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Research Paper

Immunobiological efficacy and immunotoxicity of novel synthetically prepared fluoroquinolone ethyl 6-fluoro-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate

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ABSTRACT

The present study examined the cytotoxicity, anti-cancer reactivity, and immunomodulatory properties of new synthetically prepared fluoroquinolone derivative 6-fluoro-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate (6FN) *in vitro*. The cytotoxicity/toxicity studies (concentrations in the range 1–100 μM) are focused on the cervical cancer cells HeLa, murine melanoma cancer cells B16, non-cancer fibroblast NIH-3T3 cells and reconstructed human epidermis tissues EpiDerm™. The significant growth inhibition of cancer cells HeLa and B16 was detected. The cytotoxicity was mediated *via* apoptosis-associated with activation of caspase-9 and -3. After 72 h of treatment, the two highest 6FN concentrations (100 and 50 μM) induced toxic effect on epidermis tissue EpiDerm™, even the structural changes in tissue were observed with concentration of 100 μM. The effective induction of RAW 264.7 macrophages cell-release of pro- and anti-inflammatory T_H1, T_H2 and T_H17 cytokines, with anti-cancer and/or anti-infection activities, respectively, has been revealed even following low-dose exposition.

1. Introduction

Generally, fluoroquinolones (FQ) are broad spectrum antibiotics, active against different gram positive and gram negative bacteria, specifically by targeting bacterial DNA gyrase and bacterial topoisomerases (Ahmed and Daneshtalab, 2012; Blasi et al., 2006; Hotinski et al., 2015; Ichiyama et al., 2001; Leibovitz, 2006). The fluorine substitution at the 6-position in all FQ confers greater antibacterial potency and a broader spectrum of activity than that, revealed by nalidixic acid and other nonfluorinated quinolones (*e.g.* cinoxacin, oxilinic acid) (Owens and Ambrose, 2000, 2005; Shalit et al., 2002). Next, the quinolones have been reported to display different biological activities, such as antitumor, anti-HIV-1 integrase, anti-HCV-NS3 helicase and anti-NS5B-polymerase activities. Evidently, the members of quinolone family of drugs comprised three biological activities, mainly antibacterial, anticancer, and the antiviral effectivity (Ahmed and Daneshtalab, 2012; Hasinoff et al., 2012; Owens and Ambrose, 2005; Scatena et al., 2010; Stieglitz et al., 2015). In particular, some members of this drug family display high activity against bacterial topoisomerases, but also against eukaryotic topoisomerases and are toxic to cultured mammalian cells, and *in vivo* and *ex vivo* tumour models. Thus, these cytotoxic quinolones represent an exploitable source of new anticancer agents with the ability to induce apoptosis and cell cycle arrest in various cancer cell lines alone, or in combination with other chemotherapeutic agents (Abdel-Aziz et al., 2013; Alibek et al., 2012; Sissi and Palumbo, 2003). Paul et al. (Paul et al., 2007) demonstrated in their review and meta-analysis, that quinolone prophylaxis administered to cancer patients significantly reduces all-cause mortality.

Several of quinolone derivatives have been explored as an antibiotics and therapeutics for various cancers. The ciprofloxacin and some ciprofloxacin derivatives, analogues and complexes revealed potent *in vitro* anti-tumour activity against several cancer cell lines as leukemia, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, breast cancer (Abdel-Aziz et al., 2013; Suresh et al., 2013; Yadav et al., 2015). Moreover, *in vitro* experiments have demonstrated the bioimmunological efficacy of fluoroquinolones as biological responce modifiers, the agents engaged in the inter- and

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intra-cellular signalling of immune cells, with direct influence on the interleukins' and growth factors' release (Dalhoff and Shalit, 2003; Hamilton-Miller, 2001; Riesbeck and Forsgren, 1994). Modulating effect on immune cells resulting either into the induction, or inhibition of the production and secretion of various cytokines and chemokines had been several times reported with different fluoroquinolones (Dalhoff, 2005; Dalhoff and Shalit, 2003; Kuwahara et al., 2005; Kwiatkowska et al., 2013; Ono et al., 2000; Riesbeck, 2002; Williams et al., 2005; Yadav et al., 2012; Yadav et al., 2015).

It has been demonstrated that trovafloxacin at therapeutic doses suppresses the production of cytokines and growth factors *i.e.* IL-1α, IL-1β, IL-6, IL-10, TNF- α and GM-CSF in monocytes. Ciprofloxacin and pefloxacin at doses lower than 100 mg/L reduced the production of IL-1, ciprofloxacin and ofloxacin at concentrations 25 mg/L reduced the synthesis of TNF- α. The inhibition of mRNA of IL-1α, IL-1β, IL-6, IL-8 and TNF- α has been revealed with grepafloxacin (Khan et al., 1998).

Previously, we have suggested as potentially bioimunologically active formulas 1,4-dihydro-4-oxoquinoline substituted at 4-pyridone and/or benzene moieties. These were assayed for photochemical, cytotoxic and phototoxic activities *in vitro,* the highest cytotoxic/antiproliferative activity on human promyelocytic leukemia cell line has been observed with fluoroquinolone ethyl 6-fluoro-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate (6FN) (Barbierikova et al., 2011; Füzik et al., 2007; Rimarcik et al., 2011).

In this study, the ability of 6FN to inhibit the cell growth of human leukemia HL-60 cells, human cervical cancer cell line HeLa and murine melanoma cancer cells B16 has been evaluated. Next, the 6FN induction of apoptosis and the mechanism of cell death have been studied. The experimental design included also evaluation of 6FN cytotoxicity/toxicity on non-cancer fibroblast NIH-3T3 cells and human derived non-transformed epidermal keratinocytes EpiDerm™. In addition, we investigated the immunomodulatory activity of 6FN on murine macrophage RAW 264.7 cells, based on the cell proliferation and induced release of pro-inflammatory and anti-inflammatory interleukins, growth factors and determination of oxidative stress.

2. Material and methods

2.1. Reagents

The ethyl 6-fluoro-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate (6FN) (Fig. 1) has been prepared by Rimančík et al. (Rimarcik et al., 2011) according to modified procedure described in Černuchová et al. (Cernuchova et al., 2004) starting from 2-nitro-4-fluoroaniline and diethyl ethoxymethylenemalonate. Elemental analysis were satisfactory for C, H, N within +/− 0.3% limit. IR and UV spectra are discussed in Rimarčík et al. (Rimarcik et al., 2011).

The stock solution of 6FN was dissolved in 100% dimethylsulfoxide (DMSO), and further diluted in the cell culture medium. The final concentration of DMSO added to cells never exceeded 0.1% (vol/vol). DMSO was purchased from Merck (Slovakia). Albumine bovine pure was from Koch (Germany), Nonidet NP 40 Substitute from Fluka

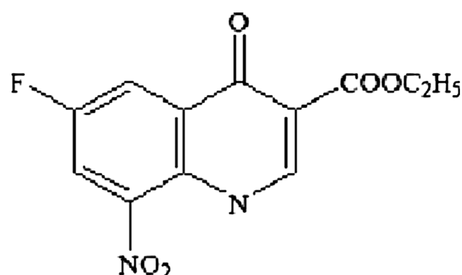

Fig. 1. Chemical structure of the ethyl 6-fluoro-8-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate.

(Germany) and Ultra Safe Blue from Syngene (UK). CellROX® Green was purchased from Invitrogen (USA). Dulbecco's modified eagle medium (DMEM) was from Invitrogen (Germany), fetal bovine serum (FBS) were from PAA Laboratories GmbH (Austria). Fetal calf serum (FCS) was from Biocom (Slovakia). ViaLight™ plus kit has been purchased from Lonza (USA). Quantikine ELISA® Mouse M-CSF, was provided by R & D Systems (USA); Platinum ELISA: Mouse IL-12, Mouse GM-CSF, Mouse IL-6, Mouse IL-17, Mouse IL-2 and Instant ELISA®: Mouse IL-1β, Mouse TNF-α, Mouse IL-10, were purchased from Affymetrics eBioscience, Bender MedSystems GmbH (Austria). Free radicals kit was from Sedium R & D (Czech Republic). Concanavalin A was purchased from Sigma-Aldrich (USA).

Propidium iodide, Triton-X 100, Trypan blue, RNase A, cisplatin (*cis*-diamminedichloroplatinum(II), GoodView™, DNA ladder marker, reduced Nicotinamide adenine dinucleotide (NADH), Ethylenediaminetetraacetic acid (EDTA), Thiazoyl Blue Tetrazolium Bromide, agarose, glycine, Phosphate buffered saline (PBS), Bromphenol Blue sodium salt, glycerol, DL-Dithiothreitol, Hoechst 33258, penicilin and streptomycin were obtained from Sigma-Aldrich (Slovakia). 96% ethanol, 85% H_3PO_4 p.a., 36% HCl p.a., formaldehyde (36–38%) p.a., NA_2-HPO_4. $12H_2O$, NaCl, KCl, glucose p.a., Sodium pyruvate were from Lachema (Czech Republic). Proteinase K was obtained from Biotech (Slovakia). Tris(hydroxymethyl)aminomethane (Tris) ultrapure was from PAN Biotech (Germany).

2.2. Cell cultures and 3D human skin model

RAW 264.7 cell line murine macrophages (ATCC® TIB-71™, ATCC, UK) were used for immunobiological studies. The cells were cultured in complete DMEM (DMEM (high glucose), 10% heat inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin) for 24 h (until approx 80 percentage confluence) at 37 °C in an *atmosphere* of 5% CO_2 and at 90–100% relative *humidity*. Cell viability has been assessed using the trypan blue exclusion method.

Human promyelocytic leukemia cells HL-60, human cervical cancer cell line HeLa, murine melanoma cancer cells B16 and non-cancer murine fibroblast cell line NIH-3T3 (all obtained from ATCC, Rockville, MD, USA) were used for cytotoxicity tests. The cells were grown at 37 °C in RPMI medium (HL-60) or DMEM medium (HeLa, B16 and NIH-3T3 cells) in humidified 5%-CO_2 and 95%-air atmosphere. The medium was enriched with 10% FBS (HeLa cells) or heat-inactivated FCS (HL-60, B16 and NIH-3T3 cells), penicillin G (100 U/mL) and streptomycin (100 μg/mL). The HL-60 cells grew in suspension and were subcultivated three times a week. The suspension of these cells was then resuspended and the total number of cells and their viability were measured. Before a uniform monolayer of HeLa, B16 and NIH-3T3 cells was formed, cells were detached from the surface of the culture dish by a 0.25% solution of trypsin, and were subcultivated two-three times a week. The cells were plated on the Petri dishes (diameter 60 mm) and incubated for 24 h prior to the experiments. Cell viability was determined by a trypan blue exclusion test.

Reconstructed human three-dimensional skin constructs EpiDerm™ (EPI-200) were used for toxicity test. The reconstructed human epidermis tissues (EpiDerm™) were obtained from MatTek Corp. (Bratislava, Slovakia). Upon arrival, the EpiDerm™ tissues were transferred to 6 well plates (Sarstedt, Slovakia) containing 0.9 mL Maintenance Media (a proprietary media provided by MatTek Co., and included with the tissues). The tissues were then equilibrated overnight at 37 °C, 5% CO_2 in a humidified incubator prior to exposure to test substances.

2.3. Immunobiological activity of 6FN

Immunobiological activity of 6FN has been evaluated *via* stimulation induced proliferative activity and trigerred release of pro-inflammatory and anti-inflammatory interleukins, growth factors and by

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