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Indole-3-carbinol induces cMYC and IAP-family downmodulation and promotes apoptosis of Epstein–Barr virus (EBV)-positive but not of EBV-negative Burkitt's lymphoma cell lines

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ABSTRACT

Indole-3-carbinol (I3C) is a natural product found in broadly consumed plants of the *Brassica* genus, such as broccoli, cabbage, and cauliflower, which exhibits anti-tumor effects through poorly defined mechanisms. I3C can be orally administered and clinical trials have demonstrated that I3C and derivatives are safe in humans. In this study we show that I3C efficiently induces apoptosis in cell lines derived from EBV-positive Burkitt's lymphomas (virus latency I/II), while it does not have any cytotoxic activity against EBV-negative Burkitt's lymphomas and immortalized EBV-infected lymphoblastoid cell lines (virus latency III). The effect of I3C in EBV-positive Burkitt's lymphoma is very specific, since only I3C and its C6-methylated derivative, but not other 3-substituted indoles, have an effect on cell viability. I3C treatment caused apoptosis characterized by loss of mitochondria membrane potential and caspase activation. I3C alters the expression of proteins involved in the control of apoptosis and transcription regulation in EBV-positive Burkitt's lymphoma cell lines. Among those, cMYC, cIAP1/2 and XIAP downmodulation at mRNA and protein level precede apoptosis induction, thus suggesting a role in I3C cytotoxicity. We also showed that I3C and, more particularly, its condensation dimer 3,3′-diindolylmethane (DIM) prolonged survival and reduced tumor burden of mice xenotransplanted with EBV-positive Burkitt's lymphoma Daudi cells.

In summary these results, together with previous reports from clinical trials indicating the lack of toxicity in humans of I3C and derivatives, support the use of these compounds as a new therapeutic approach for treating patients with endemic (EBV-positive) Burkitt's lymphoma.

Background

Indole-3-carbinol (I3C) is a glucobrassicine derivative that is found in plants of the Cruciferae family, particularly in members of the *Brassica* genus, such as broccoli, cabbage, and cauliflower. I3C is an orally bioactive compound that is formed by the hydrolysis of glucosinolate by the plant enzyme myrosinase [1]. Several studies have described the activity of I3C and both natural and synthetic derivatives as chemopreventive and anti-tumor agents, regulating processes such as inflammation, cell proliferation, and tumor invasion [2–4]. Indeed, I3C has been shown to inhibit proliferation and induce apoptosis of a variety of tumors and cell lines of breast, endometrium, prostate, and colon cancer (reviewed in [2]).

Several studies have assessed the molecular mechanisms involved in I3C anti-tumor activities (reviewed in [2]). It has been shown that I3C and derivatives are potent estrogen and androgen receptors antagonists and aryl hydrocarbon receptor agonists. Also,

Abbreviations: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; BCL, B cell lymphoma; BIR, baculovirus IAP repeat; BL, Burkitt's lymphoma; CFLAR, CASP8 and FADD-like apoptosis regulator; cFLIP, Fas-associated death domain protein-like interleukin-1β-converting enzyme inhibitory protein; cIAP, cellular inhibitor of apoptosis; DIM, 3,3′-diindolylmethane; EBV, Epstein–Barr virus; EBNA, Epstein–Barr virus nuclear antigen; I3C, indole-3-carbinol; LCL, lymphoblastoid cell line; LMP, Epstein–Barr virus latent membrane protein; NOD, non-obese diabetic; PARP, poly(ADP-ribose) polymerase; SCID, severe combined immunodeficiency; TCL, T cell lymphoma; XIAP, X-linked inhibitor of apoptosis.

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I3C could revert multiple drug resistance in K562 erythroleukemic cells by downregulating P-glycoprotein. I3C and oligomers are potent AKT inhibitors and disrupt AKT-dependent survival pathways. Interestingly, Takada and coworkers [5] showed that I3C suppressed NFκB activation and prevented TNF-mediated induction of NFκB-regulated genes encoding proteins involved in the control of cell proliferation and apoptosis, including cyclin D1, IAP family and BCL2 family proteins, among others.

Previous reports have proposed that the biological activity of I3C is associated with its capacity of forming oligomers in acidic media, such as the stomach milieu [2,6]. Studies in human patients revealed that I3C and derivatives can be efficiently administered orally [7,8]. Importantly, concentrations at μM range of I3C-conjugation products could be reached in plasma and accumulate in tissues after oral intake of these compounds without any significant side-effect on the health of the patients [7] and mice [9,10]. Most importantly, clinical trials assessing the effect of oral administration of I3C and its dimer 3,3′-diindolylmethane (DIM) have demonstrated their safety in humans [11]. Most interestingly, there is clinical evidence of the effectiveness of I3C in promoting regression of papillomavirus-dependent precancerous lesions of cervix [12] and larynx [13–15].

Burkitt's lymphoma is a highly aggressive form of non-Hodgkin's B-cell lymphoma that is usually found in extranodal sites or presenting as an acute leukemia [16–18]. Burkitt's lymphomas are often classified in three main clinical variants: 1) the endemic variant, which constitutes the most common childhood malignancy in equatorial Africa and that is associated with Epstein–Barr virus (EBV) infection, a member of the herpesvirus family, 2) the sporadic variant, found in other regions of the world and also frequently affecting children, and 3) the immunodeficiency-associated variant, common in AIDS patients.

Available treatment for Burkitt's lymphoma result in about 80% remission in patients with localized disease, although this success is reduced to 50% in children with more widespread disease. Furthermore, current treatment involves intensive combination chemotherapy, the need for intrathecal treatment, and risk of tumor lysis syndrome, which precludes its use in the elderly and limits access to treatment to patients in developing countries [19]. For those reasons, it is a necessity to develop new therapies that facilitate access to treatment and minimize therapy-associated toxicity.

In the present study, we have studied whether I3C and other 3-substituted indoles have anti-tumor activity on Burkitt's lymphoma. Our results show that only I3C and its derivatives are effective in reducing the viability of both latency I and II EBV-positive Burkitt's lymphomas both *in vitro* and *in vivo*, while having no effect on EBV-negative BL cell lines. Our data indicate that I3C cytotoxic activity involves cMYC and IAP family downmodulation, with subsequent induction of apoptosis. Altogether our results suggest that these compounds might provide a new therapeutic approach for treating patients with endemic (EBV-positive) Burkitt's lymphoma.

Materials and methods

Reagents and antibodies

Indole compounds were purchased from Sigma–Aldrich (St. Louis, MO). Mass spectrometry analysis of indole-3-carbinol (I3C; 1*H*-indole-3-methanol) and 3,3′-diindolylmethane (DIM) confirmed the purity (>95% and >98%, respectively) and integrity of these compounds as reported by the supplier. The rest of reagents and antibodies are described in supplementary Materials and methods.

Cell lines and cell culture

Burkitt's lymphoma B cell lines BL-2, BL-41, BL-60.2, Daudi, DG-75, Mutu-1, Raji, Jijoye, Namalwa, Rael, Akata and Ramos and the immortalized EBV-infected lymphoblastoid cell lines JY, Dana, Alewife and IB-4 were cultured in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum (FCS; Life Technologies, Madrid, Spain), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Microsatellite analyses of cell lines are provided in supplementary Table T1.

LMP1 expression analysis

Intracellular LMP1 expression was determined using a commercial fixation/permeabilization kit (Fitx&Perm; Invitrogen Life Technologies), following the manufacturer's instructions. A detailed protocol is provided in supplementary Materials and methods.

Viability assays

Cells (10^6 cells/ml; 100 μl/well) were incubated in 96-well microtiter plates and cultured in the presence of the indicated reagents. Viability was determined at 24 and 48 h by using the kit CellTiter 96© AQ_{UEOUS} Assay (Promega Biotech Iberica, Madrid, Spain), following the manufacturer's instructions. The spectrophotometric absorbance of each sample was measured at 490 nm using the BioTek Synergy Mx microplate reader (BioTek Instruments, Winooski, VT).

Apoptosis detection

Apoptosis was determined by annexin V/PI staining. Briefly, cells ($2–5 \times 10^5$ cells) were harvested and incubated with FITC-labeled annexin V and 1 μg/ml PI in binding buffer (5 mM $CaCl_2$, 10 mM Hepes, and 140 mM NaCl). After 15 min in the dark, they were analyzed by flow cytometry.

Western blot analysis

Western blot was carried out as previously described [20]. A detailed description is provided in supplementary Materials and methods. Densitometry was performed on scanned images using Quantity One® software (Bio-Rad Laboratories), and values were normalized for corresponding controls of each experiment.

Determination of mitochondrial depolarization

Mitochondrial transmembrane potential ($\Delta\Psi_m$) was assessed by staining with TMRM. Cells (10^6 cells/ml) were preincubated with 250 nM TMRM for 20 min and then incubated in the presence of the different reagents. At indicated times cells were washed with PBS and analyzed by flow cytometry using a FC 500 MPL cytofluorimeter and the CXP software version 2.1 (Beckman Coulter Inc., Fullerton, CA). An excitation wavelength of 488 nm and observation wavelength of 585 nm for red fluorescence were used for analyzing mitochondrial depolarization, which is indicated by the number of cells losing red fluorescence. The protonophore CCCP (25 μM) was used as a positive control.

Quantitative polymerase chain reaction (Q-PCR)

Total RNA was extracted from 5×10^6 BL-60.2 ($n = 3$) or Ramos ($n = 1$) cells using TRI_{ZOL} reagent and the PureLink™ RNA mini kit (Ambion, Life Technologies). Q-PCR was carried out by the Service of Genomics from IIB by means of the Applied Biosystems 7900HT

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