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Structures of isothiocyanates attributed to reactive oxygen species generation and microtubule depolymerization in HepG2 cells

Piman Pocasap[a], Natthida Weerapreeyakul[b,*], Kanjana Thumanu[c]

[a] Research and Development of Pharmaceuticals Program, Graduate School, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand
[b] Division of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002 Thailand
[c] Synchrotron Light Research Institute (Public Organization), Nakhon Ratchasima, 30000, Thailand

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ABSTRACT

The structure of the isothiocyanates (ITCs)—erucin, sulforaphane, erysolin, sulforaphene, and phenethyl isothiocyanate—were assessed as well as their respective *in vitro* anticancer activity on the hepatocellular carcinoma cell line HepG2. All of these ITCs induced both apoptotic and necrotic cell death. FTIR analysis indicated that the ITCs caused changes in cellular components comparable to vinblastine. Despite no observable effect on DNA, the ITCs all induced generation of intracellular reactive oxygen species (ROS) and suppressed microtubule polymerization. The variation in sulfur oxidation states and the presence of an aromatic ring on the ITC side chain affected microtubule depolymerization and intracellular ROS generation, leading to apoptotic and necrotic cancer cell death. Knowing the influences of structural variations of the ITC side chain would be useful for selecting the more potent ITCs (*i.e.*, erysolin) for the design and development of effective chemopreventive agents.

1. Introduction

Isothiocynates (ITCs) are a group of compounds characterized by the presence of an isothiocynate (−N═C═S) moiety on the molecule. ITCs are important contributors to biological activity. In humans, ITCs have been extensively investigated for their health-promoting benefit, especially against cancer. The consumption of cruciferous vegetables is well-correlated with the reduction in the incidence of several types of cancer [1].

As the unique phytochemical components in cruciferous vegetables, ITCs are recognized as being responsible for their chemopreventive benefits. ITCs exert their anticancer properties *via* both preventive and therapeutic activity. For preventive activity, ITCs inhibit phase I carcinogen-activating enzymes and induce phase II carcinogen-detoxifying enzymes [2]. The dual activities result in a reduction in the level of intracellular carcinogens and thus the reduced likelihood of developing cancer. As a therapeutic, ITCs could inhibit cancer cell proliferation by the induction of cancer cell cycle arrest and apoptosis. The compounds also inhibit the process of angiogenesis and metastasis [3]. ITCs are, thus, of interest as chemotherapeutic agents.

The various and variable anticancer activities of ITCs have been widely reported. Some of these studies indicated that the impact of structural variations among ITC derivatives on anticancer activity could differ with the mechanism of biological activity and the cell type assessed [4–6]. Nevertheless, the studies on the anticancer activity of ITCs related to structural variations mainly focus on enzyme induction in phase II metabolism or relevant antioxidant mechanisms [5–7]. There are few, if any, compelling studies demonstrating the relationship between the respective ITC structures and their anticancer effects. Erysolin was more potent than erucin in induction of intracellular ROS as well as increasing cancer cell death in colon cancer cell line HCT116 [8]. In another study, aromatic ITCs were more effective than aliphatic ITCs on depleting mutant p53, tumor suppressor protein, in breast cancer cell lines [9]. However, the direct link between mutant p53 and cancer cell death was not yet elucidated in the study.

In our study, the impacts of structural variation on cell death inducing activity were investigated *via* the relationship of sulforaphane analogues with three molecular targets including DNA, reactive oxygen species (ROS), and tubulin in the hepatocellular carcinoma cell line HepG2. The interaction of ITCs to these molecular targets could

Abbreviations: AR, presence of an aromatic ring; CD, circular dichroism; DAPI, 4′-6-diamidino-2-phenyl indole; DB, number of double bonds; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; FTIR, fourier-transform infrared spectroscopy; GSH, glutathione; IC_{50}, inhibitory concentration at 50%; ITC, isothiocyanate; NBP, 4-(4′-nitrobenzyl) pyridine; NR, neutral red; OxS, oxidation state of sulfur; PCA, principal component analysis; Phen ITC, phenethyl isothiocyanate; ROS, reactive oxygen species

* Corresponding author at: Division of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, 123 Mittrapap Road, Amphoe Muang, Khon Kaen University, Khon Kaen 40002, Thailand.

E-mail addresses: piman.pocasap@kkumail.com (P. Pocasap), natthida@kku.ac.th (N. Weerapreeyakul), kanjanat@slri.or.th (K. Thumanu).

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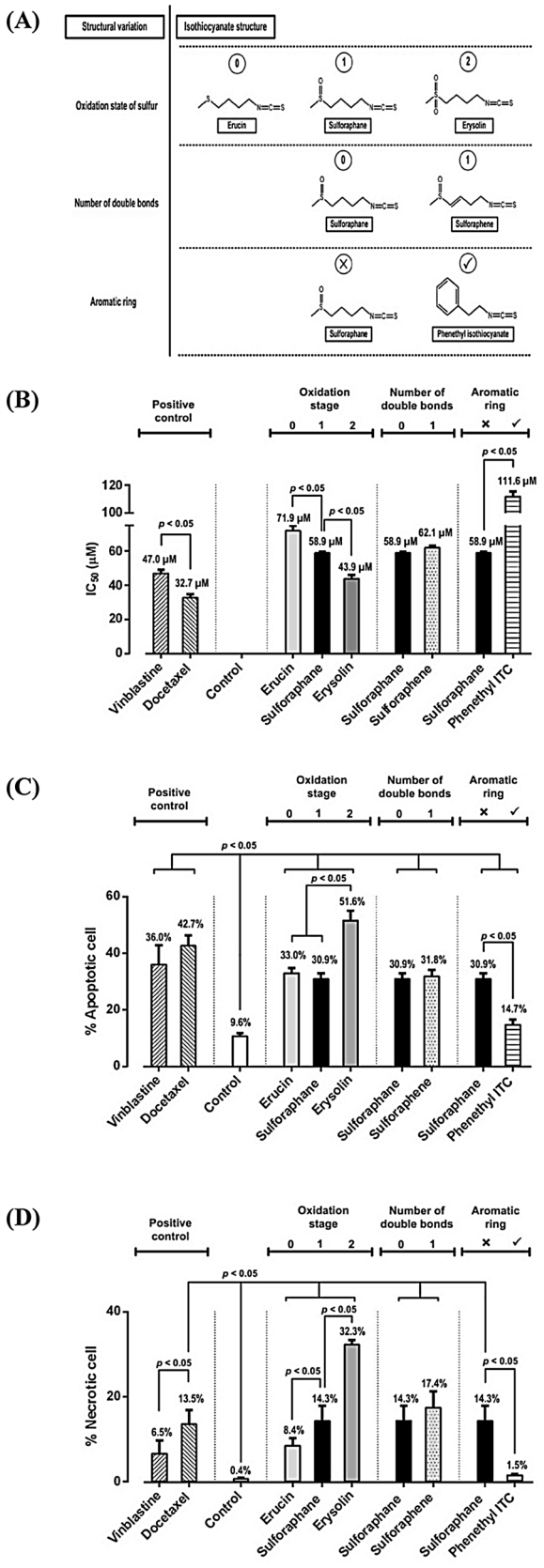

Fig. 1. Relationship of structure and anticancer activity of isothiocyanates (ITCs) against HepG2 cells. Comparison of ITCs (viz., erucin, sulforaphane, erysolin, sulforaphene, and phenethyl isothiocyanate (phenethyl ITC)) against HepG2 cells and chemotherapeutic agents (vinblastine and docetaxel) for 24 h. Note: structure (A), cytotoxicity (B), % apoptotic cells (C), and % necrotic cells (D). Cytotoxicity represented as the IC_{50}. Apoptotic and necrotic cells displayed as a percentage relative to total cell population by flow cytometry. Respective value expressed as mean ± SD ($n = 3$). $p < 0.05$, significant difference by one-way ANOVA calculated for each group with positive control (B) or control (C and D).

consequently lead to cancer cell death [10]. All of the ITCs examined in the current study are naturally occurring in cruciferous vegetables. Sulforaphane was thus used for a comparison with the other ITCs because it has been extensively studied [11]. The other ITC analogs—erucin, erysolin, sulforaphene, and phenethyl isothiocyanate (phenethyl ITC)—were selected based on the structural difference at the side chain; oxidation state of sulfur, number of double bonds, and the presence of an aromatic ring (Fig. 1). The side-chain variations are common among naturally-occurring ITCs. The C2 side chain is able to form a goitrin derivative *via* cyclization [12]. None of the selected ITCs have a hydroxyl moiety at the C-2 position, so have no goitrogenic effect. The knowledge from the study could explain the influence of structural variation of ITC derivatives on the respective chemotherapeutic action; and also provide useful information for developing more effective chemotherapeutic agents through taking advantage of structural modification.

2. Materials and methods

2.1. Materials

Sulforaphane was purchased from Calbiochem (EMD, Darmstadt, Germany). Sulforaphene was purchased from Enzo Life Science (Farmingdale, NY, USA). Erucin and phenethyl ITC were purchased from Abcam (Cambridge, MA, USA). Erysolin was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Propidium iodide (PI) was purchased from BioLegend. (San Diego, CA, USA). Anti-Tubulin antibody (ab195883) was purchased from Abcam (Cambridge, MA, USA). Bovine serum albumin was purchased from Amresco (Solon, OH, USA). Triton-X was purchased from USB Corp (Cleveland, OH, USA). Glycine and Tris were purchased from Vivantis (Selangor Darul Ehsan, Malaysia). Highly-polymerized calf thymus DNA, goat serum, RNase, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), and 4′-6-diamidino-2-phenyl indole (DAPI) were of analytical grade and purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). 4-(4′-Nitrobenzyl) pyridine (NBP) and the standard anticancer drug melphalan were purchased from Sigma–Aldrich (St. Louis, MO, USA). Docetaxel from Hospira (Lake Forest, IL, USA), vinblastine from Naprod (Mumbai, India), and cisplatin from Boryung Pharm (Gyeonggi-do, Korea) were clinical grade. These chemotherapeutic drugs were used as positive controls.

The reagents used in the cell-based assay were of molecular biological grade. The reagent and culture media (RPMI-1640), fetal bovine serum (FBS), penicillin and streptomycin, and 0.25% Trypsin-EDTA (1X) were bought from GIBCO®, Invitrogen (Grand Island, NY, USA). All other chemicals and solvents were of reagent grade and used without purification.

2.2. Cell lines and culture

The human hepatocellular carcinoma cell line HepG2 was purchased from American Type Culture Collection (ATCC# HB-8065) (Manassas, VA, USA). The HepG2 cell line was cultured in RPMI-1640, supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. The cell line was incubated at 37 °C with 95% air and 5% CO_2.