addition, APCs interact with membrane lipid rafts and enhance their affinity for binding of cABL and BCR-ABL.[9] In variance with first generation APCs, ErPC$_3$ does not cause haemolysis and can be administered intravenously.

Currently ErPC$_3$ is in advanced phase I clinical trials in CLL patients (H. Eibl, personal communication).

In order to clarify the importance of the observed induction of Rb for its cytotoxic activity, we tried to determine whether the antiproliferative activity of ErPC$_3$ would be altered by antisense-induced inhibition of Rb-expression in leukemic cells. As expected,

ОБОБЩЕНИ РЕЗУЛТАТИ И ИЗВОДИ

1. The cytotoxic effect of Erufosine in four leukemic cell lines, three lymphoma cell lines and two cell lines derived from solid human tumors was studied and data on the spectrum of its cytostatic activity were obtained. A comparative bioevaluation of the efficacy of alkylphosphocholine in aqueous-ethanolic solution and liposome suspension was performed.
    1.1. Erufosine is characterized by pronounced cytotoxicity in cells with lymphoid phenotype - SKW-3, BV-173 and in MDA-MB cells, relatively lower cytotoxic effect - in myeloid cell lines and least cytotoxicity - in HD-MY-Z lymphoid cells and cells originating from osteosarcoma SAOS-2.
    1.2. The inhibitory concentrations IC_{50} of the aqueous-ethanolic solution of Erufosine are about twice as low in all cell lines except K-562 asompared to the IC_{50} of the liposome form, with maximal cytostatic effect being equivalent at high concentrations.
2. The effect of Erufosine on the clonogenic capacity of K-562 cells was studied and evidence of an antichlonogenic effect was obtained. The liposome form achieves less pnounced inhibition of cell colony formation (up to 43% inhibition) than the aqueous-ethanol solution (up to 60% inhibition)
3. For the first time, a protective effect of erufosine on hematopoietic cells was found when used in combination with Bendamustine, Epirubicine, Ara C, Pemetrexed and Gemcitabine. Erufosine reduces cytostatic-induced myelosuppression.
4. For the first time, in vitro interactions between Erufosine and the cytostatics Bendamustine, Ara C, Epirubicine and Imatinib in leukemic and lymphoma cell lines have been obtained. Achieving synergism, additive effect or antagonism depends on the sequence of administration of the compounds, the concentrations administered and the type of cell line.
    4.1. Synergism has been established in the action of Erufosine and Imatinitb or Epirubicine, with the initial application of alkylphosphocholine; synergism is achieved in combination with Bendasmustine or Imatinib, with subsequent administration of Erufosine.
    4.2. Antagonistic interactions have been identified between Erufosine and Bendamustine or Erufosine upon initial administration of Erufosine;

antagonism is also present in the combination of Erufosine and Epirubicine, with subsequent administration of the alkylphosphocholine.

4.3.4.3. The combinations Erufosine/Ara C and Epirubicine/Erufosine are defined as irrational.

5. Erufosine alone slightly alters the distribution of cells in the different phases of the cell cycle. When used in combination with Epirubicine, the cell fraction in phase G2 is increased. When used in combination with Ara C, the cells in the sub-G1 fraction increase.
6. Erufosine affects the phosphoinositol PIP3 system and biomolecules of the apoptotic signaling pathways.
   6.1. Erufosine inhibits protein kinase B activity and increases the cyclin E/CDK2-inhibitor protein p27 expression.
   6.2. Erufosine stimulates the proapoptotic protein Bad, but this effect is followed by increased expression of the proapoptotic protein Bcl-xL.
7. Erufosine as a membrane-active compound alters the distribution of the oncoprotein BCR-ABL and protein kinase B in lipid rafts. Erufosine reduces BCR-ABL expression, which may explain the cytostatic effect in myeloid cell lines expressing BCR-ABL oncoprotein.
8. 8. Erufosine stimulates the expression of the retinoblastoma protein Rb, increasing its level in leukemic cells, thereby helping to slow and stop proliferation.
9. Two models of cells with transient or stably suppressed Rb-expression have been developed and the importance of Rb-induction for the mechanism of action of Erufosine has been established.
   9.1. Erufosine stimulates Rb-protein expression under conditions of transient suppressed Rb-expression in BV-173 cells.
   9.2. The sensitivity of BV-173 and SKW-3 cells to erufosine decreases under conditions of suppressed Rb expression induced by antisense oligonucleotides or RNA sequences.

Scientific contributions

I. Contributions related to the determination of the effect of Erufosine on tumor-transformed and normal hematopoietic cells.

1. Data on the presence of cytotoxicity of the alkylphosphocholine erufosine on tumor-transformed cells of various origins have been confirmed. This complements the characterization of erufosine as a compound with cytostatic activity and determines the spectrum of its action in vitro.
2. For the first time, a protective effect of Erufosine on hematopoietic cells was found when used in combination with Bendamustine, Ara C, Epirubicine, Pemetrexed and Gemcitabine. Erufosine reduces cytostatic-induced myelosuppression by 10-100%. At a concentration of 30 µM, Erufosine is not toxic to mouse bone marrow cells.

II. Contributions related to the in vitro interaction between erufosine and clinically administered cytostatics.

Interaction data between erufosine and cytostatics bendamustine, ara c, epirubicine and imatinib in leukemic and lymphoma cell lines were obtained for the first time in vitro. The presence of synergism, antagonism and additive effect was determined, depending on the type of cell line, the concentration of cytostatics and the sequence of their application. These data allow the development of therapeutically useful combinations and eliminate the possibility of compiling combined regimens in which antagonism in the effects is possible. We believe that cellular test systems are an appropriate model for pre-screening the type of drug interactions, which can facilitate the selection of the components of the combined scheme and the sequence of their application.

1. Установен е синергизъм в ефектите на Erufosine при последователно приложение в следните комбинации: Bendamustine/ Erufosine; Imatinib/ Erufosine; Erufosine/ Imatinib; Ara C/ Erufosine при клетъчната линия К-562; Erufosine/ Epirubicine при клетъчните линии HD-MY-Z, K-562, BV-173, AR-230.
2. Antagonism between Erufosine and cytostatics was determined in the combinations: Erufosine/Bendamustine; Ara C/Erufosine at the AR-230 cell line; Erufosine/Ara C; Epirubicine/Erufosine in cell line K-562.

III. Contributions to elucidating the mechanism of action of erufosine.

1. For the first time in K-562 cells, data were obtained on the inhibitory effect of Erufosine on protein kinase B and the increase in the expression of cyclin E / CDK2-inhibitor protein p27. These results confirm available literature data on the effect of other alkylphosphocholins on protein kinase B expression in other cell lines. They help to elucidate the mechanism of action of erufosine in relation to the antiproliferative effect and to stop the progression of the cell cycle in the G1 phase.
2. The effect of Erufosine on Bcl-2 family proteins was determined for the first time. Erufosine stimulates the expression of the proapoptotic protein Bad, which promotes cell apoptosis. The subsequent overexpression of the antiapoptotic protein Bcl-xL is likely to be a protective cellular mechanism to restore the induced cellular imbalance between apoptotic and antiapoptotic pathways. This may be the probable reason for the resistance of the surviving K-562 cells to erufosine at concentrations in the range of 12.5 - 50 μM.
3. For the first time, the effect of Erufosine on the relationship between BCR-ABL oncoproteins and lipid shelves in the cell membrane was determined. Recruitment of BCR-ABL in lipid fractions containing lipid rafts was found. By this mechanism, erufosine inhibits the interaction of the oncoprotein with its cellular substrates. In parallel, a decrease in BCR-ABL expression was observed under the action of erufosine, which is an important mechanism for its cytotoxic effect achieved in myeloid cells expressing the fusion oncoprotein.
4. For the first time, the effect of erufosine on the expression of the retinoblastoma protein Rb, a cell cycle regulator, was determined. Rb protein induction has been shown to be important for the cytotoxicity of Erufosine as it modulates the Rb signaling pathways involved in the mechanism of action of alkylphosphocholine.
   - 4.1. Erufosine increases the level of Rb in leukemic K-562 cells, which affects the regulation of the cell cycle and contributes to the inhibition of tumor proliferation.
   - 4.2. The significance of Rb induction was clarified by cell models developed by us (by known technology) with transient Rb deficiency and stably suppressed Rb expression, which have an original character. The induction of Rb plays a key role in the modulation of signal transduction induced by Erufosine, which is a significant original contribution to elucidating the pharmacodynamics of the

new antineoplastic agent Erufosine with missing myelosuppressive activity and stimulating normal hematopoiesis.

4.2.1. MTT test and CFU analysis revealed a decrease in the sensitivity of leukemic populations of BV-173 and SKW-3 cell lines to the action of erufosine after suppression of Rb expression.

4.2.2. Erufosine treatment largely restored the initial level of retinoblastoma protein expression in the BV-173 cell line.

Pharmacodynamic studies to determine the cytotoxic effect of Erufosine and its safety profile on hematopoietic cells suggest that Erufosine as a representative of alkylphosphocholines is a promising antitumor compound with membrane-active properties and inhibitory or stimulatory effects on various biologic cells..

AUTHOR'S PUBLICATIONS IN CONNECTION WITH THE DISSERTATION

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