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Synthesis and evaluation of an imidazole derivative–fluorescein conjugate

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ABSTRACT

The murine double minute (MDM2) oncogene a negative regulator of protein 53 (p53) tumor suppressor, is found overexpressed in many different types of cancer and the interaction between MDM2 and p53 has become the target of intensive research. MDM2 inhibitors represent a promising class of p53 activating compounds that may be effective in cancer treatment and diagnostic imaging. Nutlins, a family of *cis*-imidazoline analogues and small-molecule MDM2 antagonists, have the potential use in cancer therapies. We have synthesized an imidazole derivative (Nutlin–Glycine) conjugated to the commonly used fluorophore, 6-carboxyfluorescein (FAM) and evaluated its possible use as an imaging agent. Cellular uptake studies demonstrated that the fluorescence intensity in human osteosarcoma (SJSA-1) and colon carcinoma (HCT116) cells were significantly increased with the treatment of Nutlin–Glycine–FAM when compared with FAM (control). Blocking studies also confirmed that our imidazole–fluorescein conjugate may be a good candidate for imaging tumors, suggesting the need for further in vivo evaluation by positron emission tomography.

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1. Introduction

The development of molecular imaging in the clinical setting has only just begun and could yield tremendous patient benefit in the form of earlier lesion detection, treatment response monitoring, and a truly individualized approach to treatment of cancer. In this early phase of the molecular-targeted patient-friendly cancer chemotherapy, there is a need for novel viable anticancer molecular targets. The MDM2 oncoprotein has been recently validated as a potential target for cancer drug development. MDM2 is a zinc finger oncoprotein[1–4] that has been well characterized as the principal negative regulator of the p53 tumor suppressor protein.[5] The tumor-suppressor p53 is a short-lived protein that is maintained at low, often undetectable levels in normal cells. Stabilization of the protein in response to an activating signal, such as DNA damages, results in a rapid rise in p53 level with subsequent inhibition of cell growth. MDM2 binds the p53 tumor-suppressor with high affinity and negatively and modulates its transcriptional activity and stability.[6,7] The MDM2 gene has been found amplified or overexpressed in many human malignancies with impaired p53 function.[8–13] Therefore, activation of the p53 pathway through inhibition of MDM2 has been proposed as an attractive therapeutic strategy.[14,15] Several studies have shown that disruption of the p53–MDM2 interaction by different macromolecular approaches or by the suppression of MDM2 expression can lead to the activation of p53 and tumor growth inhibition.[16,17] Inhibition with small molecules is a more attractive proposal due to the pharmacological advantages of this class of drugs in stability and oral bioavailability.

In 2004, Vassilev et al. (Hoffman-La Roche Inc., Nutley, New Jersey) first described a class of antagonists that inhibit the MDM2–p53 complex. These antagonists are a group of *cis*-imidazoline analogues designated as the Nutlins (for Nutley inhibitor).[18] These *cis*-imidazoline derivatives bind tightly into the p53 pocket of MDM2 and displace p53 from its complexes with MDM2 in vitro with IC_{50} in the 100–300 nM range. Historically, it has been difficult to develop small-molecule inhibitors of nonenzyme protein–protein interactions because of their large and shallow interfaces. Through X-ray crystallography, the MDM2–p53 complex showed a well-defined hydrophobic cleft which represented the binding site for p53. These inhibitors could disrupt the MDM2–p53 interaction by binding specifically in this cleft and liberating functional p53.[19–21] These imidazole analogues (Nutlin-1 and 2) are not commercially available except Nutlin-3, which is currently under clinical investigation. Nutlin-3 inhibits p53–MDM2 interaction with an IC_{50} of 0.09 μM.[18] It induces the expression of p53-regulated genes and exhibits potent antiproliferative activity in cells with functional mutated p53.

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Several imidazole derivatives have been developed as diagnostic agents for positron emission tomography (PET) or optical imaging.[22–26] We prepared an imidazole derivative (Nutlin-2) with glycine moiety, 'Nutlin–Glycine', according to the reported procedures with some modifications by our lab.[27–29] Using this compound as a starting material for synthesizing MDM2 selective ligands, we obtained a Nutlin analog that targets to intracellular MDM2 and renders biological activities in tumor cells with wild type p53 but not cells with a mutant p53. In this report we will also describe using this analog conjugated to the commonly used fluorophore, 6-carboxyfluorescein, to serve as an imaging moiety in cultured cells.

2. Results and discussions

The MDM2 oncoprotein has been validated as a potential target for cancer drug development. MDM2 amplification and/or overexpression occur in a wide variety of human cancers, several of which can be treated experimentally with MDM2 antagonists. Small molecules, including synthetic chalcones, norbornane derivatives, *cis*-imidazoline derivatives (Nutlins), a pyrazolidinedione sulfonamide and 1,4-benzodiazepine-2,5-diones, etc., have been reported to disrupt the p53–MDM2 binding, thereby enhancing p53 activity to elicit anticancer effects.[30] We have selected the imidazole derivative Nutlin-2, which is not commercially available, and prepared Nutlin–Glycine derivative according to the reported procedure[27–29] with some modifications to achieve high yield. The modifications we developed utilized the emerging microwave technologies and techniques to accelerate the synthesis process. The synthetic scheme for Nutlin–Glycine–FAM conjugate is illustrated in Figure 1. The compound **8**, Nutlin–Glycine was purified by high performance liquid chromatography (HPLC) and isolated in 82% yield. The final conjugate 'Nutlin–Glycine–FAM' was prepared by the reaction of Nutlin–Glycine, **8** with a known fluorophore, 6-carboxyfluorescein *N*-hydroxysuccinimide ester (6-FAM-NHS) in DMF in the presence of triethylamine at ambient temperature for overnight. The crude conjugate **9** was purified by semi preparative reversed-phase HPLC and isolated in 71% yield. Nutlin–Glycine–FAM was dissolved in DMSO and filter sterilized using a 0.2 μ mini-syringe filter prior to use.

To determine the effect of Nutlin-2 on cell growth and viability, nine cancer cell lines, five with wild-type p53 status and four with mutant p53 status, were incubated with a serial concentration of Nutlin-2 for 5 days, followed with the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt)] cell viability assay. As shown in Figure 2, there were dose-dependent antiproliferative and cytotoxic effects in cells with wild-type p53, however, the cells with mutant p53

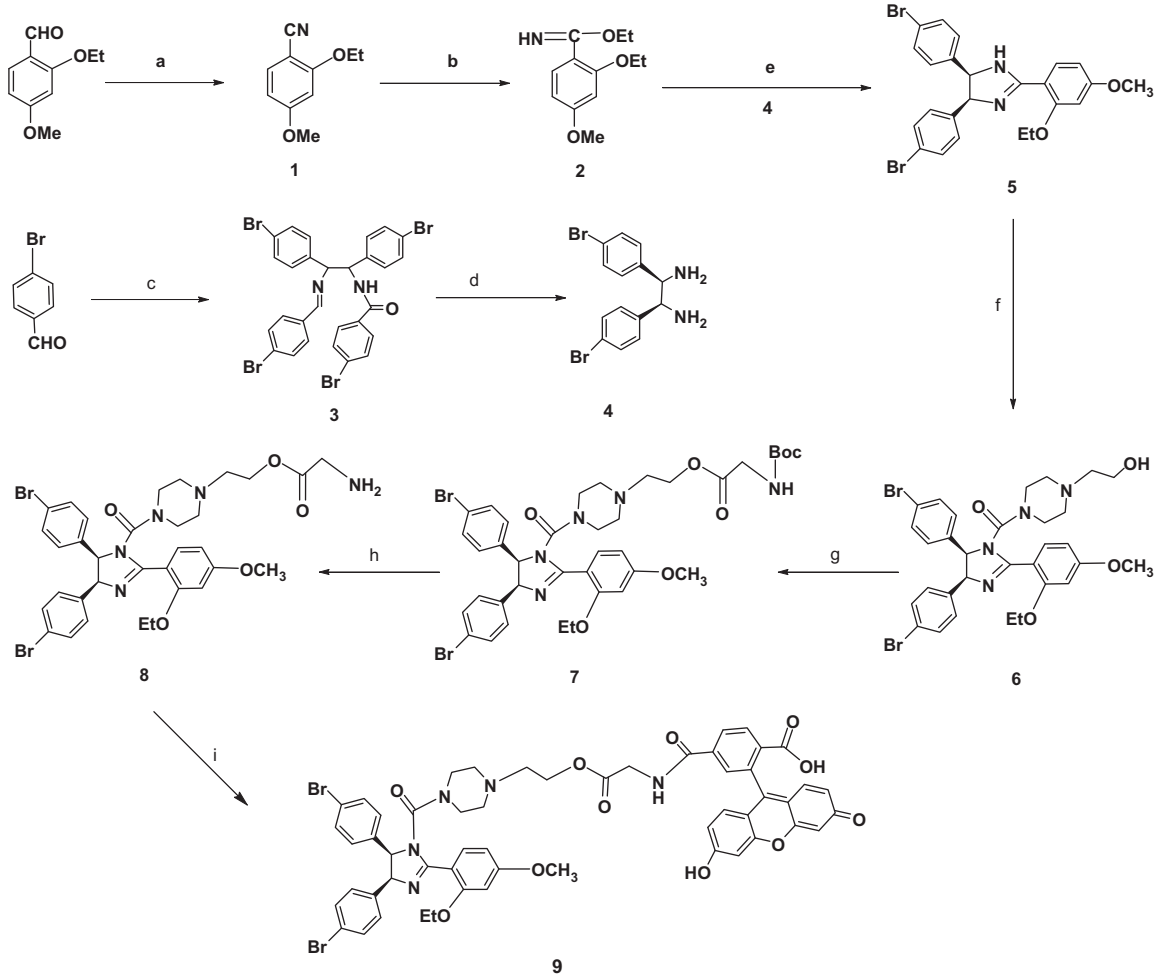

Figure 1. Synthetic scheme of Nutlin–Glycine–FAM conjugate. Reagents and conditions: (a) 30% NH_4OH, IBA, rt, 4 h, 81%; (b) $AcCl_2$, EtOH, rt, overnight, 63%; (c) NH_4OAc, microwave, 90 °C, 2 h, 67%; (d) (i) H_2SO_4, microwave, 80 °C, 3 h, (ii) 10(M)NaOH, rt, 2 h, 66%; (e) EtOH, Et_3N, DMAP, **4**, microwave, 80 °C, 4 h, 75%; (f) Phosgene, DMAP, Et_3N, DCM, rt, 1 h, piperzinyl ethanol, 73%; (g) Glycine-Boc, DCM, DMAP, DCC, rt, overnight, 73% ; (h) TFA, DCM, rt, 1 h, HPLC, 82%; (i) FAM-NHS, DMF, Et_3N, rt, overnight, HOAc, HPLC, 71%.

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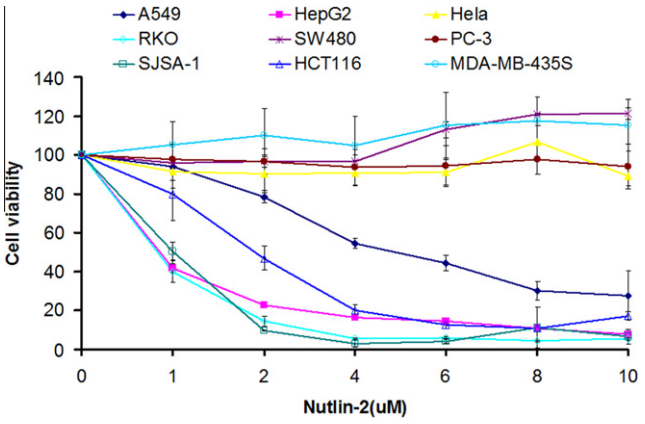

Figure 2. Antiproliferative and cytotoxic activity of Nutlin-2. Exponentially growing cultured cancer cells with wild-type p53 (A549, HCT116, HepG2, RKO, and SJSA-1) or mutant p53 (Hela, MDA-MB-435S, PC-3 and SW480) were incubated with Nutlin-2 for 5 days and the cell viability were determined by the MTS assay. The curves represent percentages of viable cells relative to untreated controls (mean ± SD, $n = 4$).

did not exhibit any impairment even incubated with the highest dose of Nutlin-2.

To investigate the effect of Nutlin-2 on cell cycle, three cancer cell lines, two with wild-type p53 and one with mutant p53, were incubated with Nutlin-2 for 48 h, and then the cells were stained with propidium iodide (PI) for cell cycle analysis by flow cytometry (Fig. 3). SJSA-1 human osteosarcoma and A549 human lung carcinoma cells (wild-type p53) showed increased G0/G1 phase fraction and sharply decreased S-phase compartment after incubation with Nutlin-2. But there was no significant change in MDA-MB-435S cells (mutant p53). Therefore, Nutlin-2 could cause growth inhibition and cell cycle arrest specifically in cancer cells with wild-type p53.

To test the effect of Nutlin–Glycine on cell growth and viability, six cancer cell lines were used for this experiment; three cell lines with wild-type p53 and three with mutant p53. Each cell line was incubated with serial dilutions of Nutlin–Glycine, Nutlin-2, or Nutlin-3 (positive controls) for 5 days, and followed by cell viability analysis. As shown in Figure 4a, there was a dose-dependent antiproliferative effect observed in cells with wild-type p53; however, the cells with mutant p53 did not exhibit any growth inhibition even when incubated with the highest dose (10 μM) of the agents (Fig. 4b). The addition of the glycine to Nutlin-2, Nutlin–Glycine, did not diminish biological activity when compared with Nutlin-2 (native) or Nutlin-3.

The Nutlin–Glycine–FAM conjugate was tested for cellular uptake in SJSA-1 cells (osteosarcoma) and HCT116 cells (colon carcinoma). As shown in Figure 5, cellular uptake of Nutlin–Glycine–FAM was significantly increased at 1 and 5 μM in SJSA-1 and HCT116 cells. The fluorophore by itself, FAM, was used as a negative control at the same concentrations. The cellular uptake of FAM was minimal and non-specific in both cell lines.

In blocking studies we used SJSA-1 and HCT116 cells. In this experiment, we used 10-fold excess of Nutlin–Glycine derivative as a blocking agent. As shown in Figure 6, Nutlin–Glycine–FAM was significantly blocked at a concentration of 5 μM when com-

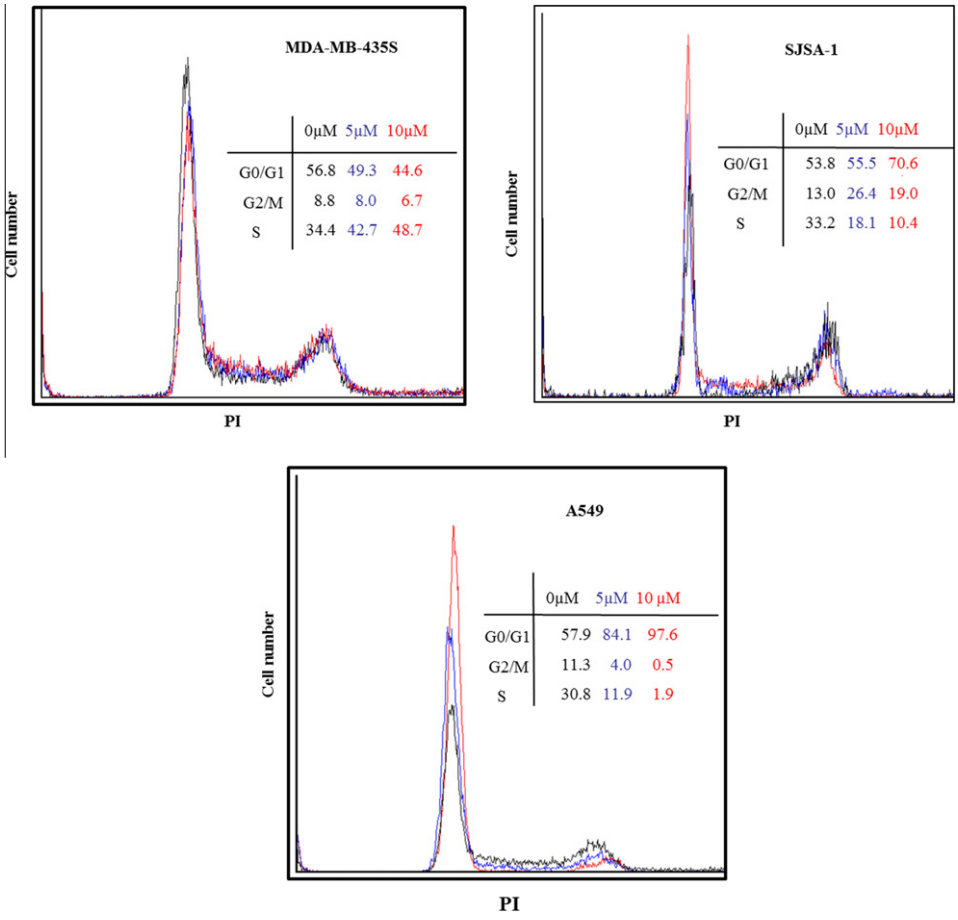

Figure 3. Cell cycle analysis with Nutlin-2. Cultured MDA-MB-435S, A549 and SJSA-1 cells were incubated with Nutlin-2 or an equivalent amount of solvent for 48 h, followed by PI staining and flow cytometry analysis. The percentages of cells in each cell cycle phase are shown in the inset above the histogram.

pared with uptake alone (no block) in both cell lines (71.71% for SJSA-1 and 77.34% for HCT116). FAM or DMSO was used as negative controls at the same concentration (5 μM). These preliminary findings warrant further investigation into the possible use of Nutlin–Glycine–FAM conjugate as an imaging agent.

3. Conclusion

We report herein an efficient synthesis and evaluation of an imidazole–fluorescein conjugate for imaging tumors by detecting MDM2 oncoprotein. The preliminary cellular uptake and also blocking studies demonstrated that Nutlin–Glycine–FAM conjugate might be useful as a tumor imaging agent and also help to identify a potential probe for further investigations for MDM2 in the future, but needs further in vivo evaluation, for example, by labeling the Nutlin–Glycine with a positron nuclide followed by positron emission tomography.

4. Materials and methods

4.1. Reagents and instrumentations

All reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI), and used without further purification. Nutlin-3 was purchased from Cayman Chemical (Ann Arbor, MI), and stored frozen as a 1 mM stock solution in DMSO. Thin layer chromatography (TLC) was performed on pre-coated Kieselgel 60 F254 (Merck) glass plates. Proton and Carbon NMR spectra were recorded on a Brucker 300 or 500 MHz spectrometer using tetramethylsilane as an internal reference and hexafluorobenzene as an external reference, respectively, at The University of Texas M. D. Anderson Cancer Center. The mass spectra were obtained on a LCQ Fleet mass spectrometer using electrospray ionization (ESI) technique. Microwave synthesis was performed on a Biotage Initiator Eight microwave synthesizer (Uppsala, Sweden). High performance liquid chromatography (HPLC) was performed on a 1200 series pump (Agilent, Germany), with UV detector operated at 254 or 494 nm, using a semi-preparative and analytical C-18 reverse phase column, Luna SCX 100A (5 μm, 250 × 10 mm) or (5 μm, 150 × 4.6 mm).

4.2. Chemistry

4.2.1. 2-Ethoxy-4-methoxybenzonitrile, 1

2-Hydroxy-4-anisaldehyde (2.0 g, 11.10 mmol) was dissolved in 30% ammonium hydroxide (15 mL) and 5 mL of acetonitrile (3:1), which resulted in the formation of a turbid solution. To this turbid solution, 2-iodoxybenzoic acid (6.22 g, 22.20 mmol) was added slowly with constant stirring at room temperature for 4 h. The yellowish-brown solution becomes colorless which indicates completion of the reaction (TLC). The reaction mixture was filtered and evaporated under high vacuum, and the residue was dissolved in dichloromethane (100 mL). The solution was washed with water (3 × 100 mL). The organic phase was dried ($MgSO_4$), evaporated to dryness and purified on a silica gel column using 2% methanol in dichloromethane. The pure compound **1** (1.6 g) was obtained in 81% yield. ^{1}H NMR **1** ($CDCl_3$) δ: 7.46 (1H, d, J = 8.4 Hz, ArH), 6.53–6.43 (2H, m, ArH), 4.11 (2H, q, CH_2), 3.85 (3H, s, CH_3), 1.51 (3H, t, J = 7.1 Hz, CH_3). MS: (M+1) calculated 178.08, found 178.11.

4.2.2. Ethyl 2-ethoxy-4-methoxybenzimidate, 2

Compound **1** (1.0 g, 5.64 mmol) was dissolved in 5 mL of anhydrous ethanol in a dry flask. Acetyl chloride (3.2 mL, 45.15 mmol) was added slowly with stirring at 0 °C for 1 h and continued at room temperature for overnight. The reaction mixture was quenched with 1(N) sodium bicarbonate solution (0.5 mL). The crude product was extracted into dichloromethane (100 mL) and the organic solution was washed with water (3 × 100 mL) and brine. The residue was purified on a silica gel column using 3% methanol in dichloromethane. The pure compound **2** (0.9 g) was obtained in 63% yield. ^{1}H NMR **2** ($CDCl_3$) δ: 8.69 (1H, br s, NH), 7.78 (1H, d, J = 7.8 Hz, ArH), 6.51–6.48 (2H, m, ArH), 4.29 (2H, q,

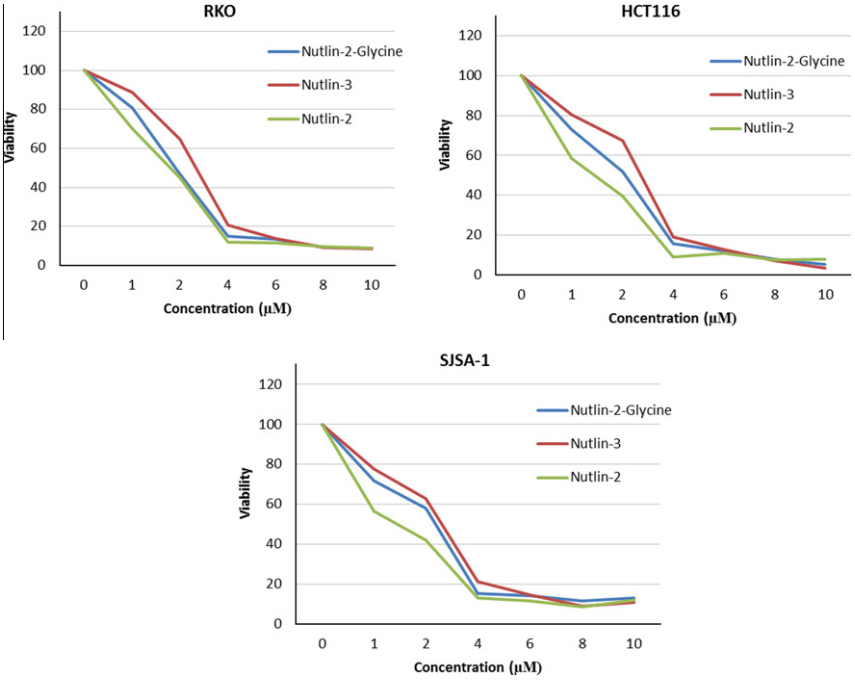

Figure 4a. Antiproliferative and cytotoxic activity of Nutlin analogs. Exponentially growing cultured cancer cells with mutant p53 (Hela, MDA-MB-435S, SW480) were incubated with Nutlin-2, Nutlin-3 and Nutlin–Glycine derivative for 5 days and the cell viability were determined by the MTS assay. The curves represent percentages of viable cells relative to untreated controls (mean ± SD, n = 4).

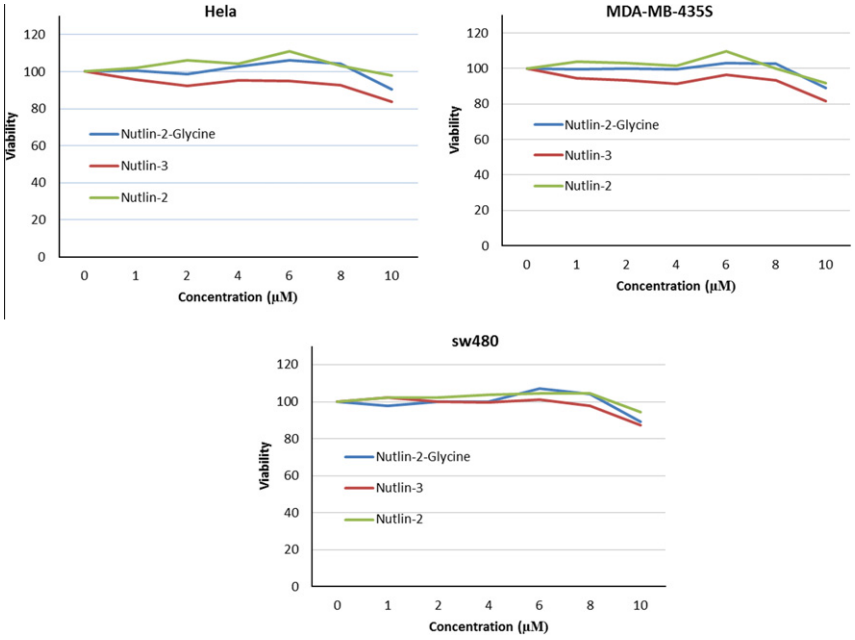

Figure 4b. Antiproliferative and cytotoxic activity of Nutlin analogs: Cultured cancer cells with wild-type p53 (HCT116, RKO, and SJSA-1) were incubated with Nutlin-2, Nutlin-3 and Nutlin–Glycine derivative for 5 days and the cell viability were determined by the MTS assay. The curves represent percentages of viable cells relative to untreated controls (mean ± SD, $n = 4$).

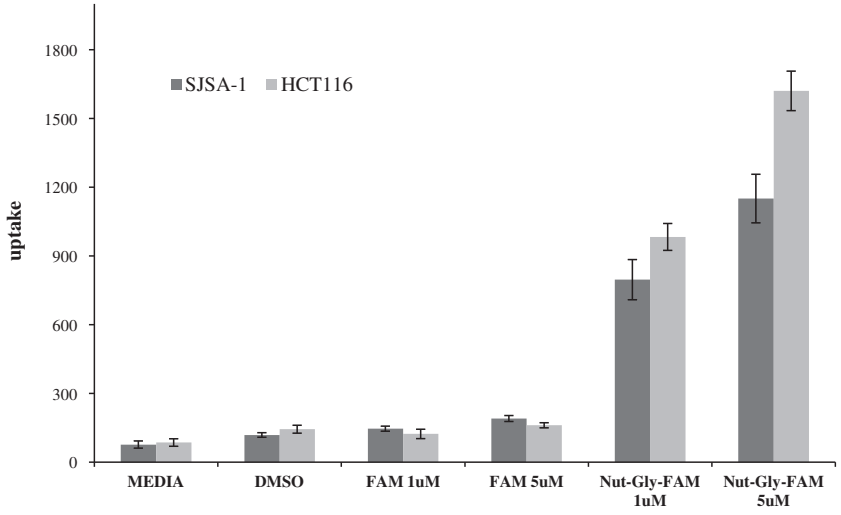

Figure 5. Cell uptake studies in cultured human osteosarcoma and colon cancer cells. Cultured SJSA-1 human osteosarcoma and HCT116 colon cancer cells were grown at 37 °C for in a tissue culture incubator. DMSO, FAM and Nutlin–Glycine–FAM were added to the separate culture media and allowed to incubate for 1 h at 37 °C, followed by cell harvest and fluorescence measurements. The culture media itself was incubated with both cell lines (no drug, no DMSO). Relative fluorescence values were plotted for the vehicle (Media or DMSO), FAM and Nutlin–Glycine–FAM at two final concentrations (1 and 5 μM). Error bars indicate standard error of the mean for 3 runs.

CH_2), 4.13 (2H, q, CH_2), 3.82 (3H, s, CH_3), 1.50 (3H, t, $J = 7.2$ Hz, CH_3), 1.39 (3H, t, $J = 7.2$ Hz, CH_3). MS: (M+1) calculated 224.26, found 224.31.

4.2.3. 4-Bromo-N-(2-(4-bromobenzylideneamino)-1,2-(4-bromophenyl)ethyl)benzamide, 3

4-Bromobenzaldehyde (1.0 g, 5.41 mmol) and ammonium acetate (2.08 g, 27.05 mmol) in water (5 mL) was heated with microwaves in a sealed tube at 90 °C for 2 h. The reaction mixture was cooled and precipitate was filtered off. The white precipitate was washed with water and evaporated to dryness. The crude product is crystallized from ethyl acetate–methylcyclohexane solvent mixture to give 2.6 g of the expected product **3** in the form of white crystals (67% yields). ^{1}H NMR **3** ($CDCl_3$) δ: 8.61 (1H, s, CH), 8.01 (1H, br s, NH), 7.88 (6H, d, $J = 8.2$ Hz, ArH), 7.69 (2H, d, $J = 7.8$ Hz, ArH), 7.58 (2H, d, $J = 7.1$ Hz, ArH), 7.47 (2H, d, $J = 7.8$ Hz, ArH), 7.15 (4H, d, $J = 6.5$ Hz, ArH), 5.33 (1H, d, $J = 7.1$ Hz, CH), 5.18 (1H, d, $J = 7.5$ Hz, CH). MS: (M+1) calculated 721.08, found 721.90

4.2.4. 1,2-Bis(4-bromophenyl)ethane-1,2-diamine, 4

Compound **3** (2.5 g, 3.47 mmol) was taken in 3 mL of sulfuric acid and 10 mL of water in a sealed tube and heated with microwaves at 80 °C for 3 h. The residue was cooled and precipitate was filtered off. The crude precipitate was dissolved in 10 (M) sodium hydroxide solutions and stirred for further 2 h at room

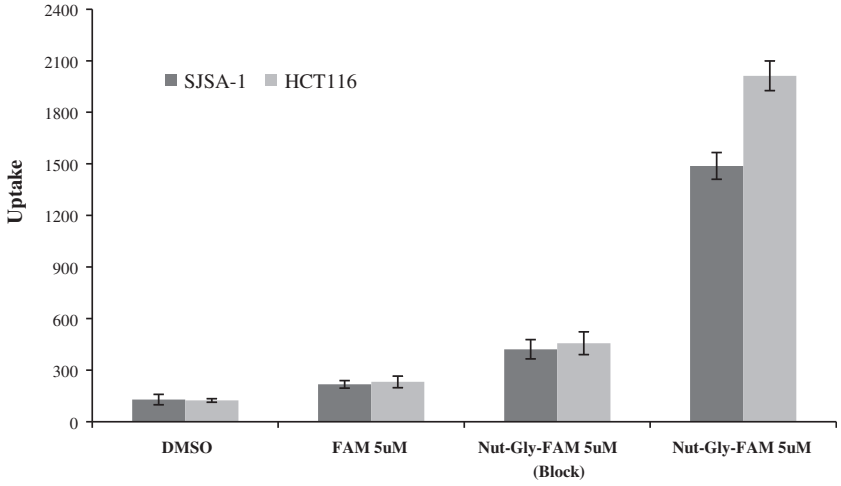

Figure 6. Cell blocking studies in cultured human osteosarcoma and colon cancer cells. Cultured SJSA-1 and HCT116 cancer cells were grown at 37 °C for in a tissue culture incubator. Nutlin–Glycine was added to the separate culture media and allowed to incubate for 1 h at 37 °C prior treatment with Nutlin–Glycine–FAM and further allowed to incubate for 1 h, followed by cell harvest and fluorescence measurements. FAM and DMSO were incubated with both cell lines for 1 h as negative controls. Relative fluorescence values were plotted for the vehicle (DMSO), FAM and Nutlin–Glycine–FAM at a final concentration (5 μM). Error bars indicate standard error of the mean for 3 runs.

temperature. After that the residue was dissolved in dichloromethane (150 mL) and washed with water (3 × 150 mL). The organic phase was dried, evaporated to dryness and purified on a silica gel column using 3% isopropanol in ethyl acetate as eluent. The mixture of epimers **4**, 0.85 g was obtained in 66% yield. ^{1}H NMR **4** ($CDCl_3$) δ: 7.46 (4H, d, J = 8.1 Hz, ArH), 7.20 (4H, d, J = 8.1 Hz, ArH), 3.98 (4H, s, NH_2), 1.38 (2H, s, CH). MS: (M+1) calculated 371.08, found 371.10.

4.2.5. 4,5-Bis(4-bromophenyl)-2-(2-ethoxy-4-methoxyphenyl) dihydroimidazole, 5

Compound **2** (0.45 g, 2.01 mmol), triethylamine (0.28 mL, 2.01 mmol), 4-dimethylamino pyridine (0.12 g, 1.0 mmol) and *N*-(4-Bromobenzoyl)-*meso*-1,2-di(4-bromophenyl)-1,2-diaminoethane, **4** (0.75 g, 2.01 mmol) was taken in 6 mL of anhydrous ethanol in a sealed tube and heated with microwaves at 80 °C for 4 h. The reaction mixture was quenched with 1(N) sodium bicarbonate solution (0.3 mL). The crude product was extracted into dichloromethane (100 mL) and the organic solution was washed with water (3 × 100 mL) and brine. The organic phase was dried ($MgSO_4$), evaporated to dryness and the crude residue was purified on a silica gel column and using 2% methanol in dichloromethane as eluent. This afforded product **5** as yellowish color powder (0.8 g, 75% yield). ^{1}H NMR **5** ($CDCl_3$) δ: 8.26 (1H, d, J = 8.7 Hz, NH), 7.21 (4H, d, J = 8.1 Hz, ArH), 6.86 (4H, d, J = 8.4 Hz, ArH), 6.64 (2H, d, J = 6.6 Hz, ArH), 6.34 (1H, d, J = 6.1 Hz, ArH), 5.36 (2H, br s, CH), 4.16 (2H, q, CH_2), 3.88 (3H, s, CH_3), 1.42 (3H, t, J = 6.9 Hz, CH_3). MS: (M+1) calculated 531.26, found 531.28.

4.2.6. 4,5-Bis(4-bromophenyl)-2-(2-ethoxy-4-methoxyphenyl) dihydroimidazolyl)(4-(2-hydroxyethyl)piperazinyl)methanone, 6

Compound **5** (0.75 g, 1.41 mmol) was dissolved in anhydrous dichloromethane (6 mL) in a dry flask under argon atmosphere and triethylamine (0.2 mL, 1.41 mmol) was added followed by the addition of 4-dimethylamino pyridine (86 mg, 0.71 mmol) at room temperature. Phosgene (0.29 g, 2.82 mmol, 20% in toluene) was added dropwise into the mixture and stirred for 1 h at room temperature and evaporated. The residue was further dried under high vacuum for 1–2 h. The mixture was dissolved in 3 mL of anhydrous dichloromethane and added dropwise to a solution of 2-piperazin-1-yl-ethanol (1.8 g, 14.1 mmol) in anhydrous dichloromethane (5 mL) at 0 °C over 10–20 min. The reaction mixture was stirred at 0 °C for 1 h and warmed to room temperature for 30 min. The reaction mixture was worked up with 0.3 mL of water, extracted with dichloromethane (100 mL) and washed with water (3 × 100 mL) and brine. The organic extract was dried and purified on a silica gel column and using 5% methanol in dichloromethane as eluent to isolate **6,** Nutlin-2 (0.71 g) in 73% yield. ^{1}H NMR **6** ($CDCl_3$) δ: 7.55 (1H, d, J = 8.4 Hz, ArH), 7.22 (4H, dd, J = 8.1 Hz, ArH), 6.85 (4H, dd, J = 8.4 Hz, ArH), 6.56 (1H, d, J = 8.7 Hz, ArH), 6.51 (1H, s, ArH), 5.61 (1H, d, J = 9.6 Hz, CH), 5.43 (1H, d, J = 9.6 Hz, CH), 4.28–3.91 (2H, m, CH_2), 3.88 (3H, s, CH_3), 3.59 (2H, t, J = 5.4 Hz, CH_2), 3.18 (4H, br s, CH_2), 2.44 (2H, t, J = 5.1 Hz, CH_2), 2.16 (4H, br s, CH_2), 1.45 (3H, t, J = 6.9 Hz, CH_3). ^{13}C NMR **6** ($CDCl_3$) δ: 16.6, 43.2, 44.4, 56.6, 56.9, 58.6, 60.1, 60.9, 62.6, 68.4, 69.5, 109.2, 111.3, 112.1, 124.4, 125.3, 128.4, 128.7, 130.2, 130.6, 131.5 (2), 1.33.4, 135.1 (2), 141.5, 145.3, 156.2, 159.1, 164.9 and 169.3. High resolution MS: M+1, calculated 687.4341, found 687.4342.

4.2.7. 2-(4,5-Bis(4-bromophenyl)-2-(2-ethoxy-4-methoxyphenyl)dihydroimidazole-carbonyl)piperazinyl)ethyl 2-(*tert*-butoxycarbonylamino) acetate, 7

Compound **6** (0.5 g, 0.73 mmol) and *N*-(*tert*-butoxycarbonyl)glycine (0.19 g, 1.09 mmol) were dissolved in anhydrous dichloromethane (10 mL) in a dry flask under argon atmosphere and *N*,*N*′-dicyclohexylcarbodiimide (0.45 g, 2.19 mmol) was added followed by the addition of 4-dimethylamino pyridine (89 mg, 0.73 mmol) at room temperature. The reaction mixture was stirred for overnight when TLC showed no starting material remained. Solvent was evaporated and crude residue was purified on a silica gel column using 3% methanol in dichloromethane to isolate **7** (0.45 g) in 73% yield. ^{1}H NMR **7** ($CDCl_3$) δ: 8.11 (1H, br s, NH), 7.90 (1H, d, J = 8.1 Hz, ArH), 7.65 (4H, d, J = 8.3 Hz, ArH), 7.01 (4H, dd, J = 8.1 Hz, ArH), 6.54 (1H, d, J = 8.0 Hz, ArH), 6.52 (1H, s, ArH), 5.41 (1H, d, J = 6.4 Hz, CH), 5.28 (1H, d, J = 7.1 Hz, CH), 4.22 (2H, t, J = 7.3 Hz, CH_2), 4.18 (2H, br s, CH_2), 4.03 (2H, q, CH_2), 3.79 (3H, s, CH_3), 3.41 (4H, t, J = 6.1 Hz, CH_2), 3.02 (2H, t, J = 6.3 Hz, CH_2), 2.46 (4H, t, J = 6.0 Hz, CH_2), 1.36 (3H, s, CH_3), 1.33 (3H, t, J = 7.1 Hz, CH_3). MS: M+1, calculated 844.60, found 844.61.

4.2.8. 2-(4,5-Bis(4-bromophenyl)-2-(2-ethoxy-4-methoxyphenyl)dihydroimidazole-carbonyl)piperazinyl)ethyl-2-aminoacetate, 8

Compound **7** (0.3 g, 0.36 mmol) was dissolved in dichloromethane (5 mL) under argon atmosphere. Trifluroacetic acid (0.5 mL) was added dropwise in to the mixture and stirred for 30 min at 0 °C and 1 h at room temperature when TLC showed no starting material remained. Solvent was evaporated and crude residue was purified on a semi preparative high-performance liquid chromatography (HPLC). Purification was performed on a Luna SCX 100A column (5 μm, 250 × 10 mm). The flow was 4 mL/min, with the mobile phase starting from 90% solvent A (0.1% trifluoroacetic acid in water) and 10% solvent B (0.1% trifluoroacetic acid in acetonitrile) to 10% solvent A and 90% solvent B at 20 min. The fraction containing Nutlin–Glycine, **8** was collected and dried in 82% yield (0.22 g). ^{1}H NMR **8** ($CDCl_3$) δ: 7.96 (1H, d, J = 8.4 Hz, ArH), 7.61 (4H, d, J = 8.1 Hz, ArH), 7.13 (4H, dd, J = 8.4 Hz, ArH), 6.64 (1H, d, J = 8.0 Hz, ArH), 6.50 (1H, s, ArH), 5.45 (1H, d, J = 6.1 Hz, CH), 5.33 (1H, d, J = 6.4 Hz, CH), 4.31 (2H, t, J = 7.1 Hz, CH_2), 4.26 (2H, s, CH_2), 4.06 (2H, q, CH_2), 3.81 (3H, s, CH_3), 3.40 (4H, t, J = 7.2 Hz, CH_2), 2.91 (2H, t, J = 6.1 Hz, CH_2), 2.56 (4H, t, J = 6.5 Hz, CH_2), 1.59 (2H, br s, NH_2), 1.34 (3H, t, J = 6.9 Hz, CH_3). ^{13}C NMR **8** ($CDCl_3$) δ: 16.4, 42.3, 45.2, 52.0, 54.5, 55.6, 56.4 (2), 60.2, 63.3, 66.1, 74.7, 108.6, 113.0, 116.1, 122.5, 123.1, 127.1, 127.7, 131.4, 131.9, 135.5, 136.2 (2), 139.8, 141.3, 145.5, 151.1, 158.9, 164.2, 165.7, 166.8, and 171.4. High resolution MS: M+1, calculated 744.4934, found 744.4937.

4.2.9. Nutlin–Glycine–FAM conjugate, 9

A solution of compound **8** (50 mg, 0.067 mmol) and triethylamine (11 μL, 0.077 mmol) in anhydrous *N*,*N*-dimethylformamide (0.3 mL) was cooled to 0 °C for 10–20 min under argon filled balloon. 6-carboxyfluorescein *N*-hydroxysuccinimide ester (48 mg, 0.10 mmol) in anhydrous *N*,*N*-dimethylformamide (0.1 mL) was added dropwise and stirred in the dark at ambient temperature for overnight. The reaction mixture was quenched by adding 100 μL of 5% acetic acid in water. The solvent was evaporated to dryness under high vacuum. The purification of the crude product was carried out on a semi preparative high-performance liquid chromatography (HPLC) and above solvent system at 4 mL/min flow rate. The fraction containing Nutlin–Glycine–FAM conjugate was collected, dried and stored in the dark at −20 °C until use. The pure conjugate **9**, 52 mg was obtained in 71% yield and was characterized by high resolution mass spectroscopy (HRMS). HRMS (M+1): calculated 1100.1675, found 1100.1678.

4.3. Cell viability/cytotoxicity assay

Human cancer cells containing a wild-type p53 (A549, HCT116, HepG2, RKO, and SJSA-1) and a mutant p53 (Hela, MDA-MB-435S, PC-3 and SW480) were grown in the recommended medium supplemented with 10% fetal bovine serum (Invitrogen, San Diego, CA) in a humidified environment with 5% CO_2 at 37 °C. Drugs were dissolved in DMSO and stored as 1 mmol/L stock solutions in small aliquots at −20 °C. Cells were seeded into 96-well plates and treated with Nutlin-2, Nutlin-3 and Nutlin–Glycine derivative at serial concentrations of 0–10 μM, respectively. After 5 days treatment, cell viability was assessed by the MTS-based Cell Titer 96 Aqueous One Solution Reagent (Promega, Madison, WI) according to manufacturer's protocol.

4.4. Cell cycle analysis

Cell cycle was determined by flow cytometry analysis with propidium iodide (PI) staining (BD Pharmigen). One million cells were seeded in 75-cm^2 flasks and the subsequently treated were executed with the compounds indicated in Section 2. After the treatment, cells were harvested, washed twice by PBS, fixed with 70% ethanol, and incubated overnight at 4 °C. After thawing, cells were washed twice in cold PBS and suspended in PI (50 μg/mL), 0.1 mg/mL RNase A, 0.05% Tritin X-100 solution, followed with an incubation at 37 °C for 45 min. Finally, 2 mL of PBS was added, the cells were pelleted at 1000 rpm for 5 min and re-suspended in 500 μL of PBS for flow cytometer analysis with a BD LSR II cytometer.

4.5. In vitro uptake studies

Wild type p53 human osteosarcoma cells (SJSA-1) and colon cancer cells (HCT116) were grown in a 24-well plate at a density of 400,000 cells/well. They were allowed to attach for 24 h prior to treatment. FAM and Nutlin–Glycine–FAM conjugate were directly added to the separate culture media at a concentration of 1 and 5 μM and incubated for 1 h at 37 °C. DMSO (5 μM) or culture media itself was also incubated for 1 h at 37 °C. Cells were then washed three times with pre-warmed PBS (37 °C), harvested by scraping, and counted and diluted to 100,000 cells/mL. Approximately 10,000 cells per well were seeded in 96-well black-wall plates, and the fluorescence intensity in cells was measured in a fluorometric plate reader (FLUOstar OPTIMA, Durham, NC) with a 485/520 nm filter at an optical magnification of 20×. For each sample tested a total of 3 runs were assayed.

4.6. Cell blocking studies

In this experiment, wild type p53 human osteosarcoma cells (SJSA-1) and colon cancer cells (HCT116) were used at a density of 600,000 cells/well. They were allowed to attach for 24 h prior to treatment. Nutlin–Glycine derivative (10-fold excess) was added to a separate culture media at a concentration of 5 μM prior treatment with Nutlin–Glycine–FAM for 1 h at 37 °C. Nutlin–Glycine–FAM (5 μM) was added into this media and further incubated for 1 h at 37 °C. FAM and DMSO were also incubated for 1 h at a concentration of 5 μM as negative controls. Cells were then washed, harvested and approximately 10,000 cells per well were seeded in 96-well black-wall plates, and the fluorescence intensity in cells was measured in a fluorometric plate reader with a 485/520 nm filter. For each sample tested a total of 3 runs were assayed.

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References and notes

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