Available online at www.sciencedirect.com
SCIENCE DIRECT
Leukemia Research 30 (2006) 136–144
Leukemia Research
www.elsevier.com/locate/leukres

Cytotoxic effect of a lipophilic alkylating agent after incorporation into low density lipoprotein or emulsions: Studies in human leukemic cells

Michèle Masquelier [a,*], Bo Lundberg [b], Curt Peterson [c], Sigurd Vitols [a]

[a] *Department of Medicine, Division of Clinical Pharmacology, Karolinska Institute/Karolinska University Hospital, S-171 76 Stockholm, Sweden*
[b] *Department of Biochemistry and Pharmacy, Åbo Akademi University, BioCity, P.O. Box 66, 20520 Åbo, Finland*
[c] *Division of Clinical Pharmacology, Department of Medicine and Care, Faculty of Health Sciences, Linköping University, SE-581 85 Linköping, Sweden*

Received 11 April 2005; received in revised form 17 June 2005; accepted 18 June 2005
Available online 8 August 2005

Abstract

The use of low density lipoprotein (LDL) as drug carrier in acute myeloblastic leukemia chemotherapy is attractive due to high LDL uptake by leukemic cells. Lipid-based formulations, such as liposomes or microemulsions are promising alternatives. In the current study, we incorporated *N*-trifluoroacetyl-adriamycin-14-valerate (AD32), a lipophilic derivative of daunorubicin (DNR), and WB4291, a lipophilic alkylating agent, into LDL or lipid microemulsions and evaluated their cytotoxic activities towards leukemic cell lines using as references DNR and melphalan. The incorporation of AD32 into LDL or emulsion resulted in complexes with poor cytotoxicity. WB4291-LDL and WB4291-emulsion exerted, on the other hand, promising cytotoxic effects towards parental and resistant K562 and HL60 cell lines.

Keywords: LDL; Lipid emulsion; Acute myeloid leukemia; DNR; Melphalan

1. Introduction

In cancer research, many approaches have been dedicated to increase the selectivity of antineoplastic agents by associating them to a carrier that could guide them to the target cells [1,2]. The potential of LDL as a carrier was stressed by many rapports showing that tumor cells possess a higher low density lipoprotein (LDL) uptake than the corresponding normal cells [3–5]. A particular enhancement of LDL receptor-mediated degradation has been reported in leukemic cells from patients with acute myeloid leukemia (AML) (up to 100-fold), underlining the potential of LDL as a therapeutic carrier in AML. Two main drawbacks have held up the clinical development of LDL as a drug carrier: firstly, the low stability of the complex in plasma, and secondly, a poor drug loading which increases the necessity of using a highly potent substance. However, we have shown that it is possible to incorporate a lipophilic alkylating agent, WB4291, into LDL at a high molar ratio (up to 1000 drug molecules per LDL particle) and that the complex behaves like LDL after iv injection in rabbits [6]. Moreover, this complex showed LDL receptor-mediated cytotoxicity towards WEHI-3B cells, a murine myelomonocytic leukemia cell line expressing LDL receptors. The effects were counteracted by an excess of LDL but not by methylated LDL, which does not recognize LDL-receptors. Finally, the complex showed promising therapeutic effects in mice with experimental leukemia, although it has never been tested on human leukemic cells.

WB4291 is a lipophilic mitoclomine derivative that was synthesized in the beginning of the seventies. It is an alkylating agent possessing a bischloroethylamine group that exerts a cytotoxic effect through the formation of DNA-DNA or DNA-protein crosslinking via the two chloroethyl functional groups [7]. Further, drug development was hindered because of poor water solubility.

LDL is a lipid microemulsion surrounded by a polar shell containing Apoprotein B, a large protein that binds to LDL receptors [8]. The isolation of LDL from human plasma is a time-consuming and relatively expensive pro-

* Corresponding author. Tel.: +46 851774461; fax: +46 8331343.
E-mail address: michele.masquelier@medks.ki.se (M. Masquelier).

0145-2126/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.leukres.2005.06.012

cess, also involving the risk of transmitting blood contaminants. The use of an artificial LDL is therefore an attractive alternative. Maranhao and co-workers demonstrated that a protein-free emulsion with a lipid composition resembling LDL behaves like native LDL after injection in patients with acute leukemia [9]. They suggest that this microemulsion acquires ApoE from lipoproteins circulating in blood and is then taken up into cells via the ApoB, E receptor. As an alternative formulation for LDL, we therefore incorporated WB4291 into lipid emulsion droplets with triolein as oil core and purified egg yolk phosphatidylcholine as the principal emulsifier stabilized with polysorbate 80 and polyethylene glycol-phosphatidylethanolamine. Submicron-sized oil-in-water lipid emulsions are attractive alternatives as drug delivery systems and have been successfully used to incorporate lipophilic drugs, such as paclitaxel [10]. A mixture of polysorbate 80, DL-tocopherol acetate, tocopherol polyethylene glycol succinate (TPGS) and tributyrin was also tested as stabilisator.

In this study, we tested the cytotoxicity of drug-LDL and drug-emulsion complexes towards the human leukemic cell lines, K562 and HL60 and some resistant subclones. K562 cells have a high uptake of LDL which is increased two to five times in the resistant subclones [11]. We compared the cytotoxicity of WB4291 complexes with that of *N*-trifluoroacetyladriamycin-14-valerate (AD32, also named valrubicin) complexes. We have earlier shown that AD32 is incorporated into LDL with a much lower yield and leaks slowly from the complexes in the presence of plasma [12]. Due to the insolubility of WB4291 and AD32 in aqueous solutions, the cytotoxicity of these complexes was compared to the cytotoxicity of the closely related drugs melphalan and daunorubicin (DNR).

2. Materials and methods

High purity egg phosphatidylcholine (EPC) was purchased from Sigma (St. Louis, MO) and used without purification after control of the purity by TLC. Triolein (TO), phosphatidylethanolamine (PE), polyoxyetylenesorbitan (polysorbate 80), oleoyl alcohol, MTT and carbonyldiimidazole were also from Sigma. Polyethyleneglycol-phosphatidylethanolamine (PEG-PE) was synthesized by reaction of PEG 2000 with carbonyldiimidazole followed by the addition of phosphatidylethanolamine (Allen et al., 1991); DL-tocopherol acetate was purchased from Carl Roth KG (Karlsruhe, Germany) and tocopherol polyethyleneglycol succinate (TPGS) from Peboc division of Eastman Chemical Ltd. (Llangefni, Anglesey, UK). Naphtylnitrogen mustard (code number designation WB4291, Fig. 1) was a kind gift of Boehringer Ingelheim (UK). AD32 was kindly provided by Dr Mervyn Israel (Memphis, TN, USA). Daunorubicin was purchased from Aventis Pharma (Bridgewater, NJ, USA) and melphalan from GlaxoSmithKline (PA, USA). Melphalan oleate was synthesized by adding 0.1 mmol of dicyclohexylcarbodiimide (Sigma) to a mixture of 0.1 mmol oleoyl alcohol and 0.1 mmol melphalan in chloroform while stirring at room temperature overnight. The product was purified by preparative silica gel TLC using chloroform:methanol (90:10) as eluent.

2.1. Cell lines

The human erythroleukemia cell line K562 and six drug resistant subclones (K562/Vcr30, K562/Vcr150, K562/DNR44, K562/DNR75, K562/Ida, K562/Nov), the human myeloid leukemia cell line HL60 and the resistant subclone (HL60/Nov) were kind gifts from Dr Astrid Gruber (Department of Hematology, Division of Medicine, Karolinska University Hospital, Solna, Sweden). Drug resistance was developed by continuous exposure to increasing concentrations of the drugs and the cells were maintained in medium containing 30 and 150 nM vincristine (K562/vcr30 and K562/Vcr150), 44 and 75 ng daunorubicin/ml (K562/DNR44 and K562/DNR75), 20 ng idarubicin/ml (K562/Ida) and 100 ng mitoxantrone/ml (K562/Nov and HL60/Nov). Cells were cultured twice a week in growth medium consisting of RPMI 1640 cell culture medium supplemented with 10% foetal bovine serum (Invitrogen) and 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (All from Gibco, Life Technologies Ltd., Paisley, Scotland).

2.2. Clinical sample acquisition

Heparinized peripheral blood was obtained from two untreated patients with de novo AML after informed consent (patient A, AML-M2, white blood cell count 75×10^9/l, leukemic cells 77% and patient B, AML-M4 white blood cell count 82×10^9/l, leukemic cells 66%). Mononuclear cells were isolated by centrifuging 5 ml blood on 3 ml Lymphoprep (Nycomed, Norway) at $550 \times g$ for 15 min at 4 °C. After two washes with PBS the cell number was determined using a Coulter counter Z2 (Beckman Coulter, Fullerton, CA, USA) and the cells were incubated with drugs for determination of cytotoxicity or drug uptake. The study was approved by the ethic's committee at Karolinska Institute.

2.3. Incorporation of WB4291 and AD32 into LDL

Human LDL (density 1.02–1.063 g/ml) was isolated from plasma from healthy blood donors by sequential ultracentrifugation as described by Havel et al. [13]. WB4291 and AD32 were incorporated into LDL as previously described with minor modifications [14]. Briefly, after dialysis with 0.3 mM NaEDTA pH 7.4, LDL (at 5 mg/ml) was transferred into a glass tube containing 10 mg sucrose/mg LDL (40% sucrose solution). The solution was rapidly frozen in liquid nitrogen and lyophilised overnight. AD32, dissolved in methylene chloride, or WB4291 dissolved in diethylether was added to the dried LDL and the mixture was incubated for 1 h at room temperature. The solvent was then evaporated