RAPID COMMUNICATION

Kinetic Analysis of the Toxicity of Pharmaceutical Excipients Cremophor EL and RH40 on Endothelial and Epithelial Cells

LÓRÁND KISS,[1,2] FRUZSINA R. WALTER,[1] ALEXANDRA BOCSIK,[1,2] SZILVIA VESZELKA,[1] BÉLA ÓZSVÁRI,[3] LÁSZLÓ G. PUSKÁS,[3] PIROSKA SZABÓ-RÉVÉSZ,[2] MÁRIA A. DELI[1]

[1]Laboratory of Molecular Neurobiology, Institute of Biophysics, Biological Research Centre of the Hungarian Academy of Sciences, Szeged H-6726, Hungary

[2]Department of Pharmaceutical Technology, University of Szeged, Szeged H-6720, Hungary

[3]Avidin Ltd., Szeged H-6726, Hungary

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ABSTRACT: Cremophor EL and RH40 are widely used excipients in oral and intravenous drug formulations such as Taxol infusion to improve drug dissolution and absorption. Studies indicate that Cremophors, especially EL, have toxic side effects, but few data are available on endothelial and epithelial cells, which form biological barriers and are directly exposed to these molecules. Human hCMEC/D3 brain endothelial and Caco-2 epithelial cells were treated with Cremophor EL and RH40 in the 0.1–50 mg/mL concentration range. Cell toxicity was monitored by real-time cell microelectronic sensing and verified by lactate dehydrogenase release and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, and morphological methods. Cremophors caused dose- and time-dependent damage in both cell types. In endothelial cells, 0.1 mg/mL and higher concentrations, in epithelial cells, concentrations of 5 mg/mL and above were toxic, especially at longer incubations. Cell death was also proven by double fluorescent staining of cell nuclei. Immunostaining for tight junction proteins claudin-4 and -5 showed barrier disruption in cells treated by surfactants at 24 h. In conclusion, Cremophor EL and RH40 in concentrations corresponding to clinical doses caused endothelial and epithelial toxicity. Endothelial cells were more sensitive to surfactant treatment than epithelial cells, and Cremophor EL was more toxic than RH40 in both cell types. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:1173–1181, 2013

Keywords: absorption enhancer; Caco-2 cells; cell toxicity; Cremophor; endothelial; epithelial; excipient; real-time cell microelectric sensing; surfactant; toxicokinetics

INTRODUCTION

Excipients are necessary for effective drug dissolution of pharmacons and their transport across biological barriers.[1] Nonionic surfactants such as Tweens and Cremophor EL and RH40 are widely employed to improve dissolution and delivery of drugs.[2] Their toxicity profile is better than that of ionic surfactants,[3] still during their application, side effects often occur. Cremophor EL and RH40 are polyoxyethylene castor oil derivatives used to formulate several drugs such as antineoplastic paclitaxel, teniposide, and aplidine; immunomodulatory cyclosporine A; sedative diazepam; and antiretroviral lopinavir and ritonavir.[2,4]

Cremophor EL is applied in infusions, such as Taxol, in oral capsules, such as Gengraf, as well as in solutions.[2] Adverse effects observed for Cremophor EL[4] include anaphylactoid hypersensitivity reactions due to complement activation in human plasma.[5] Axonal demyelination[6] and acute toxicity of the heart and thymocytes[7,8] were found in rats. Cremophor EL induced damage in isolated human cancer cells[9] and human cell lines including Caco-2.[10] These toxic effects are related to inhibition of cardiac mitochondrial respiration,[11] cell membrane perturbation,[4,12] and promotion of oxidative stress.[8,13]

Cremophor RH40 is used in microemulsions,[14] in self-emulsifying drug delivery systems,[15] in buccoadhesive tablets,[16] and in oral formulations, such as

Correspondence to: Mária A. Deli (Telephone: +36-62-599602; Fax: +36-62-433133; E-mail: deli.maria@brc.mta.hu)

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Neoral (Novartis, East Hanover, NJ). Recently, our group has used Cremophor RH40 as an absorption enhancer in a nasal drug delivery system.[17] Cremophor RH40 is intensely investigated as a pharmaceutical excipient, but there is little data on its toxicity profile in cultured cells.[18,19]

Despite the use of Cremophors in clinically applied drugs and in other formulations, their toxicity on biological barriers is not fully understood. To our knowledge, there is no published information about the effect of Cremophors on brain endothelial cells, which are directly affected during infusions of drugs and may be related to their side effects.

The aim of the study was to evaluate the toxic effects of Cremophor EL and RH40 on culture models of two important biological barriers. Brain endothelial cells, applied in blood–brain barrier studies,[20,21] and Caco-2 human intestinal epithelial cells, a model of small intestinal barrier,[22] were tested with Cremophors in clinically relevant doses by real-time cell microelectronic sensing (RT-CES), a new label-free method, colorimetric end-point viability assays, and cell morphology.

MATERIALS AND METHODS

Chemicals

All reagents were purchased from Sigma–Aldrich Ltd., Budapest, Hungary, unless otherwise indicated.

Cell Culture

Human cell lines brain microvascular endothelial hCMEC/D3 cells,[23] intestinal epithelial Caco-2 cells (ATCC HTB-37), and primary rat brain endothelial cells were used. D3 cells were grown in Endothelial Basal Medium-2 (EBM-2) containing Endothelial Growth Medium-2 (EGM-2) BulletKit (Lonza, Basel, Switzerland) supplemented with 2.5% fetal bovine serum in a humidified 37°C incubator with 5% CO_2. Caco-2 cells were grown in Eagle's minimal essential medium (Gibco, Invitrogen, Budapest, Hungary and Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% sodium–pyruvate, and 50 μg/mL gentamicin. Primary cultures of cerebral endothelial cells were prepared from 2-week-old rats[24] and used for morphological studies. Cell lines were seeded in culture dishes at a density of 5×10^4 cells/cm^2, and the medium was changed every 2 days. Cells were subcultured with 0.05% trypsin–EDTA solution. For RT-CES, 96-well E-plates with built-in gold electrodes (Roche, Budaörs, Hungary and Basel, Switzerland) were used. Surfaces were coated with 0.05% rat-tail collagen before cell seeding.

Treatment

Cremophor EL and RH40 (pharmaceutical grade; BASF, Lampertheim, Germany) were tested at 0.1–50 mg/mL concentrations. Triton X-100 detergent (10 mg/mL) was used in toxicity assays as a reference compound to cause cell death.

Real-Time Cell Microelectronic Sensing

This technique dynamically monitors living cells.[25,26] The xCELLigence system (Roche) utilizes impedance derived from interaction between cells and electrodes of E-plates to noninvasively quantify cell proliferation and viability in real time. Culture medium (80 μL) was added to each well for background readings, then 80 μL cell suspension was dispensed at the density of 1×10^4 cells/well. Cells were grown until confluency. Impedance was measured every 2 min following treatments. The cell index (CI) at each time point was defined as $(R_n - R_b)/15$, where R_n is the cell-electrode impedance of the well when it contains cells, and R_b is the background impedance of the well with the medium alone. The CIs were normalized to the latest time point before the treatment of each group ($CI_n/CI_{\text{before treatment}}$) or presented as percent of nontreated control group [$(CI_n/CI_{\text{average of control group}}) \times 100$]. CI values reflect cell number, adherence, cell growth, and health.[25,26]

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Dye Reduction and Lactate Dehydrogenase Release Assays

Colorimetric cell viability assays were performed as described above.[27] The yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye is converted by viable cells to purple formazan crystals; therefore, decrease in dye conversion reflects cellular damage. Human endothelial hCMEC/D3 and Caco-2 epithelial cells were seeded on 96-well plates. The confluent monolayers were treated with Cremophors for 1 and 24 h. After treatment, cells were washed with phosphate buffered saline (PBS) solution and incubated with 0.5 mg/mL MTT solution in Dulbecco's modified Eagle's medium for 3 h. Crystals were diluted with dimethyl sulfoxide, and dye conversion was determined by absorbance measurement at 570 nm. Nontreated control group was considered as 100% viable.

Cell death and membrane damage were investigated by detecting the release of intracellular lactate dehydrogenase (LDH) enzyme to the phenol-red-free culture medium. Cells in 96-well plates were treated by Cremophors for 1 and 24 h. The LDH levels in culture supernatants were determined by a kit (Cytotoxicity Detection Kit LDH; Roche). Cytotoxicity was calculated as a percentage of the total LDH release from cells treated with 1% Triton X-100 detergent.