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***In vitro* cytotoxicity and *in vivo* efficacy, pharmacokinetics and metabolism of pyrazole-based small-molecule inhibitors of Mdm2/4-p53 interaction**

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Abstract

Purpose—The interaction of p53, with its negative regulators Mdm2/4 has been widely studied [1]. In p53^{+/+} cells, expression of Mdm2/4 leads to p53 turnover, inhibition of downstream transcription, decreasing cell cycle arrest or apoptosis. We report *in vitro* cytotoxicity and *in vivo* efficacy, pharmacokinetics, and metabolism of YH264, YH263 and WW751, three proposed small molecule inhibitors of the Mdm2/4-p53 interaction.

Methods—MTT cytotoxicity assays were performed and alterations in proteins were examined using Western blots. Mice were dosed 150 mg/kg YH264 or YH263 iv or po QDx5. Mice were iv dosed 88 mg/kg, 57 mg/kg, or 39 mg/kg WW751 for three, five, or five days. YH264, YH263 and WW751 and metabolites were quantitated by LC-MS/MS.

Results—IC₅₀ values for YH264, YH263 and WW751 against p53 wild type HCT 116 cells after 72 h of incubation were 18.3 ± 2.3 μM, 8.9 ± 0.6 μM, and 3.1 ± 0.2 μM respectively. Only YH264 appeared to affect p53 expression *in vitro*. None of the compounds affected the growth of HCT 116 xenografts in C.B-17 SCID mice. YH264 plasma half-life was 147 min; YH263 plasma half-life was 263 min; and WW751 plasma half-life was less than 120 min.

Conclusions—Despite dosing the mice at the maximum soluble doses, we could not achieve tumor concentrations equivalent to the intracellular concentrations required to inhibit cell growth *in vitro*. YH263 and WW751 do not appear to affect p53/Mdm2 and none of the three were active in a subcutaneous HCT116 p53^{+/+} xenograft model.

Introduction

The interaction of p53 with Mdm2 is one of the most widely studied protein-protein interactions and many small molecule inhibitors of this interaction have been proposed [1]. The nuclear transcription factor and tumor suppressor p53 plays a major role in cells under

stress by stimulating a variety of biological responses including DNA repair, cell cycle arrest, and apoptosis. Loss of p53 function by either mutation or deletion is found in nearly 50% of all cancers [2]. The expression of Mdm2 (murine double minute 2) leads to the turnover of p53 by inhibiting its transcriptional activity. Overexpression of Mdm2 is found in nearly 7% of tumors; the most common being soft tissue tumors (20%) which include, Ewing's sarcoma, lipomas, liposarcomas, and malignant fibrous histiocytomas [3]. Mdm2 and p53 interact primarily through their N-terminal domains and regulate one another through feedback loops [4, 5]. Mdm2 regulates p53 in two ways: it alters p53 stability by decreasing the half-life and decreases its activity as a transcription factor. Even a small decrease in p53 reduces Mdm2 protein and results in an increase in p53 activity. Mdm4 (MdmX) is a structural homolog of Mdm2 that interacts with Mdm2 through its ring domains and cooperates with Mdm2 in regulating p53 function [6]. The use of small molecules to prevent Mdm2/4-p53 interaction and reactivate the function of p53 is a promising therapeutic strategy. However, few small molecule inhibitors have been developed that are active in cells and have appropriate pharmacokinetic parameters. Several small molecules have progressed to clinical trials including Nutlin-3 and its derivatives (RG7112 and RG7388) MI-319, CGM097, and DS-3032b [6]. YH264, YH263, and WW751 are three small molecule inhibitors that were developed at the University of Pittsburgh and had nM activity in protein binding assays, were characterized by co-crystal structures, and had low μ M activity in the NCI 60 cell screen [7–9, 11, 12]. In this manuscript we have assessed the activity of YH264, YH263, and WW751 *in vitro* and *in vivo*. Our objective was to characterize the uptake of these small molecules in HCT 116 p53^{+/+} colon cancer cells, and the *in vitro* cytotoxicity on both HCT116 p53^{+/+} and HCT 116 p53^{-/-} cells. *In vivo*, we evaluated the efficacy, pharmacokinetics and metabolism of YH264, YH263 and WW751 in C.B-17 SCID mice bearing HCT 116 p53^{+/+}, xenografts.

Material and Methods

Drugs and Reagents

YH263, YH215, YH264, YH245, WW751, and YH_WW (Fig. 1) were developed by Dr. Alex Dömling. Control mouse plasma was purchased from Lampire Biological laboratories, Inc. (Pipersville, Pa). Acetonitrile (HPLC-grade) and water (HPLC-grade) were purchased from Fisher Scientific (Fairlawn, NJ). Formic Acid (Reagent –Grade was purchased from Sigma-Aldrich Co. (St. Louis, MO). Nitrogen gas and liquid nitrogen were purchased from Valley National Gases, Inc. (Pittsburgh, PA). Cremophor EL, and methylthiazolyldiphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO). Sterile saline (0.154 M NaCl), sterile water, and phosphate-buffered saline (PBS) were purchased from Baxter Healthcare Corporation (Deerfield, IL). Ethanol (200 Proof) was purchased from Pharmaco Products (Brookfield, CT). Nitrogen gas for the mass spectrometer was purified with a Parker Balston Nitrogen Generator (Haverhill, MA).

Dosing Solutions

Dosing solutions were prepared by dissolving YH264 or YH263 at a final concentration of 15 mg/mL in cremophor EL:ethanol:saline (1:1:8, v/v/v). Dosing solutions for WW751 were prepared by dissolving WW751 at a final concentration of 8.8 mg/mL in cremophor

EL:ethanol:saline (1:1:12, v/v/v). Mice received 0.01 mL/g exact fasted body weight of the dosing solutions or the appropriate vehicle.

Mice

Specific-pathogen-free, female C.B-17 SCID mice (4–6 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA) and allowed to acclimate for one week to the animal facilities at the University of Pittsburgh Cancer Institute. Mice were maintained in microisolator cages and handled in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and on a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Ventilation and airflow in the animal facility were set to >12 changes/h. Room temperature was regulated at 22 ± 2 °C, room humidity was between 30–70%, and the rooms were kept on automatic 12-h light/dark cycles. Mice received ProLab ISOPRO RMH 3000, Irradiated Lab Diet (PMI Nutrition International, Brentwood, MO) and water *ad libitum* except on the evening prior to dosing for the pharmacokinetic studies, when all food was removed and withheld until 4 h after dosing. Sentinel animals remained free of specific pathogens throughout the study period, implying that the study animals were free of specific pathogens.

Tumor Cell Lines

HCT 116 colon adenocarcinoma cell line (p53^{+/+}) were obtained from ATCC (Manassas, VA); HCT 116 p53^{-/-} cells were kindly provided by Dr. Bert Vogelstein [10, 11]. Cells were cultured in RPMI-1640 medium with L-glutamine (BioWhittaker Inc., Walkersville, MD), containing 10% heat-inactivated fetal bovine serum and 100 units of penicillin/mL and 100 µg/mL of streptomycin (Biofluids, BioSource, Rockville, MD) in an incubator with 95% air, 5% CO₂, and 95% humidity at 37°C.

MTT Assay

HCT 116 cells (5×10^5 cells) in logarithmic growth were plated into each well of 96-well culture plates and allowed to acclimate for 24 h. Compound was added to wells such that the final concentrations ranged between 0.3–100 µM in medium containing 3% DMSO. After 72 h, 50 µl of 1 mg/mL MTT was added to each well and incubated for 4 h. At the end of the incubation, medium containing drug and MTT was removed from each well, and 150 µl DMSO was added, followed by shaking for 5 min. The absorbance at 570 nm was read on a Tecan Safire 2 microplate reader (Tecan US Inc., Morrisville, NC). Results were compared to wells containing vehicle-treated cells and expressed as % inhibition. The IC₅₀ was calculated from triplicate experiments using the Hill equation and the computer program ADAPT 5 (D'Argenio and Schumitzky, 1997).

Cellular Accumulation of YH264, YH263 and WW751

HCT 116 cells (1×10^7 cells in logarithmic growth) were incubated for 0, 0.5, 1, 2, or 4 h in 3 mL of complete medium containing 18 µM, 9 µM and 3.5 µM of YH264, YH263 or WW751, respectively. After incubation, cells were harvested, split into two samples of 1.5 mL each, and overlaid in microcentrifuge tubes containing 0.5 mL of silicon oil (Silicones, lot no. SF1250, General Electric Production Division, Waterford, NY). The tubes were

centrifuged at $13,000 \times g$ for 6 min. After centrifugation, the top 1 mL of medium was removed and stored in cryovials at -80°C until analysis. The remaining medium and silicon oil were carefully removed without disturbing the cell pellets. The sides of the tubes were cleaned with cotton-tipped applicators, and the cell pellets were stored at -80°C until analysis.

Preparation of Cell Lysates and Western Blot Assay

HCT 116 cells (1×10^7) were plated in culture dishes and allowed to attach overnight. At 24 h, the compounds were added at the concentrations indicated: YH264 (60 μM), YH263 (30 μM), or WW751 (15 μM) for 4 h. Doxorubicin at 1 μM served as a positive control for DNA damage. Vehicle samples were processed the same way as the drug treated samples to control for baseline handling stress. At the end of incubation, cells were washed twice with PBS, scraped, and spun down at $13,000 \times g$ for 5 min to collect the cell pellet. Cell pellets were lysed in 200 μL $1\times$ cell lysis buffer (Cell signaling #9803) supplemented with protease inhibitors (Halt protease inhibitor, Thermo scientific). The protein concentration in each lysate supernatant was measured using Bio-Rad Protein assay. Samples (10 μg of protein/sample) were run on a 4–15% tris-HCl gel (Criterion ready gel). The separated proteins were transferred onto a nitrocellulose membrane and blocked with Odyssey blocking buffer. The membranes were then incubated with antibodies against Mdm2 (1:500, Santa Cruz sc-812), p-53 (1:500, Santa Cruz sc-126), p-21 (1:1,000, Cell signaling, 12D1), and GAPDH (1:10,000 Millipore MAB374) overnight at 4°C . The blots were probed with Licor Goat anti-rabbit IRDye 800 CW (1:10,000 for Mdm2 and p-21), goat-anti mouse IRDye 680 CW (1:15,000 for p-53), or goat anti-mouse IRDye 800 CW (1:10,000 for GAPDH). The membranes were read on the LICOR Odyssey and the fluorescent signal of the bands was expressed as integrated intensity (kcounts/ mm^2). Mdm2, p53 and p21 protein expressions were normalized to GAPDH protein expression and the change in expression with each treatment was compared to the protein ratio to GAPDH in the vehicle control.

Tumor Implantation

HCT 116 cells (5×10^6 cells in logarithmic growth) were injected subcutaneously into the right flank of passage female C.B-17 SCID mice. When tumors reached approximately 500 mm^3 , passage mice were euthanized with CO_2 , tumors were harvested aseptically, and ~25 mg fragments were implanted on the right flank of study mice. When the tumors in the study mice were approximately 200 mm^3 , the animals were stratified to treatment groups, such that there were no differences in mean body weights or tumor volumes between the groups (ANOVA and Kruskal-Wallis, $p = 0.95$).

Determination of Maximum Tolerated Doses

The single maximum tolerated doses of YH263, YH264 and WW751 were determined in Balb/C mice using the UP and Down method after single bolus doses administered by lateral tail vein injection [14]. The starting doses for YH263 and YH264 were 150 mg/kg (15 mg/mL in 1:1:8 cremophor:ethanol:saline (v/v/v)) and for WW751 was 200 mg/kg (20 mg/mL in 1:1:12:: cremophor:ethanol:saline (v/v/v)).

Efficacy Study

Female C.B-17 SCID mice bearing HCT 116 xenografts were dosed with either YH264, YH263 (150 mg/kg iv or po or 100 mg/kg iv) or WW751 (88 mg/kg, 57 mg/kg or 39 mg/kg iv) or vehicle (1:1:8 cremophor:ethanol:saline) for QDx5, and body weights and tumor volumes were recorded twice weekly. Tumors were measured with digital calipers, and tumor volumes were calculated using the formula: $TV = (L \times W^2)/2$, where L is the longest diameter of the tumor and W is the shortest diameter perpendicular to L. Mice were followed for 16, 6 and 16 days after the last administered dose of YH264, YH263, or WW751, respectively, so that tumor growth could be monitored. To calculate doubling time, the tumor volume of each mouse was normalized to day 0 (first day of dosing).

Pharmacokinetic Study

C.B-17 SCID mice bearing HCT116 xenografts were fasted overnight and treated either iv or po with 150 mg/kg of either YH264 or YH263. Three mice per time point were euthanized by CO₂ inhalation at 5, 15, 30, 60, 120, 240, 360, and 1440 min after iv administration of YH264 and 1440 min after iv vehicle administration. Mice treated po with YH264 were euthanized at 5, 15, 30, 60, 120, 360 min after administration. The YH264 treated mice that were euthanized at 1440 min had urine collected at 0–6 h and 6–24 h as were the 1440 min vehicle-treated mice. These mice were housed in separate metabolism cages that were washed with 15 mL of sterile water after final collections of urine. Three mice per time point were euthanized at 5, 15, 30, 60, 120, 360, 960, and 1440 min after iv administration of YH263 and 1440 min after iv vehicle administration. After po administration of YH263 mice were euthanized at 15, 60, 120, 360, and 960 min post administration. The YH263 treated mice that were euthanized at 960 min had urine collected at 0–16 h while the 1440 treated mice had urine collected at 0–6 and 6–24 h as were the 1440 min vehicle-treated mice. Blood was collected by cardiac puncture using 3 cc heparinized syringes. Tumor was collected, weighed, and snap-frozen in liquid nitrogen. Blood samples were gently mixed and then centrifuged at $13,000 \times g$ for 4 min to obtain plasma and packed red blood cells. All samples, including urine, and cage washes, were stored at -80°C until analysis. WW751 was administered to mice at 88 mg/kg iv and the mice were euthanized at 5, 60 and 180 min after administration.

Chromatography

The LC system consisted of an Agilent (Palo Alto, CA, USA) 1100 autosampler and binary pump, a Phenomenex (Torrance, CA, USA) Synergi Polar-RP (4 μm , 2 mm $\times$ 100 mm) column kept at ambient temperature, and a gradient mobile phase. Mobile phase solvent A was 0.1% (v/v) formic acid in acetonitrile, and mobile phase solvent B was 0.1% (v/v) formic acid in water. The initial mobile phase was 40% solvent A and 60% solvent B pumped at a flow rate of 0.3 mL/min. Between 0 and 12 min, the percentage of solvent A was increased linearly to 80%. At 12.1 min, the flow rate was increased to 0.5 mL/min, which was maintained until 17 min. Between 17 and 17.1 min, the percentage of solvent A was decreased linearly to 40% and held until 24 min, at which time the next sample was injected into the LC system. For WW751, the initial mobile phase was 35% solvent A and 65% solvent B pumped at a flow rate of 0.3 mL/min. Between 5.6 and 9 min, Between 9.1

min and 15min, the percentage of solvent A was decreased linearly to 35% and then held until 24 min, at which time the next sample was injected into the LC system.

Mass Spectrometry

Mass detection was performed on a Micromass Quatromicro triple-stage bench top mass spectrometer (Waters Corporation, Milford, MA) with electrospray ionization in positive ion monitoring mode. The settings of the mass spectrometer were as follows: capillary voltage, 4.0 kV; cone voltage, 30 V; source temperature, 120 °C; desolvation temperature 450 °C. The cone and desolvation gas flows were 0 and 550 L/h, respectively. The collision voltage used was 20 V. Quadrupoles 1 and 2 each had low mass and high mass resolution set at 12. The dwell time was 0.15 s, and the interscan delay was 0.1 s. The MRM m/z transitions monitored were 480.0 to 436.0 for YH264 and 508.0 to 462.0 for YH263. Their respective internal standard m/z transitions were 462.0 to 418.0 for YH245 and 490.0 to 443.0 for YH215. The LC system and mass spectrometer were controlled by Waters Micromass MassLynx software (version 4.0), and data were collected with the same software. Under these conditions, the retention times of YH264, YH263 and their internal standards YH245 and YH215 were approximately 8.7, 11.1, 8.5 and 11.0 min, respectively. Standard curves of YH264 and YH263 at concentrations of 30–10000 ng/mL in plasma were prepared in triplicate. The analyte-to-internal standard ratios were calculated by dividing the area of each analyte peak by the area of the respective internal standard peak for that sample. Standard curves were fitted by linear regression, weighted by $1/y^2$, followed by back calculation of concentrations. Based on the structure of the parent compound, we screened for masses corresponding to expected metabolic transformations, such as hydroxylation, glucuronidation, and an O-glucuronide. Concentrations of metabolites present in plasma and tumor are expressed as ng-equivalents/mL to either YH264 or YH263. WW751 mass detection was performed on the same system as described for YH263 and YH264, but for the cone voltage which was 40 V. The MRM m/z transitions monitored were 629.0 to 85.5 for WW751 and 647.0 to 68.0 for YH-WW (internal standard). Under these conditions, the retention times of WW751 and internal standard were approximately 3.8 and 4.1 min, respectively. Standard curves of WW751 at concentrations of 30–10,000 ng/mL in plasma were prepared and analyzed as described above.

Preparation of calibration standards and quality control samples

Stock solutions were prepared at 1.0 mg/mL in acetonitrile and stored at –80 °C in the dark. On the assay day, this solution was diluted (in steps of 10-fold) with acetonitrile to obtain the lower calibration working solutions of 0.1, 0.01, and 0.001 mg/mL. Using these working solutions calibration samples were prepared at 0, 30, 100 300, 1,000, 3,000, 5,000, and 10,000 ng/mL in control mouse plasma. Quality Control (QC) stocks were prepared separately at 20, 250, and 8000 ng/mL and stored at –80 °C in the dark..

Sample Preparation

Plasma, tissue culture medium, HCT 116 cell pellets, and urine were diluted with control mouse plasma to fit within the upper limit of quantification. Tumor samples were homogenized in PBS (3 mL for each g) and then diluted with control mouse plasma to fit within the assay range. A 10 µl internal standard mixture (1 µg/mL of YH245 and 10 µg/mL

of YH215 in acetonitrile) was added to a 50 μ L sample of plasma, medium, cell pellet, urine, or tumor homogenate in a 1.5-mL microcentrifuge tube. After adding 500 μ L of acetonitrile and vortexing for 1 min, samples were centrifuged at $12,000 \times g$ at room temperature for 5 min. Supernatants were transferred to 12 mm $\times$ 75 mm borosilicate glass tubes and evaporated to dryness under a gentle stream of nitrogen at 37 $^{\circ}$ C. Each dried residue was re-suspended in 100 μ L of acetonitrile: water: formic acid (80:20:0.1, v/v/v), and 5 μ L of sample was injected into the LC-MS/MS. Samples containing WW751 were processed similarly except the internal standard was YH-WW (10 μ L of 1 μ g/mL).

Pharmacokinetic Analysis

The terminal half-life, clearance, and volume of distribution at steady-state for plasma and tumor were determined by standard non-compartmental methods, with PK Solutions 2.0 (Summit Research Services, Montrose, CO; <http://www.summitPK.com>). The maximum concentration ($C_{\max}$) and time to reach the maximum concentration ($t_{\max}$) were determined by visual inspection of the plasma concentration vs time curves. Area under the concentration vs time curve ($AUC_{0 \text{ to } t}$) was calculated by the trapezoidal rule with extrapolation to infinity ($AUC_{0 \text{ to } \infty}$). Compartmental analysis for YH264 was performed using a two compartment open model comparing po and iv administration and also using an uncoupled tumor in ADAPT5 (D'Argenio DZ, Schumitzky A, Wang X (2009) ADAPT 5 user's guide: pharmacokinetic/pharmacodynamic systems analysis software. Biomedical Simulations Resource, Los Angeles). Because of the variability in the plasma concentrations of YH263, compartmental modeling was not performed.

Results

In Vitro Cytotoxicity

The IC_{50} values for YH264, YH263 and WW751 against p53 wild type HCT 116 cells after 72 h of incubation were $18.3 \pm 2.3 \mu$ M, $8.9 \pm 0.6 \mu$ M, and $3.1 \pm 0.2 \mu$ M respectively (Fig. 2 A–C). The IC_{50} for YH264 was higher in the p53 null cells than in the wild type HCT 116 cells (28 vs 18 μ M). The IC_{50} 's for the other compounds were not different when p53 null HCT 116 cells were used and were 10.2 vs 8.9 and 3.3 vs. 3.1 μ M for YH263 and WW751, respectively.

Cellular Accumulation of YH263, YH264 and WW751

HCT 116 cells accumulated all three compounds in a similar manner and peak concentrations in the cells were reached at 4 h after the addition of the compounds to medium (Fig. 2D). Assuming that the volume of 10^7 cells is approximately 10 μ L, the accumulation in the cells relative to medium was approximately 60-fold in YH264-treated cells, approximately 200-fold in YH263-treated cells, and approximately 200-fold in WW751 exposed cells. The concentrations measured in the cell pellets by 4h during uptake were $>500 \mu$ M for YH263 and YH264 and $>200 \mu$ M for WW751 (Fig. 2D).

Western Blots

When HCT 116 p53^{+/+} cells were exposed to doxorubicin or the Mdm2/4 inhibitors in culture for 4 h at 3 times the 72 h IC_{50} concentrations, Mdm2 expression was only increased

by 31% in the doxorubicin treated cells and 19% in the YH264 treated cells when compared to the expression in the vehicle treated control cells (Fig. 2 E and F). YH264 and doxorubicin also increased the expression of p53 and p21 in the wild type cells by 4.7- and 4.5-fold and 4.6- and 7-fold, respectively, compared to their baseline expression in the vehicle treated p53^{+/+} cells (Fig. 2 E and F). No changes were apparent in Mdm2, and p53 was only increased 1.8- and 1.6-fold, respectively, after treatment with YH263 or WW751 in these cells. In the p53^{-/-} cells Mdm2 expression was slightly less after treatment with doxorubicin or the inhibitors than was present in the vehicle treated cells. No p53 was detected and p21 expression was also slightly reduced from that observed in the vehicle treated cells.

Determination of MTD

The single dose iv MTD of both YH263 and YH264 were determined to be the highest soluble dose at 150 mg/kg (15 mg/mL) when administered to both male and female BALB/c mice.

For WW751, the single dose iv MTD was 88 mg/kg. When Balb/C mice were administered 200 mg/kg WW751 as a slow iv bolus, the mice died within 0.5 min of dosing. The dose was then reduced by 1/3 to 133 mg/kg, resulting in mice dying within 2 min of dosing. Further dose reduction by 1/3 to 88 mg/kg did not result in lethality. During the first 4 h after dosing 88 mg/kg, mice had reduced activity and lethargy and did not move when prompted, but the mice recovered normal activity and behavior by 24 h after dosing. The mice maintained body weight and did not exhibit further toxicity during the 16 days of observation. No gross lesions were observed upon necropsy.

Efficacy Studies

Treatment of C.B-17 SCID mice, bearing HCT 116 xenografts, with 150 mg/kg iv or po or 100 mg/kg iv or po of YH263 daily for five consecutive days had no significant effect on tumor growth (Fig. 3B). Over the course of treatment, the mean body weight of the mice receiving YH263 did not decrease by more than 6% of their initial body weight over the 5 days of treatment (Fig 3A) and following treatment, the body weights of the mice treated with YH263 or vehicle were not different from those of the untreated control mice.

Treatment at 150 mg/kg iv or po of YH264 daily for five days did not result in any decrease in tumor growth (Fig. 3C and D) and the body weight loss in the animals during treatment was of the same magnitude as observed with YH263. When mice bearing HCT 116 xenografts were treated with 88 mg/kg iv of WW751 daily, treatment was stopped after the third dose due to loss of body weight and decreased activity following treatment (Fig. 3E and F). Mice treated with 88 mg/kg lost 16.8% body weight by day 4. These mice did not regain body weight back to their starting body weight during the 21 days of observation. Mice treated with 57 mg/kg, or 39 mg/kg iv daily for five consecutive days had body weight losses of 12% and 6%, respectively. Oral administration of 200 mg/kg WW751 resulted in a 13.5% loss of body weight during treatment. None of these animals exhibited the loss of activity observed in the mice treated with 88 mg/kg WW751 iv. Treatment for 5 days with WW751 had no effect on tumor growth (Fig 3F).

Pharmacokinetics of YH264

After iv administration of 150 mg/kg YH264 to mice bearing HCT 116 xenografts, peak plasma concentration ($C_{\max}$) of YH264 was $1451 \pm 676 \mu\text{g/mL}$ ($3020 \pm 1411 \mu\text{M}$) at 5 min, while tumor $C_{\max}$ was $43.7 \pm 12.0 \mu\text{g/g}$ ($91 \pm 0.2 \mu\text{M}$) at 240 min. YH264 plasma concentrations declined rapidly while the tumor concentration remained relatively constant out to 24 h (Fig.4A). Four metabolites were detected in plasma: 2 hydroxylated metabolites, a hydroxylated glucuronide, a glucuronide, but at concentrations $\sim 1/10,000$ of YH264 in plasma, assuming equal ionization efficiency (Fig.4B). All metabolites were detectable in plasma out to 360 min. The plasma half-life of YH264 was 147 min, clearance was 1.2 mL/min/kg, and the volume of distribution was approximately 0.255 L/kg. No metabolites were detected in tumor. Urinary excretion of YH264 was less than 0.003% of the dose after iv administration. After po administration of 150 mg/kg to mice bearing HCT 116 xenografts, plasma $C_{\max}$ of YH264 was $72.1 \mu\text{g/mL}$ ($151 \mu\text{M}$) at 120 min. The O-glucuronide, the glucuronide, and the hydroxylated metabolites were not detectable until 60 min. (Fig. 4C) Plasma half-life was 219 min, apparent clearance was 5.7 mL/min/kg, and the apparent volume of distribution was approximately 1.80 L/kg. YH264 was not detected in tumor after po administration. The bioavailability of YH264 after po administration of 150 mg/kg was 12.6%. The non-compartmental data are summarized for YH264 in Table 1. YH264 plasma and tumor data was modeled using an uncoupled tumor model in ADAPT5 and the parameters are presented in Table 2.

Pharmacokinetics of YH263

After iv administration of 150 mg/kg YH263 to mice bearing HCT 116 xenografts, plasma $C_{\max}$ of YH263 was $995 \pm 249 \mu\text{g/mL}$ ($1960 \pm 491 \mu\text{M}$) at 5 min, while the tumor $C_{\max}$ was $20.5 \pm 0.5 \mu\text{g/g}$ ($40 \pm 0.1 \mu\text{M}$) at 120 min (Fig 5A). YH263 plasma concentrations declined rapidly along with an O-glucuronide and two hydroxylated metabolites.(Fig. 5B). YH263 was also metabolized to YH264 and its metabolites. The peak concentration of YH264 was $5.9 \pm 1.5 \mu\text{g/mL}$ ($12 \pm 3 \mu\text{M}$) at 120 min, and its glucuronide, O-glucuronide and hydroxylated metabolites were also present in plasma. YH263 and its major metabolite YH264 had estimated plasma half-lives of 263 and 219 min, respectively. Clearance of YH263 was 3.3 mL/min/kg while the volume of distribution was approximately 1.24 L/kg. No metabolites of YH263 or YH264 were detected in tumor. Urinary excretion of YH263 after iv dosing was less than 0.005% of the dose. Oral administration of 150 mg/kg of YH263 to mice resulted in a $C_{\max}$ of $8.2 \pm 0.5 \mu\text{g/mL}$ ($16 \pm 1 \mu\text{M}$) at 120 min (Fig.5C) and a half-life of 222 min. Again, YH264 was the major metabolite, with a plasma $C_{\max}$ of $0.47 \pm 0.13 \mu\text{g/mL}$ ($0.9 \pm 0.2 \mu\text{M}$) at 360 min. Other metabolites of YH263 were only detectable out to 120 min. Apparent plasma clearance of YH263 was 68.7 mL/min/kg while the apparent volume of distribution was approximately 22.0 L/kg. Peak tumor concentration of YH263 was $5.18 \mu\text{g/g}$ ($10.2 \mu\text{M}$) at 360 min. Metabolites observed in tumor included YH264 at concentrations approximately 10-fold lower than YH263 and other metabolites of YH263 at even lower concentrations (Fig.5D). Bioavailability of YH263 was 10.9% after po administration at 150 mg/kg. The non-compartmental data for YH263 are summarized in Table 1.

Pharmacokinetics of WW751

Only very preliminary pharmacokinetics were performed on WW751 due to its apparent toxicity in mice. After 88 mg/kg iv, the plasma C_{max} of WW751 was only 100 $\mu\text{g/mL}$ (158 μM) and concentrations decreased very rapidly, suggesting a half-life of less than 120 min. Peak tumor concentration was 5.2 $\mu\text{g/g}$ (8.3 μM).

Discussion

p53 is involved in numerous cell processes including regulation of the cell cycle, apoptosis, senescence, metabolism, angiogenesis, DNA repair and even innate immunity. This tumor suppressor is inactivated in about 50% of tumors either due to mutation or deletion and in some other tumors, its function is inhibited by overexpression of Mdm2. Inhibitors of Mdm2/p53 binding can overcome this negative regulation by interfering with the binding and this inhibition is considered to be an attractive strategy for development of new cancer therapeutics.

Several Mdm2 inhibitors are currently in clinical trials including RG-7112, RO5503781, MK-8242, MI-888, CGM097 and DS-3032b [6, 15]. The results of RG7112 as both a single agent and in combination with AraC in AML were recently reported. RG7112 demonstrated single agent activity and 17% complete responses were observed in the Phase Ib study with Ara C [15].

The three small molecules presented in this paper, YH264, YH263, and WW751, were purported to be inhibitors of Mdm2/p53 based on previous protein binding and co-crystal structure studies, and are all based on the 6-chloroindole structure as described by Popowicz, Dömling and Holak [9]. When evaluated initially for disruption of Mdm2-p53 binding *in vitro*, all had nM activity in protein binding assays, were characterized by co-crystal structures, and had low μM activity in the NCI 60 cell screen [12, 13]. In the studies described here, we evaluated the activity of these compounds against HCT116 colon carcinoma cells because we could compare their activity against the parental HCT-116 line expressing wild type p53^{+/+} and with the activity against the mutant HCT-116 cell line that is p53^{-/-}. In agreement with the results of the NCI 60 screen, all three compounds were cytotoxic *in vitro* with IC_{50} s in the mid μM range. The carboxylic ethyl ester of YH263 may have increased its penetration into cells and resulted in a lower IC_{50} of 8.9 μM , compared to 18 μM for YH264 with a free carboxylic acid. As was shown in Figure 2, the IC_{50} s of these compounds were only slightly lower in the HCT-116 cell line that was p53^{+/+} compared to the IC_{50} s in the HCT-116 p53^{-/-} cell line. This suggested that these compounds may affect other pathways within the cells and not target p53/Mdm2. Conventional Mdm2 inhibitors, such as Nutlin-3 and MI-43, have IC_{50} values that are at least 10-fold higher in HCT-116 p53^{-/-} cells than in the wild type cells [21].

The uptake of these inhibitors into the wild type HCT-116 cells in culture was rapid and the estimated cellular concentrations were between 60 and 200 times higher than the observed medium concentrations. The cellular uptake was lower for YH264 than for YH263, presumably due to the lack of charge and increased lipophilicity afforded by the carboxy ethyl ester of YH263. For all compounds, the medium concentrations of the compounds

decreased between 0 and 240 min from their initial concentrations of 18 μM , 9 μM and 3.5 μM for YH264, YH263 and WW751 respectively, but then the medium concentrations of YH263 and YH264 remained relatively constant over the remaining observation period. Medium concentrations of WW751 continued to decrease with time so that the concentrations at 24 h were only about 0.02 μM .

Examination of the expression of p53, Mdm2, and downstream p21 by Western blots (Figure 2 E and F) did not support the conclusion that YH263 or WW751 were inhibitors of Mdm2 and YH264 appeared to be a weak inhibitor compared to the previous studies in which nM activity in protein binding assays and co-crystal structures suggested the compounds were inhibitors of p53Mdm2/4. For our studies we used doxorubicin as a positive control because doxorubicin induces DNA damage and the sensitivity of this colon cancer cell line to doxorubicin appears to be associated with a dose dependent increase in both p53 and p21 [17]. Induction of p53 after treatment with doxorubicin was shown to be controlled at the posttranslational level in another colon cancer cell line RKO [18]. Further, HCT-116 p53^{-/-} cells were reported to be approximately 2-fold less sensitive to doxorubicin [17], in line with the 2-fold differences observed in these p53^{-/-} cells in response to doxorubicin in our *in vitro* studies. Only one of the compounds, YH264, appeared to increase p53 expression in a range equivalent to that of doxorubicin. After treatment with either doxorubicin or YH264, the increase in p53 was accompanied by a similar increase in the expression of p21. We assume that this increase in p21 following treatment with doxorubicin is due to the increase in p53 we observed and occurs by the pathways that have previously been documented in colon cancer cell lines [17–20]. Mdm2 inhibitors, such as Nutlin-3 and MI-43, increase the expression of p53, p21, Mdm2 and DR5 expression in p53 wild type colon cancer cell lines [20–21]. The compounds, YH263, YH264 and WW751, did not increase the expression of Mdm2.

When administered to SCID mice bearing HCT-116 colon xenografts iv for 5 days, YH263 and YH264 did not affect tumor growth. WW751 was toxic at its maximum soluble dose and the drug dose had to be decreased to 57 mg/kg in order to complete the 5 days of intravenous dosing, which did not result in a decrease in tumor growth.

When the plasma concentrations of these 3 compounds were evaluated and compared with tumor concentrations, the plasma C_{max} for each compound was ~50 X higher than their respective tumor C_{max} . In spite of tumor concentrations at least 2-fold higher than the *in vitro* IC₅₀ (18 μM , medium concentration) for YH264, the only compound to show changes in p53 *in vitro*, the tumor concentrations were much lower than the observed cell concentrations obtained *in vitro* (>500 μM) for this compound. Based on these calculations it was not surprising that neither this compound nor its analogue (YH263) was efficacious against the HCT116 xenografts. The more potent *in vitro* compound, WW751, was much more toxic to mice than either YH264 or YH263 and the concentrations that could be achieved in the plasma and the tumor of the mice were also below the cell concentrations achieved in the cell pellets during the *in vitro* uptake studies (Fig 2D).

After iv administration, both YH264 and YH263 had plasma half-lives greater than 3 h in mice, but neither compound was well absorbed after po administration, with po

bioavailabilities of less than 13%. Based on the limited metabolism studies of these compounds, both YH264 and YH263 appear to be susceptible to metabolism by both phase I and phase II metabolic enzymes. YH263 was hydrolyzed to form YH264 and further hydroxylation and glucuronidation of these compounds was observed, suggesting metabolism as a major route of elimination. Further supporting metabolism as a major route of elimination, very little of either compound was excreted unchanged in the urine.

Based on the data presented here, these 6-chloroindole compounds do not target Mdm2-p53 interactions in HCT116 cells. Only YH264 resulted in changes in p53 concentrations, but did not affect Mdm2. Modifications in the basic structure will be required to improve the selectivity targeting of Mdm2 and p53 and to increase the metabolic stability and po bioavailability of future compounds in order to develop analogues that will be active in animal models and can be advanced to the clinic.

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Abbreviations

PBS	phosphate-buffered saline
LC-MS/MS	liquid chromatography/tandem mass spectrometry
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
AUC	area under the drug concentration versus time curve
C_{max}	maximum peak concentration

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Figure 1.
Chemical structures of YH263, YH215, YH264, YH245, WW751 and YH_WW.

F

Sample #	1	2	3	4	5	6	7	8	9	10
Cell line	p53 wt					p53 null				
Treatment	Vehicle	Dox 1 uM	YH264 60 uM	YH263 30 uM	WW751 15 uM	Vehicle	Dox 1 uM	YH264 60 uM	YH263 30 uM	WW751 15 uM
Mdm2/GAPDH	0.97	1.28	1.15	1.00	0.93	0.72	0.62	0.64	0.63	0.63
p53/GAPDH	0.10	0.45	0.47	0.17	0.13	0.00	0.00	0.00	0.00	0.00
p21/GAPDH	0.08	0.56	0.37	0.14	0.13	0.13	0.14	0.11	0.11	0.11

Figure 2.

IC₅₀'s of YH263, YH264 and WW751 against HCT 116 colon cancer cells, uptake in HCT 116 wt cells, and protein alterations by Western blot.

A–C: The IC₅₀'s for each compound were determined by MTT assay after 72 h of compound exposure in medium. Both wild type HCT 116 p53^{+/+} and HCT 116 p53^{-/-} cells were tested simultaneously. Each of these plots is a representative of 3 assays conducted using triplicate wells at each concentration of the compound. A: YH264; B: YH263; and C: WW751.

D: Cells and media were processed as described under accumulation. The IC_{50} concentrations were respectively 9, 18 and 3.5 μM for YH263, YH264 and WW751. Cells and medium were processed as described in the methods under cell accumulation. ○ YH664 in cells; □, YH263 in cells; , , WW751 in cells; •, YH264 medium; ■, YH263 medium. ▲ WW751 medium.

E&F: Western Blots of YH263, YH264 and WW751 against HCT116 p53 wild type cells and HCT 116 p53^{-/-} cells after 4 h of exposure. HCT116 p53^{+/+} and p53^{-/-} cells were exposed to the Mdm2/4-p53 inhibitors: at 3× the IC_{50} concentration of YH264 (60 μM), YH263 (30 μM), WW751 (15 μM) or doxorubicin (1 μM) for 4 h. Cells were harvested, lysed and Western blots run as described in the Methods. Densities are expressed as the ratio to the GAPDH loading control band in F

Figure 3.

Mean tumor volumes and body weights of the mice bearing HCT-116 sc xenografts during and following 5 days of treatment with YH263, YH264 or WW751, respectively. A and B: YH263 mean body weights $\pm$ standard deviation for groups of 5 mice and mean tumor volumes $\pm$ standard deviation. C and D: YH264 at its maximum soluble dose of 150 mg/kg iv or po mean body weights $\pm$ standard deviation for groups of 5 mice and mean tumor volumes $\pm$ standard deviation. E and F: WW751 at doses of 88, 57 and 39 mg/kg iv and 200 mg/kg po. The dose of 88 mg/kg iv could only be administered for 3 days because of toxicity.

Mean body weights $\pm$ standard deviation for groups of 5 mice and mean tumor volumes $\pm$ standard deviation

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Figure 4.

Plasma and tumor concentrations vs time curves of YH264 and metabolites. A. Comparison of plasma and tumor concentrations with time after iv administration of 150 mg/kg YH264. B. Plasma concentrations of YH264 and metabolites expressed as YH264 equivalents/mL after iv administration of YH264 at 150 mg/kg. C. Plasma concentrations of YH264 and metabolites expressed as YH264 equivalents/mL after po administration of YH264 at 150 mg/kg.

Figure 5.

Plasma and tumor concentrations vs time curves of YH263 and metabolites and WW751. A. Comparison of plasma and tumor concentrations with time after iv administration of 150 mg/kg of YH263. ○, plasma; □, tumor. B. Plasma concentrations of YH263, YH264 and other metabolites expressed as YH263 equivalents/mL after IV administration of YH263 at 150 mg/kg. ○, plasma YH263; □, YH264; △, YH263-OH; ▽, YH264-O-B; *, YH263-O-glucuronide. C. Plasma concentrations of YH263, YH264 and other metabolites expressed as YH263 equivalents/mL after PO administration of YH263 at 150 mg/kg. ○, YH263;

□, YH264; ○, YH264-OH-A; △, YH264-glucuronide; *, YH263-O-glucuronide. D: Tumor concentrations of YH263, YH264 and other metabolites expressed as YH263 equivalents/mL after PO administration of YH263 at 150 mg/kg. ○, YH263; □, YH264; △, YH264-OH-A; △, YH264-glucuronide; *, YH263-O-glucuronide. E: Plasma and tumor concentrations vs time curves of WW751 after iv administration of 88 mg/kg WW751. ○, plasma; □, tumor.

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Table 1
Non-compartmental plasma pharmacokinetic parameters for YH264, YH263 and WW751.

Compound	Dose (mg/kg)	Route	C _{max} (µg/mL)	t _{max} (min)	t _{1/2} (min)	AUC _{0 to t} (µg·min/mL)	AUC _{0 to ∞} (µg·min/mL)	CL (mL/min/kg)	Vd (mL/kg)	F
YH264	150	IV	1451.5	5	147	124943	125032	1.2	255	1.0
YH264	150	PO	72.1	120	219	15708	26362	5.69	1797	0.126
YH263	150	IV	994.7	5	263	45477	45834	3.3	1240	1.0
YH263	150	PO	8.2	120	222	2111	2184	69	21966	0.05
WW751	88	IV	100.1	5	120	***	***	***	***	***

Table 2

Compartmental parameters for YH264 fitting plasma (2 compartments) and tumor (uncoupled compartment) concentrations. Data were modeled using a 2-compartment open linear model with maximum likelihood estimation. R^2 plasma= 0.814; R^2 tumor = 0.314.

Parameter	Value	%CV
K_e	0.0134	26.28
V_c	59.7	24.97
K_{cp}	0.048	23.18
K_{pc}	0.006	21.32
K_{ct}	0.0002	15.14
K_{tc}	0.004	25.89
V_t	1	NE