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Effect of indole ethyl isothiocyanates on proliferation, apoptosis, and MAPK signaling in neuroblastoma cell lines

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Abstract—Several indole ethyl isothiocyanate (**IEITC**) analogs were designed, synthesized, and screened to evaluate their cytotoxicity against neuroblastoma (**NB**) cells in-vitro. In **NB**, predominantly a tumor of early childhood, survival remains low despite aggressive treatments. Therefore, novel treatment strategies are greatly needed. The objective of the present study was to study the therapeutic potential of **IEITC** by analyzing the cytotoxic, anti-proliferative, and apoptotic effects on **NB** cell lines. 7-Methyl-indole-3-ethyl isothiocyanate (**7Me-IEITC**) proved to be cytotoxic to various **NB** cell lines (SMS-KCNR, SK-N-SH, SH-SY5Y, and IMR-32) with an IC_{50} at 2.5–5.0 μM, while primary control cells (lung fibroblasts) were not affected. **7Me-IEITC** led to the activation of apoptotic markers caspase-3, -8, and -9, caused activation of pro-apoptotic p38 MAPK and SAP/JNK, and down-regulated pro-survival factor AKT in SMS-KCNR cells. Moreover, **7Me-IEITC** displayed anti-proliferative effects (IC_{50} at 600 nM) and caused an arrest in cell cycle progression. This wide effect of **7Me-IEITC** on **NB** cell signaling and survival suggests that it could be developed as a therapeutic agent against neuroblastoma.

Neuroblastoma (**NB**), predominantly a tumor of early childhood, is the most common extracranial solid tumor. Two-thirds of the cases occur in children younger than 5 years of age. **NB** accounts for 7–10% of all childhood cancers; in the majority of patients older than 1 year of age the disease is fatal.[1] There are approximately 500–1000 new cases of **NB** in the U.S. each year.[2] Treatment methods currently available include surgery, radiation therapy, chemotherapy, and autologous stem-cell transplantation.[3–5] However, despite intensive multimodality treatment, more than 50% of children with high-risk disease relapse, due to drug-resistant residual disease.[6–8] Eradication of refractory microscopic disease remains one of the most significant challenges in the treatment of the high-risk **NB** and innovative treatments for children with neuroblastoma need to be developed.

Isothiocyanates (**ITCs**) are currently being investigated as anti-tumor agents and in animal models **ITCs** have been shown to inhibit chemically induced tumor genesis in the lung, stomach, colon, liver, esophagus, bladder, and mammary glands.[9] Natural **ITC**s exist as glucosinolates in plants and their release is catalyzed by myrosinase enzymes. Several mechanisms for the activities of **ITC** in cancer treatment have been proposed, such as (i) induction of apoptosis and G2/M cell cycle block,[10] (ii) inhibition of phase-I and -II carcinogen-activating enzyme,[9] (iii) reduction of NF-kB binding to DNA,[11] (iv) inhibition of histone deacetylase,[12] and (v) up-regulation of thioredoxin reductase-1.[13] Various other effects such as disruption of microtubulin polymerization[14] and disruption of the mitochondrial membrane potential have been reported.[15] Interestingly, various **ITCs** such as naphthyl ITC (**NITC**), phenethyl ITC (**PEITC**), and benzyl ITC (**BITC**) (Fig. 1a) inhibit activation and/or function of factors implicated in emergence of multi-drug resistance.[16]

Naturally occurring non-**ITC** indole derivatives exhibit potent anti-proliferative activity, induce apoptosis, and cause cell cycle arrest in many human solid and non-so-

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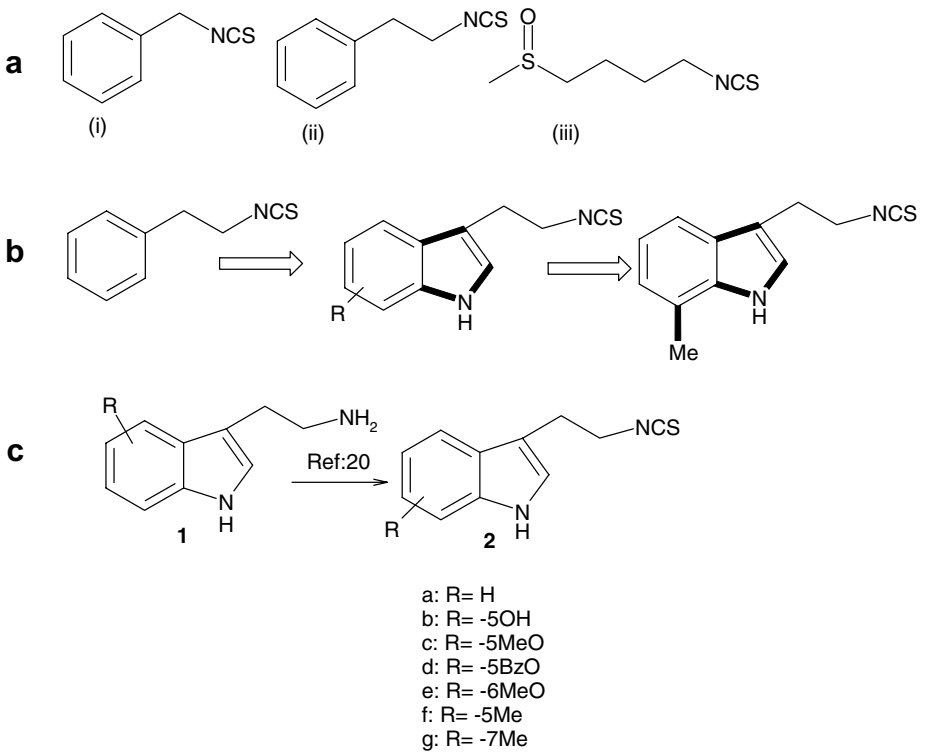


Figure 1. Naturally occurring ITC; design and structure of novel indole ethyl ITC (IEITC): (a) various naturally occurring **ITC**: (i) BITC; (ii) PEITC; (iii) sulforaphane . (b) Design of novel **IEITC** including **7Me-IEITC** (**2g**). (c) Synthesis of **IEITC**: Tryptamines, Thiophosgene (1.1 equiv), 20% $NaHCO_3$, EtOAC + H_20, 1 h; yield: 70–75% after purification through preparative thin layer chromatography.

lid tumors.[17] The objective of the present study was to identify an isothiocyanate class (based on an indole scaffold) with improved anti-cancer activity. We observed that indole ethyl isothiocyanates (**IEITCs**) are structurally very close to benzyl isothiocyanate (**BITC**) and phenyl ethyl isothiocyanate (**PEITC**) that display anti-cancer activity.[16,18,19] The present report describes the syntheses and cytotoxic activities of seven **IEITC** analogs in SK-N-SH **NB** cell lines to determine if **IEITC**s are potential anti-**NB** drugs. We analyzed the effect of the highly cytotoxic compound **7Me-IEITC** on the viability of four **NB** cell lines. In addition, we investigated the therapeutic potential of **7Me-IEITC** by analyzing its effects on caspase activation, activation of pro-apoptotic markers (JNK, p38), suppression of pro-survival marker Akt, and on cell proliferation and cell cycle progression in SMS-KCNR **NB** cells.

Various commercially available tryptamine derivatives were converted to corresponding isothiocyanates in one step (70–75% yield after purification) by following a recent protocol.[20] The derivatives (Fig. 1c) were characterized by IR, NMR, and mass spectrometry. As an initial approach to evaluation of the cytotoxicity of indole ethyl ITC analogs, all derivatives were screened against the SK-N-SH **NB** cell line[21] in a cell viability assay (CellTiter 96® AQ_{ueous} Cell Assay; Promega Corp., Madison, WI).[22] An interesting **IEITC** structure–activity relationship emerged. Substituting a phenyl ring (**PEITC**; Fig. 1a) for an indole ring (compound **2a**) leads to a significant increase in cytotoxicity (Fig. 2). The nature and position of further substitutions on the indole moiety affected the cytotoxic activity significantly: we observed that polar groups such as –OH (**2b**) and –OMe (**2c**) did not significantly increase the cytotoxicity (**2a**), whereas Me– (**2e** and **2g**) and Benzyloxy (BzO) (**2d**) substituents dramatically increased the cytotoxic activity against **NB** cells (IC_{50} below 2.5 μM; Fig. 2). We hypothesize that non-polar group substitutions at the 5- or 7-positions are essential for the biological activity of this family of compounds.

Compounds **7Me-IEITC** (**2g**) and **5BzO-IEITC** (**2d**) were subsequently screened against three additional neuroblastoma cell lines[21] (SMS-KCNR, IMR-32, and SH-SY5Y; Fig. 3). Both compounds **2d** and **7Me-IEITC** (**2g**) dose-dependently reduced the viability of all **NB** cell lines. **7Me-IEITC** (**2g**) was selectively cytotoxic for **NB** cells. The viability of primary lung fibroblasts (LF, passage 10), which like **NB** cell lines possess a high metabolism and growth rate and, thus, were used as controls, was not affected by **7Me-IEITC** treatment (Fig. 3). Even though **7Me-IEITC** (**2g**) displayed IC_{50} cytotoxicity values (50% death as compared to the untreated control) between 2.5 and 5.0 μM in various **NB** cell lines, fibroblasts were not significantly affected even at drug concentrations as high as 20 μM (Fig. 3).

To define the cellular response of **NB** cells to treatment with **7Me-IEITC** (**2g**) we next analyzed the expression and/or activation of cellular markers that are hallmarks of pro-survival (Akt), pro-apoptotic signaling (JNK, p38 MAPK) or directly indicate apoptotic responses (such as caspase-3, -8, -9, and PARP-1).

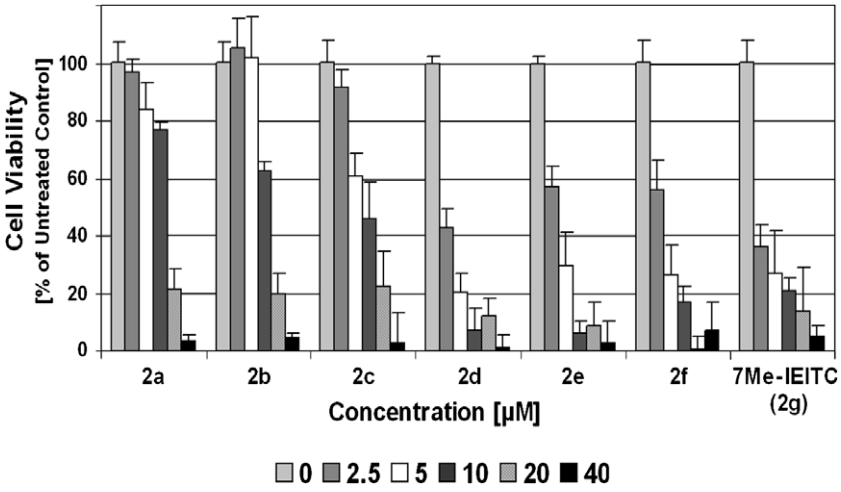


Figure 2. Comparative analysis of the cytotoxic effect of various IEITC analogs in a human NB cell line (SK-N-SH). SK-N-SH **NB** cells were treated with various concentrations (2.5–40μM) of **IEITC**s **2a**–**2g** for 48 h. A viability assay (Promega Corp., Madison, WI) was carried out.[21] Experiments were performed in triplicate; data are expressed as means of the triplicate determinations ($X \pm$ SD) of a representative experiment in % cell viability of untreated cells [100%].

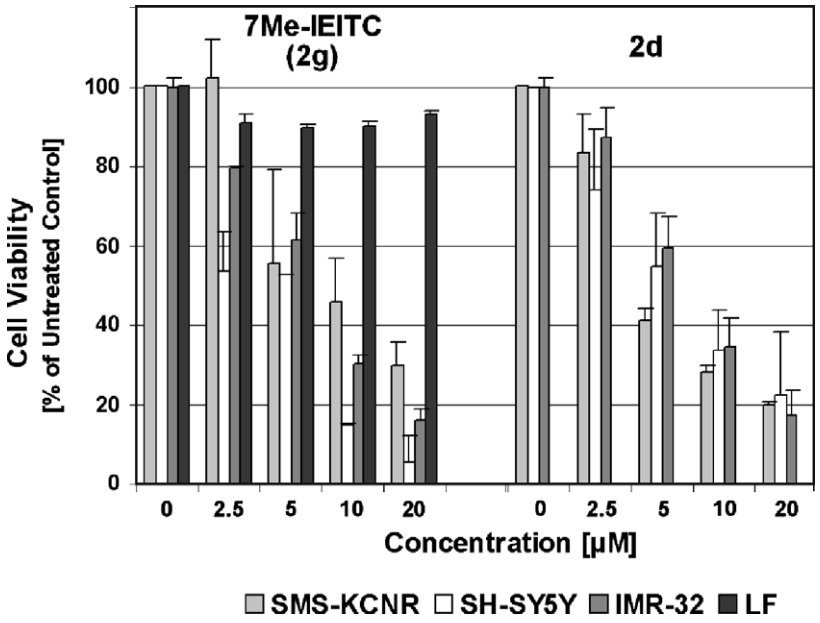


Figure 3. Comparative analysis of the cytotoxic effect of 5BzO-IEITC and 7Me-IEITC in various NB cell lines. **NB** cells (SMS-KCNR, SH-SY5Y, and IMR-32) and primary fibroblasts (LF, control cells) were treated with various concentrations (2.5–40 μM) of **5BzO-IEITC** (**2d**) or **7MeIEITC** (**2g**) for 48 h and the MTS viability assay was carried out. Experiments were performed in triplicate; data are expressed as means of the triplicate determinations ($X \pm$ SD) of a representative experiment in % cell viability of untreated cells [100%].

The effect of **7Me-IEITC** (**2g**) on the activity of JNK, p38 MAPK, and Akt was studied by immunoblotting of PAGE separated lysates of treated cells using antibodies[23] specifically recognizing the inactive, as well as the phosphorylated active form of these proteins. **7Me-IEITC** (**2g**) caused a rapid (within 1 h), strong and sustained activation (peak at 18 h) of p38 and JNK, along with a delayed up-regulation of non-phosphorylated p38 and JNK (Fig. 4a). This figure also shows that SMS-KCNR cells show a high basal level of activation (phosphorylation) of the pro-survival factor Akt in untreated cells, which within 36 h of **7Me-IEITC** (**2g**) treatment was down-regulated.

Akt plays important role in cell survival and proliferation and has been strongly implicated in development of resistance against chemotherapy agents such as Paclitaxel, Cisplatin, Vincristine, and Rapamycin in various human solid tumors.[24] In contrast, JNK and p38 MAPK are involved in the apoptotic response to cytotoxic agents.[25] Activation of p38 and JNK has been observed in human breast cancer cells treated with AplidinTM[26] a depsi-peptide molecule undergoing Phase-II clinical trials. JNK mediates the apoptosis induced by DNA-damaging drugs such as Etoposide (VP-16) and Camptothecin in human myeloid leukemia cells[27] and of Vinblastine in KB3 lung carcinoma cells.[28]

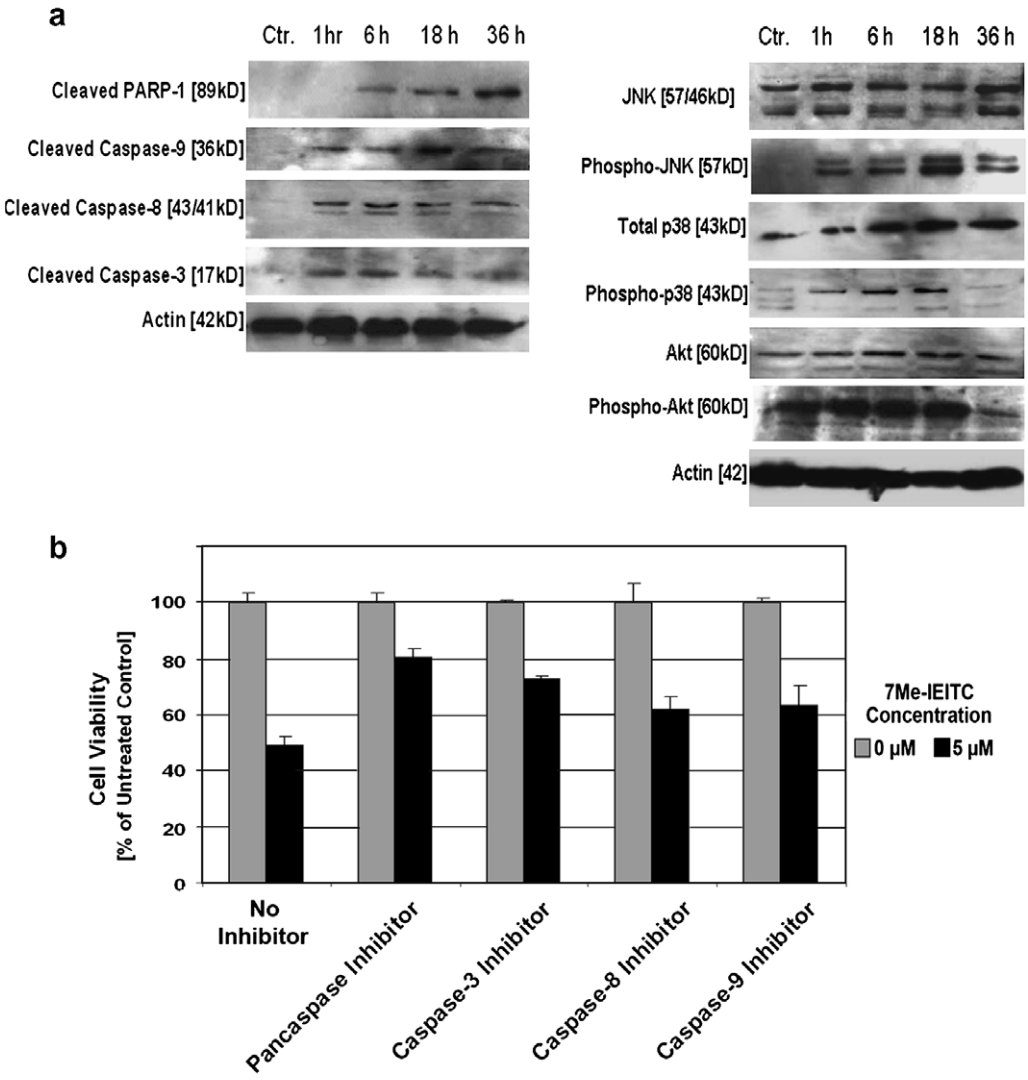


Figure 4. (a) Expression of apoptotic and pro-survival markers in NB cells after 7Me-IEITC treatment: SMS-KCNR **NB** cells were treated with 3 μM **7Me-IEITC** (**2g**). Detection of proteins in the lysates of treated and untreated cells by PAGE and Western blot analysis was carried out as described.[23] *Caspase activation:* primary antibodies against pro- and activated caspase-3, -8, -9, and inactivated/cleaved PARP-1. As an internal standard for equal loading, blots were probed with an anti-beta-Actin antibody. *Kinase activation:* primary antibodies against phosphorylated and inactive JNK, p38, and AKT. (b) Effect of various caspase inhibitors on the cytotoxicity of 7Me-IEITC in SMS-KCNR cells: SMS-KCNR **NB** cells were pre-incubated with 40 μM caspase inhibitors (Z-VAD-FM/pancaspase, Z-DEVD-FMK/caspase-3, Z-IETD-FMK/caspase-8, and Z-LEHD-FMK/caspase-9) for 2 h. **7Me-IEITC** (5.0 μM) was added to the cells for 48 h and cell viability assessed. Experiments were performed in triplicate; data are expressed as means of the triplicate determinations ($X \pm$ SD) of a representative experiment in % cell viability of untreated cells [100%].

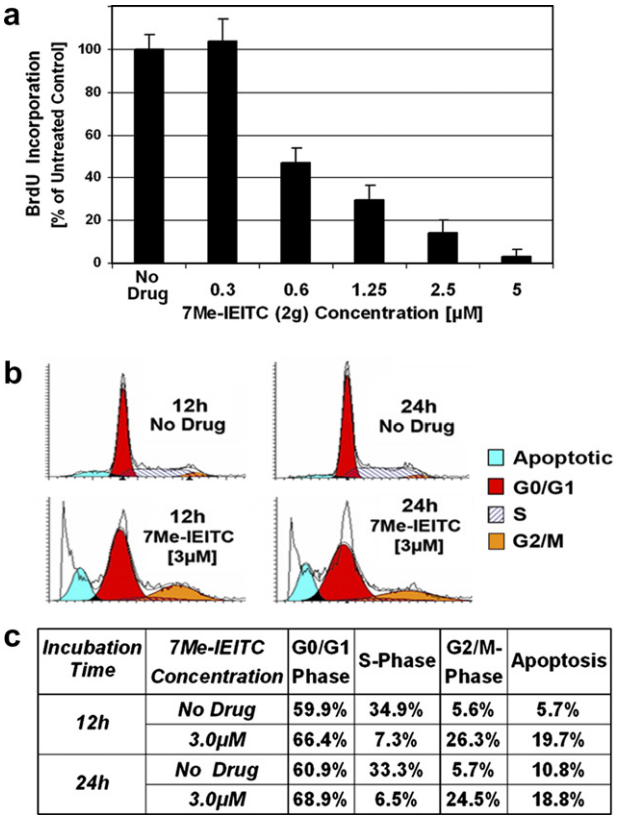
In MDA-MB-231 breast cancer cells, Taxol induced apoptosis via JNK, which caused inactivation of the anti-apoptotic Bcl-2 protein.[29] Taxol has also been shown to increase p38 MAPK, ERK, and JNK activities in human breast cancer cells.[30] Similarly, our present study suggests that **7Me-IEITC** (**2g**) suppresses pro-survival signaling and induces pro-apoptotic signaling in SMS-KCNR **NB** cells (Fig. 4a).

Apoptosis is executed by caspases: initiator caspases (such as caspase-2, -8, -9, and -10) function mainly as upstream apoptotic signals. Once activated, the initiator caspases cleave and activate downstream effector caspases (such as caspase-3, -6, and -7), which are responsible for the cleavage of many intracellular proteins, leading to the morphological and biochemical changes associated with apoptosis.[31,32] Accordingly, immunoblotting of lysates of SMS-KCNR **NB** cells confirmed that **7Me-IEITC** (**2g**) treatment resulted in strong activation/cleavage of caspase-3, -8, and -9 (Fig. 4a) and PARP-1, another hallmark of apoptosis. The direct consequence of the induction of apoptosis by **7Me-IEITC** (**2g**) is the reduction of viability in **NB** cells as demonstrated in Figure 4b. We employed various caspase inhibitors (Calbiochem, La Jolla, CA), which were added to NB cell cultures 2 h prior to treatment with **7Me-IEITC** (**2g**) (at IC_{50} concentration of 5.0 μM). Cytotoxicity of the drug was reduced by ~2/3 using a specific pancaspase inhibitor (Z-VAD-FMK), by ~1/2 using a specific caspase-3 inhibitor (caspase-3), and by 1/3 with specific caspase-8 (Z-IETD-FMK) or -9 (Z-LEHD-FMK) inhibitors (Fig. 4b).

The strong activation of caspase-8 in SMS-KCNR cells by **7Me-IEITC** (**2g**), evident in our Western blotting and cytotoxicity/inhibitor studies, is of special significance. Caspase-8 regulates the survival and invasive capacity of neuroblastoma cells.[33] Suppression of caspase-8 expression results in metastasis of neuroblastoma in vivo, and reconstitution of caspase-8 expression in deficient neuroblastoma cells suppressed their metastasis.[33] Our experiments indicate that **7Me-IEITC** (**2g**) exhibited the potential to activate caspase-8 and, thus, can potentially be considered to correct aberrations in caspase-8 expressions of aggressive neuroblastoma cells.

As described in the previous section **7Me-IEITC** (**2g**) acts as a cytotoxic drug and leads to a protein expression profile characteristic for apoptotic events. To investigate if **7Me-IEITC** (**2g**) affects the proliferation of **NB** cells (particularly at the drug concentration of 3 μM, when cell viability is only partially reduced), we performed BrdU incorporation assays and cell cycle analysis. The BrdU/Proliferation assay is a colorimetric assay[34] and spectroscopic data directly correlate to the amount of BrdU incorporated into the DNA, which in turn represents proliferation. Figure 5a demonstrates that **7Me-IEITC** (**2g**) dose-dependently reduced SMS-KCNR proliferation. Even at low drug concentrations (600 nM) BrdU incorporation was dramatically suppressed when compared to untreated cells (Fig. 5a). Cell cycle analysis[35] revealed that **7Me-IEITC** (**2g**) treatment of **NB** cells at 3.0 μM leds to a high percentage of cells in the apoptotic sub-G0/G1 (Fig. 5b) as early as 12 h following treatment. The apoptotic sub-G0/G1 population represents cells with significant DNA damage. This observation directly correlates with the reduction of SMS-KCNR viability by **7Me-IEITC** (**2g**) in concentrations between 2.5 and 5.0 μM (Fig. 3). With respect to the cycling cells, **7Me-IEITC** (**2g**) increased the number of cells in G0/G1 and caused a prominent arrest in the G2/M phase of the cell cycle (Fig. 5b) in this asynchronous **NB** cell population. Accordingly, the block of cell cycle progression resulted in a diminished cell count in the S-phase. Even though not the objective of the present report, further studies emphasizing cancer related cell cycle features,[36,37] could focus on the specific checkpoints in G0/G1 and G2/M phase affected by **7Me-IEITC** (**2g**) treatment. This would require to study the expression profile of cell cycle regulators (cyclin-dependent kinases and cyclins),[38,39] in synchronized NB cultures.

Conclusion. For the present report several indole ethyl isothiocyanate (**IEITC**) analogs were designed, synthesized, and screened in viability assays against neuroblastoma (**NB**) cells in vitro. The nature and position of substitutions on the indole moiety significantly affected the cytotoxic activity. We observed that the cytotoxicity of **IEITC**s with non-polar groups such as –Me and –Benzyloxy (BzO) was significantly higher than that of **IEITC**s with polar groups such as –OH and –OMe. Substitution at the 5- and 7-position (**2d** and **2g**) resulted in an additional improvement of the cytotoxic activity against **NB** cells. The present report suggests that **7Me-IEITC** is a potent and growth-suppressing agent in various **NB** cell lines (stromal S-type SMS-KCNR as well as neuronal N-type SH-SY5Y, SK-N-SH, and IMR-32). **7Me-IEITC** inhibited **NB** (SMS-KCNR) cell proliferation and cell viability along with caspase activation, inhibition of survival marker Akt, and activation of pro-apoptotic p38/JNK MAPKs and could be developed as treatment for neuroblastoma.



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Incubation Time	7Me-IEITC Concentration	G0/G1 Phase	S-Phase	G2/M-Phase	Apoptosis
12h	No Drug	59.9%	34.9%	5.6%	5.7%
	3.0μM	66.4%	7.3%	26.3%	19.7%
24h	No Drug	60.9%	33.3%	5.7%	10.8%
	3.0μM	68.9%	6.5%	24.5%	18.8%

Figure 5. 7Me-IEITC effect on cell proliferation and cell cycle progression in NB cells. (a) BrdU Incorporation: NB cells (SMS-KCNR) were treated with various concentrations (300 nM to 5 μM) of **7Me-IEITC** (**2g**) for 48 h. The proliferation assay was carried out as described.[34] Experiments were performed in triplicate; data are expressed as means of the triplicate determinations ($X \pm SD$) in % cell proliferation of untreated cells [100%]. (b and c) Cell cycle analysis by FACS: SMS-KCNR **NB** cells were treated with 3.0 μM **7Me-IEITC** (**2g**) for 12 or 24 h. Cell cycle analysis of treated and untreated cells was carried out.[35] Data are presented as the relative fluorescence intensity of cell sub-populations in the two-dimensional FACS profile (b) or in % of cells in a given sub-population (c).

Acknowledgments

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Compound **2b**: ^1H NMR ($CDCl_3$) δ 7.978 (br s, 1H, NH), 7.29–7.27 (d, 1H, J = 3.6 Hz, Ar), 7.10–7.09 (d, 1H, J = 2.4 Hz, Ar), 6.968–6.961 (d, 1H, J = 2.1 Hz, Ar), 6.853–6.816 (dd, 1H, J = 2.4 Hz, Ar), 3.737–3.692 (t, 2H, J = 6.9 Hz, CH2-Ar), 3.101–3.056 (t, 2H, J = 6.3 Hz, CH2NCS); MS (ES): *m/z* 219 $[M+H]^+$.
Compound **2c**: ^1H NMR ($CDCl_3$) δ 7.93 (br s, 1H, NH), 7.22–7.215 (d, 1H, J = 1.5 Hz, Ar), 7.049 (s, 1H, Ar), 6.939–6.932 (d, 1H, J = 1.8 Hz, Ar), 6.862–6.825 (dd, 1H, J = 2.4 Hz, Ar), 3.83 (s, 3H, OCH3), 3.746–3.701 (t, 2H, J = 6.9 Hz, CH2-Ar), 3.118–3.073 (t, 2H, J = 6.6 Hz, CH2NCS); MS (FAB) *m/z*: 233 $[M+H]^+$, 255 $[M+Na]^+$.
Compound **2d**: ^1H NMR ($CDCl_3$) δ 8.01 (br s, 1H, NH), 7.43–7.40 (d, 2H, J = 7.2 Hz, Ar), 7.351–7.187 (m, 4H, Ar), 7.024–7.0 (d, 1H, J = 7.2 Hz, Ar), 6.91–6.88 (d, 1H, J = 9 Hz, Ar), 5.059 (s, 2H, OCH2), 3.69–3.65 (t, 2H, J = 6.6 Hz, CH2-Ar), 3.076–3.031 (t, 2H, J = 6.6 Hz, CH2NCS); MS (FAB) *m/z*: 308 $[M+H]^+$, 331 $[M+Na]^+$.
Compound **2e**: ^1H NMR ($CDCl_3$) δ 8.09 (br s, 1H, NH), 7.29–7.265 (d, 1H, J = 1.8 Hz, Ar), 7.049 (s, 1H, Ar), 6.94–6.93 (d, 1H, J = 2.4 Hz, Ar), 6.857–6.819 (dd, 1H, J = 2.1 Hz, Ar), 3.863 (s, 3H, OCH3), 3.79–3.701 (t, 2H, J = 6.9 Hz, CH2-Ar), 3.12–3.07 (t, 2H, J = 7.2 Hz, CH2NCS); MS (FAB) *m/z*: 233 $[M+H]^+$, 255 $[M+Na]^+$.
Compound **2f**: ^1H NMR ($CDCl_3$)δ 8.11 (br s, 1H, NH), 7.93 (br s, 1H, NH), 7.41–7.44 (d, 1H, J = 6.9 Hz, Ar), 7.217–7.21 (d, 1H, J = 2.1 Hz, Ar), 7.033–7.131 (m, 2H, Ar), 3.733–3.752 (t, 2H, J = 5.7 Hz, CH2-Ar), 3.159–3.176 (t, 2H, J = 5.1, CH2NCS), 2.65 (t, 3H, CH3); MS (FAB) *m/z*: 216 $[M]^+$, 239 $[M+Na]^+$.
Compound **2g**: IR (DCM): 3145, 2940, 2167, 2040, 1629, 1479, 1045 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.08 (br s, 1H, NH), 7.39–7.41 (d, 1H, J = 6.6 Hz, Ar), 7.25–7.26 (d, 1H, J = 2.7 Hz, Ar), 7.041–7.13 (m, 2H, Ar), 3.76–3.77 (t, 2H, J = 5.1 Hz, CH2-Ar), 3.15–3.17 (t, 2H, J = 5.1, CH2NCS), 2.52 (t, 3H, CH3); MS (ES) *m/z*: 219 $[M+H]^+$.
21. Cell culture: LF1 (primary human lung fibroblasts) and **NB** cell lines SK-N-SH, SH-SY5Y, IMR-32 were obtained from American Type Culture Collection (Manassas, VA). SMS-KCNR (**NB**) cells were provided by John Maris (CHOP, Philadelphia, PA). All cells were seeded at 5×10^5cells/T75 cell culture flask (Corning, New York, NY) and cultured to ~80% confluency according to the suppliers recommendation.
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23. *Western blot analysis:* Cells were seeded into 100 mm^2 tissue culture dishes (5×10^5 cells/dish), cultured to ~80% confluency, and treated as indicated, rinsed in PBS, pH 7.4, scraped off, spun down in a microcentrifuge (10,000*g*, 5 min), and pellets resuspended in lysis buffer (1% NP-40, 20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM activated sodium orthovanadate, 10 μg/mL Aprotinin, 10 μg/mL Leupeptin, and Inhibitor Cocktail P-2714; Sigma–Aldrich, St. Louis, MO). Lysates were rocked at 4 °C for 5 min, sonicated (10 pulses 5 s), centrifuged at 140,000*g* for 10 min, and the protein concentration of the supernatant quantitated (BioRad protein estimation kit, Hercules, CA). The samples were boiled in the presence of 5× SDS–PAGE sample buffer and 50 μg total protein/lane was separated on 12% SDS–polyacrylamide gels and blotted onto PVDF membranes. The blots were blocked with 5% nonfat dried milk in PBST for 1 h at room temperature and incubated overnight at 4 °C with antibodies (purchased from Cell Signaling Technology, Beverly, MA) at a 1:1000 dilution in 5% BSA in PBST on a rotating platform. After washing in PBST the blots were incubated with secondary antibody (peroxidase-conjugated antibodies; Amersham Pharmacia Biotech, Piscataway, NJ). The bands were visualized by enhanced chemiluminescence and autoradiography (F-Bx810 Film, Pheonix, Hayward, CA).
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34. *Cell proliferation* was assessed by a BrdU assay, which measures incorporation of the pyrimidine analog, 5-bromo-2′-deoxyuridine (BrdU), during DNA synthesis (Roche Applied Science, Indianapolis, IN) according to the manufacturer's recommendations. Briefly, cells (5×10^3/well) were plated into 96-well flat-bottomed plates (Corning, Inc., Corning, NY) and allowed to attach overnight before treatment as indicated in fresh complete medium. BrdU (10 μM final concentration) was added and the cells grown for additional 6 h. After various wash steps the cells were fixed and incubated for 2 h at 37 °C with an anti-BrdU antibody-peroxidase conjugate and immune complexes were detected by addition of a tetramethyl-benzidine substrate solution according to the manufacturer's recommendation. The reaction was stopped by adding 50 μL of 1 M sulfuric acid, and the absorbance was measured with an ELISA plate reader (Thermo Labsystems, Waltham, MA) at 450 nm. Blank wells were incubated with the anti-BrdU antibody and the background absorbance was subtracted from all other values. In this assay, the color intensity correlates directly to the amount of BrdU incorporated into the DNA, which in turn represents proliferation. Experiments were performed in triplicate; data are expressed as means of the triplicate determinations ($X \pm$ SD) of a representative experiment in % of absorbance of untreated cells [100%]; Ref: Gratzner, H. G. *Science* **1982**, *218*, 474.
35. *Cell cycle analysis:* Cell cycle analysis and quantification of apoptosis were carried out by flow cytometry. Cells were seeded into 100 mm^2 tissue culture dishes (7.5×10^5 cells/dish), allowed to attach overnight, and treated for 48 h as indicated. At the end of the incubation period detached cells were collected in 15 mL polypropylene centrifuge tubes along with the medium; culture dishes were washed once with PBS, adherent cells scraped off and combined in the same tube. After centrifugation (250*g*, 5 min) cells were fixed (ice-cold 70% ethanol for 30 min) followed by incubation with 50 μg/mL of propidium iodide and 100 μg/mL of RNase for 30 min at 37 °C in the dark. Data were acquired on a BD FACSort flow cytometer using CellQuest software (BD Immunocytometry Systems, San Jose, CA) and analyzed using ModFit LT software (Verity Software House, Inc., Topsham, ME). Ten thousand events were analyzed for each sample. Appropriate gating was used to select the single cell population NB cells. The same gate was used on all samples, ensuring that the measurements were made on a standardized cell population.
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