Anti-Cancer Drugs 2002, **13,** pp. 359–366

Preclinical report

Identification of phenyl-pyridine-2-carboxylic acid derivatives as novel cell cycle inhibitors with increased selectivity for cancer cells

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Ro 41-4439, a phenyl-pyridine-2-carboxylic acid derivative, was identified by a cell-based screening approach that exploits the differences between normal and cancer cells in their sensitivity to cytotoxic agents. This compound showed low micromolar antiproliferative activity and cytotoxicity against a broad panel of human cancer cell lines *in vitro*, and over 10-fold selectivity to cancer cells when tested in parallel with a panel of proliferating normal human cells. Cytotoxicity of Ro 41-4439 is due to arrest of cell cycle progression in mitosis followed by induction of apoptosis. Four-week treatment of nude mice bearing established mammary tumor xenografts (MDA-MB-435) with well-tolerated doses of the compound showed 73% inhibition of tumor growth. Limited exploration of structure–activity relationships involving side chain length, and aryl and pyridine rings allowed for the identification of more potent analogs. [© 2002 Lippincott Williams & Wilkins.]

Key words: Antitumor, cancer cells, cell cycle, mitosis, xenografts.

Introduction

Antimitotics represent one of the most important classes of cancer chemotherapeutics used in the clinic today.[1,2] These drugs (e.g. taxanes, vinca alkaloids) are powerful cell cycle inhibitors, and potent antiproliferative, pro-apoptotic and antitumor agents.[3] However, their clinical efficacy has been limited by a relatively low selectivity index.[1] Novel cell cycle inhibitors with increased selectivity for proliferating cancer cells may provide an improved therapeutic window and better clinical outcome in the treatment of cancer.

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Recently, we developed a cell-based *in vitro* screening approach for the identification of novel antitumor agents that exploits the differential sensitivity of normal and cancer cells. It is a three-step, high-throughput screen for tumor cell-specific antiproliferative and/or cytotoxic activity measured by a 7-day MTT assay using small panels of proliferating primary human cells and established cancer cells.[4] Application of this screening approach to a library of 110 000 compounds allowed the identification of several novel classes of compounds active against a broad panel of cancer cell lines *in vitro*. Ro 41-4439 is one of the screening hits representing a novel class of mitotic inhibitors (phenyl-pyridine-2-carboxylic acid derivatives) with enhanced selectivity toward cancer cells *in vitro* and antitumor activity against established breast cancer xenografts.

Materials and methods

Chemicals and synthesis

All reagents used in the chemical synthesis were purchased from Aldrich (Milwaukee, WI). All other chemicals were from Sigma (St Louis, MO) unless otherwise indicated in the text.

The synthesis of phenyl-pyridine-2-carboxylic acid derivatives is shown in Figure 1.

- Picolinic acid (**1**): Reference to the chemical entity: Aldrich.
- Methyl-4-chloropicolinate hydrochloride (**2**): reference to the chemical entity.[5]
- (2-Amino-ethyl)-carbamic acid tert-butyl ester (**3**): reference to the chemical entity.[6]

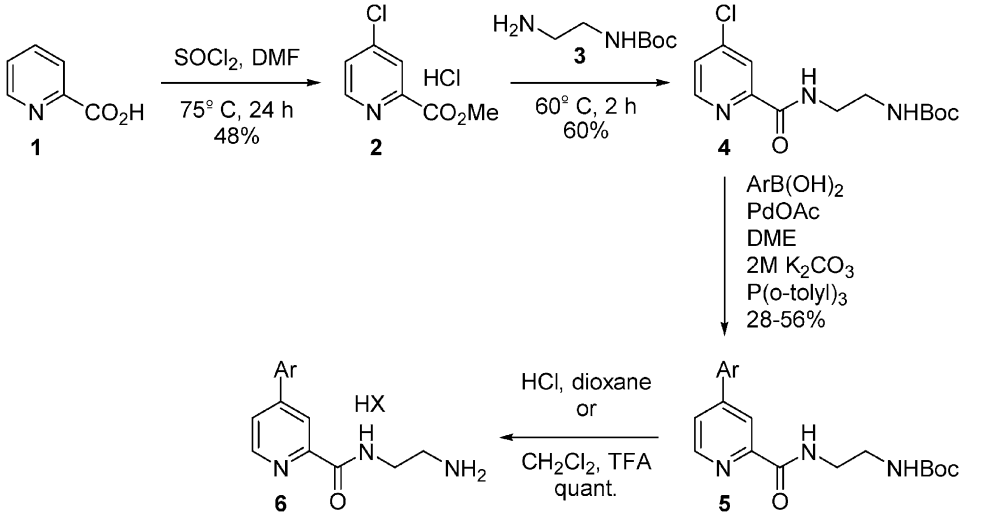


Figure 1. Synthetic scheme of phenyl-pyridine-2-carboxylic acid derivatives.

- Carbamic acid, [2-[[(4-chloro-2-pyridinyl)carbonyl]-amino]ethyl]-1,1-dimethylethyl ester (**4**): reference to the chemical entity: Ger. Offen. (1986), 51 pp. DE 3530046.
- (2-{[4-(2-Methoxy-phenyl)-pyridine-2-carbonyl]-amino}-ethyl)-carbamic acid tert-butyl ester (**5g**): ^{1}H-NMR (300 MHz, $CDCl_3$).
- 4-(2-Methoxy-phenyl)-pyridine-2-carboxylic acid (2-amino-ethyl)-amide (**6g**): ^{1}H-NMR (300 MHz, $CDCl_3$).

Cells

MDA-MB-435 cells were obtained from Dr Patricia Steeg (National Cancer Institute) upon permission of Dr Janet Price (MD Anderson Cancer Center, University of Texas).[7] RKO and SW480 cells were provided by Dr Bert Vogelstein (Johns Hopkins Oncology Center).[8] All other cell lines were purchased from ATCC (Rockville, MD). They were propagated in DMEM (SW480, RKO, HeLa and WI38), RPMI-1640 (MDA-MB-231, MDA-MB-435, DU145, PC3 and T-47D) or McCoy's 5A (HCT116) media supplemented with 10% heat-inactivated fetal bovine serum (Gibco/BRL, Rockville, MD) under 5% CO_2. Human prostate epithelial cells (PrEC) and human mammary epithelial cells (HMEC) passage 2 and 6, respectively, were purchased from Clonetics (San Diego, CA), and were propagated in the media recommended by the supplier (PrEGM BulletKit for PrEC and MEGM BulletKit for HMEC). To maintain cells in exponential growth, every 2 weeks new cultures of PrEC and HMEC cells were started from early passage frozen aliquots (500 000 cells/vial). Cell growth inhibition and cytotoxicity were measured using a 7-day MTT assay[9] as described previously.[4]

Cell cycle analysis

SW480 and RKO cells (10^6) were plated in 75-cm^2 tissue culture flasks 24 h prior to the experiment. They were incubated with the test compounds for 22 h, trypsinized and collected by low-speed centrifugation. Cell pellets were washed twice with PBS [phosphate-buffered saline (PBS)] and once with 5 ml cold 70% ethanol. Cells were collected by centrifugation (1000 *g*, 5 min) and resuspended in 0.5 ml PBS. Then 0.5 ml RNase A (1 mg/ml in PBS) was added and tubes were incubated at 37°C for 15 min. After addition of 0.1 ml propidium iodide (1 mg/ml in PBS), each sample was passed through a filter cap tube to remove cell aggregates. Samples were read in FACSort (Becton Dickinson, San Jose, CA) using the CellQuest program. Data were analyzed using ModFit software.

Metaphase spreads

Cells were seeded in flasks as for cell cycle analysis. They were incubated with the tested compounds for 18 h, trypsynized and collected by a low-speed centrifugation. Cell pellets were washed twice with PBS, resuspended in hypotonic buffer (5 ml, 0.04 M KCl, 0.025M Na citrate) and incubated at 37°C for

10 min. An equal volume of methanol:acetic acid (3:1) was added to the pellets with gentle mixing and the cells were spun down (200 *g*, 5 min). They were resuspended gently in a small volume of ice-cold methanol:acetic acid and stored at 4°C. Metaphase cell spreads were prepared by the gravity method by dropping 20 μl cell suspension on pre-cleaned glass microscope slides. They were stained with Giemsa (0.4%) for 5 min, washed with water and dried before microscopic examination.

Tubulin polymerization assay

The effect of chemical compounds on tubulin polymerization *in vitro* was tested using the Cyto-DYNAMIX screen 02 kit (Cytoskeleton, Denver, CO) following manufacturer's protocol. The kinetics of tubulin polymerization (bovine brain, 3 mg/ml, >97% pure) in 80 mM PIPES buffer, 1 mM $MgCl_2$, 1 mM EGTA and 1 mM GTP was measured at 37°C by following the absorbance at 340 nm for 60 min.

DNA fragmentation assay

Apoptosis was detected using the DNA fragmentation assay as described previously.[4] Briefly, subconfluent RKO cells in six-well tissue culture plates were treated with the test compounds for 24 h. Cells were collected by centrifugation, washed with PBS and resuspended in 0.5 ml lysis buffer (50 mM Tris–HCl, pH 8.0, 1.0 M NaCl, 10 mM EDTA and 0.5% SDS). Lysates were transferred to microcentrifuge tubes and Proteinase K (Roche Molecular Biochemicals) was added to a final concentration of 0.2 mg/ml and incubated at 37°C overnight. DNA was extracted by phenol:chloroform:isoamylic alcohol (24:24:1) and precipitated with 70% ethanol. DNA pellets were dried and dissolved in 40 μl of 10 mM Tris–HCl (pH 8.0)/0.1 mM EDTA. DNase-free RNase A (1 μl) was added to each sample and incubated for 30 min at 37°C. Aliquots of each sample were run on 2% agarose gels containing 0.5 μg/ml ethidium bromide at 80 V for 1.5 h. DNA was visualized under UV illumination.

Tumor inhibition studies

Female CD1 *nu/nu* mice (5–6 weeks old; Harlan, Correzzana, Italy) were injected s.c. in the mammary fat pad with 1.5×10^6 MDA-MB-435 cells in 0.1 ml HBSS. Drug treatment was initiated 17 days post-implantation when palpable s.c. tumors (approximately 100 mg) were established. Mice were randomized in experimental groups (10 mice/group). Animals were treated with compounds twice a day, 5 days a week for 4 weeks. Tumor growth was monitored by weekly caliper measurements. Animal body weight was measured weekly for all groups. Tumor weight (TW) was calculated as follows: $TW=1/2ab^2$, where a and b are the long and the short diameters of the tumor mass in millimeters.

Results

Identification and antitumor activity of Ro 41-4439

Ro 41-4439 (Figure 2A) was first identified in a cell-based screen designed to detect agents with increased selectivity to cancer cells.[4] In addition to the original four cancer cell lines used in the screen (SW480, RKO, MDA-MB-435 and RKO), the compound was tested for activity against a wider panel of tumor-derived cell lines representing three major solid tumor types: breast (MDA-MB-435, MDA-MB-231 and T47D), colon (SW480, RKO, HCT116 and COLO320) and prostate (DU145 and PC3), and showed IC_{50} in the range of 1.2–4.2 μM (Table 1). There was no clear correlation between the degree of inhibition and the growth rate of the cell lines, since the IC_{50} for SW480 cells with a population doubling (PD) time of 25.3 h was lower than that of the slightly faster growing RKO cells (PD=19.1 h).[4] Similarly, there was no obvious dependence on the cancer type.

Ro 41-4439 was tested for selectivity using a panel of three normal primary (WI38, PrEC and HMEC) and four cancer cell lines (DU145, MDA-MB-435, RKO and SW480) with comparable growth rates.[4] The IC_{90} values measured by a 7-day MTT assay were used to calculate the selectivity to cancer cells. It was shown previously that IC_{90} values represent a more accurate measure of the selectivity of test compounds than the IC_{50} values.[4] This selectivity expressed as a ratio between the average IC_{90} for the panel of three normal lines, and the IC_{90} for each individual cancer cell line was in the range between 10 and 20 (Figure 2A). During the 7-day incubation with Ro 41-4439, cancer cells were severely growth inhibited or killed at concentrations well below the dose needed to suppress significantly the growth of normal cells (Figure 2B).

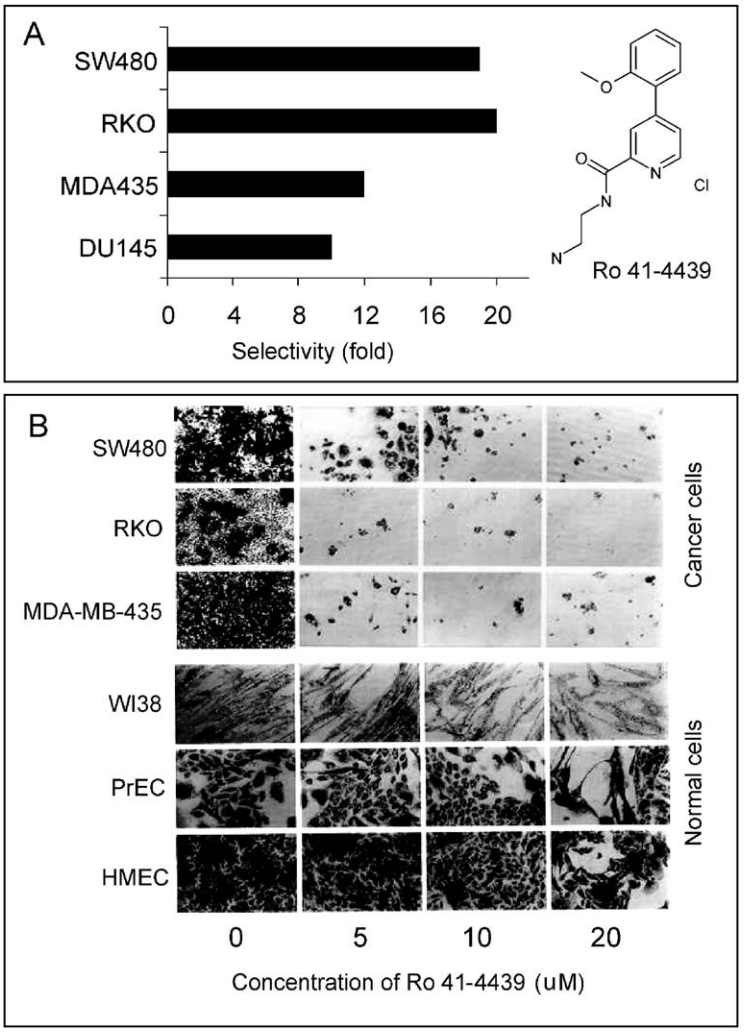


Figure 2. Selectivity of Ro 41-4439 for cancer cells. (A) Ro 41-4439 was tested on a panel of three normal (WI38, PrEC and HMEC) and four cancer cell lines (SW480, RKO, MDA-MB-435 and DU145) using a 7-day MTT assay.[4] Percent inhibition and IC_{90} values were determined for each cell line in three independent experiments. Selectivity was calculated as the ratio between the average IC_{90} of Ro 41-4439 for the panel of normal cell lines (44 μM) divided by the IC_{90} for each individual cancer line.[4] (B) Cells were seeded in 96-well plates as for the MTT assay and incubated with the indicated concentrations of Ro 41-4439 for 7 days. Viable cells were stained by incubation with MTT (1 mg/ml) for 1 h.

To gain insight into the mechanism of its antiproliferative activity, we investigated the effect of Ro 41-4439 on cell cycle progression. Treatment of SW480 cells with increasing concentrations of the compound for 22 h led to a significant increase in the fraction of G_2/M phase cells at 6 μM (48% in G_2/M) and to a complete G_2/M block at 12 μM (Figure 3A). In order to distinguish between G_2 and M phase arrest, metaphase spreads were prepared from exponentially growing SW480 cells exposed to Ro 41-4439. Treatment with 10 μM Ro 41-4439 for 18 h produced cells with highly condensed chromosomes indicative of metaphase arrest (Figure 3B). Similar mitotic arrest was observed in MDA-MB-435 and RKO cells exposed to the compound (data not shown). Since most of the known mitotic inhibitors have been shown to interfere with the microtubule dynamics,[2] we tested Ro 41-4439 for its ability to affect tubulin polymerization *in vitro*. At 100 μM, a concentration 20-fold higher than the average IC_{90} determined for most of the tested cancer cell lines *in vitro* (Table 1), the compound did not show a significant effect on the polymerization rate compared with the untreated control. Under the same conditions 3 μM Nocodazole, used as a control tubulin polymerization inhibitor, completely blocked tubulin polymerization

(data not shown). This result does not support the role of direct interference with tubulin polymerization as a cause of mitotic arrest.

Next, we tested the ability of Ro 41-4439 to induce chromosomal DNA fragmentation, one of the hallmarks of apoptosis. Subconfluent RKO cells were treated with increasing concentrations of the compound for 24 h, cells were lysed and their DNA was analyzed by agarose gel electrophoresis. A nucleosomal size DNA ladder was detected in all cells exposed to 6 μM or greater concentrations of the compound (Figure 3C) This result indicates that the inhibition of mitotic progression by Ro 41-4439 likely leads to induction of apoptosis in tumor cells.

Table 1. Antiproliferative activity of Ro 41-4439 measured by a 7-day MTT assay

Cells	IC_{50}	IC_{90}
Breast cancer		
MDA-MB-435	1.5	3.7
MDA-MB-231	4.2	9.5
T47D	1.6	4.3
Colon cancer		
SW480	1.2	2.2
RKO	1.3	2.5
HCT116	1.5	2.7
COLO 320	3.2	7.2
Prostate cancer		
DU145	3.2	4.4
PC3	4.1	9.2

We have shown previously that known cancer drugs exhibit 4 fold or greater selectivity to a panel of cancer cells versus normal proliferating cells at their IC_{90} *in vitro*. However, most of them were either non-selective or only marginally selective at the IC_{50}.[4] To evaluate the selectivity of Ro 41-4439 relative to known cancer drugs, it was tested in parallel with two other mitotic inhibitors (paclitaxel and vinblastine) using the standard four cancer/three normal cell panel (Figure 4). At the IC_{90}, all three compounds demonstrated selectivity to the cancer cells with Ro 41-4439 emerging as the most selective (16-fold selectivity). However, at IC_{50} Ro 41-4439 was the only selective compound (7-fold selectivity). Vinblastine had a selectivity=1 while paclitaxel showed a higher selectivity to the normal cell panel (selectivity <1).

To assess the *in vivo* antitumor potential of Ro 41-4439, nude mice bearing established xenografts of

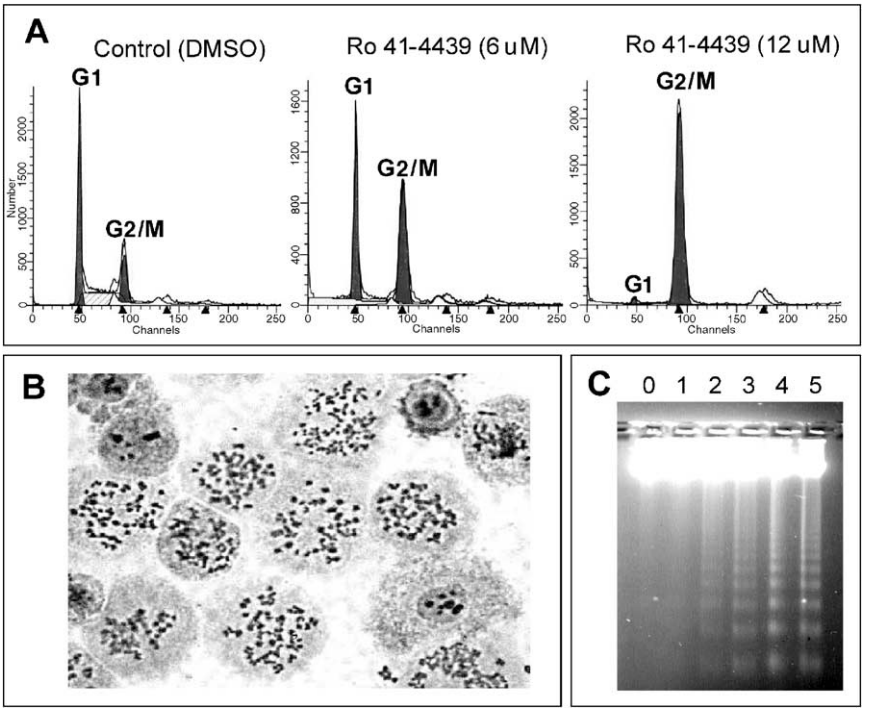


Figure 3. Ro 41-4439 is a mitotic inhibitor. (A) SW480 cells were incubated with the indicated concentrations of Ro 41-4439 or equivalent volume of the solvent DMSO for 22 h and their cell cycle distribution was analyzed by FACS. (B) Metaphase spreads from SW480 cells treated with 10 μM Ro 41-4439 as above. (C) Nucleosomal DNA ladder from RKO cells incubated with Ro 41-4439 for 24 h: lane 0, control (DMSO); lane 1, 3 μM; lane 2, 6 μM; lane 3, 12 μM; lane 4, 24 μM; lane 5, 48 μM.

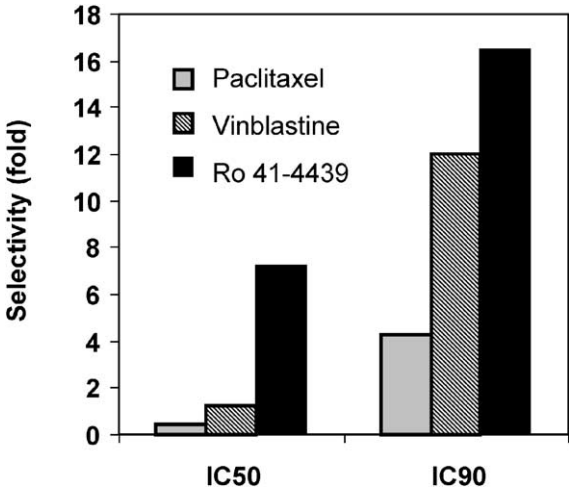


Figure 4. Cancer cell selectivity of Ro 41-4439, paclitaxel and vinblastine. Compounds were tested in a 7-day MTT assay as in Figure 2(A). Selectivity was calculated as the ratio between the average IC_{50} (paclitaxel 3.8 nM, vinblastine 3.5 nM and Ro 41-4439: 12.6 μM) or IC_{90} (paclitaxel 55.2 nM, vinblastine 41.2 nM and Ro 41-4439 44.0 μM) of the normal cell panel divided by the average IC_{50} or IC_{90}, respectively, of the cancer cell panel. A selectivity of 1 indicates no difference in IC_{50} between normal and cancer cells.

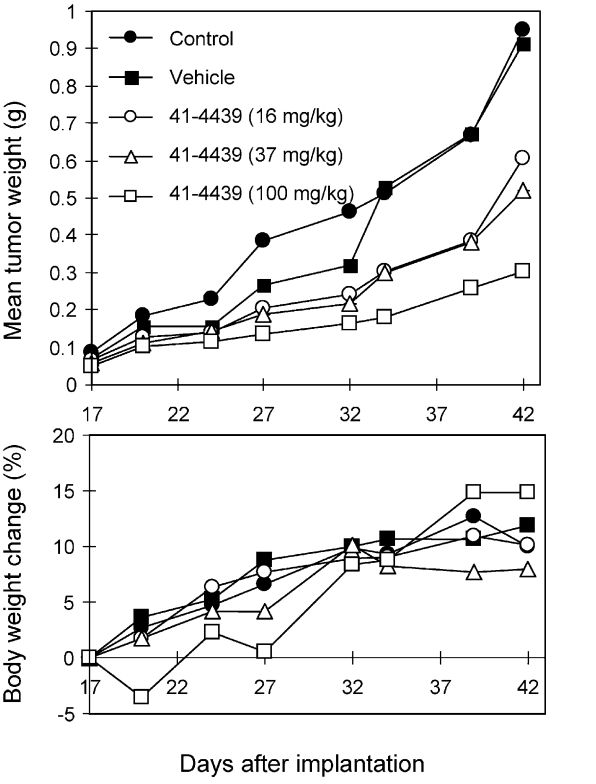


Figure 5. Antitumor activity of Ro 41-4439 Nude mice with established MDA-MB-435 xenografts were injected i.p. with the indicated doses twice a day, 5 times a week, for 4 weeks.

the human breast cancer cell line MDA-MB-435 were injected with the compound i.p. twice a day for 4 weeks. This treatment showed a dose-dependent inhibition of tumor growth (Figure 5). At the highest dose tested (100 mg/kg) Ro 41-4439 suppressed the growth of the tumor xenografts by 73%. This dose was very well tolerated since the mice in this group had body weights comparable to the vehicle controls and did not show any signs of toxicity upon necropsy.

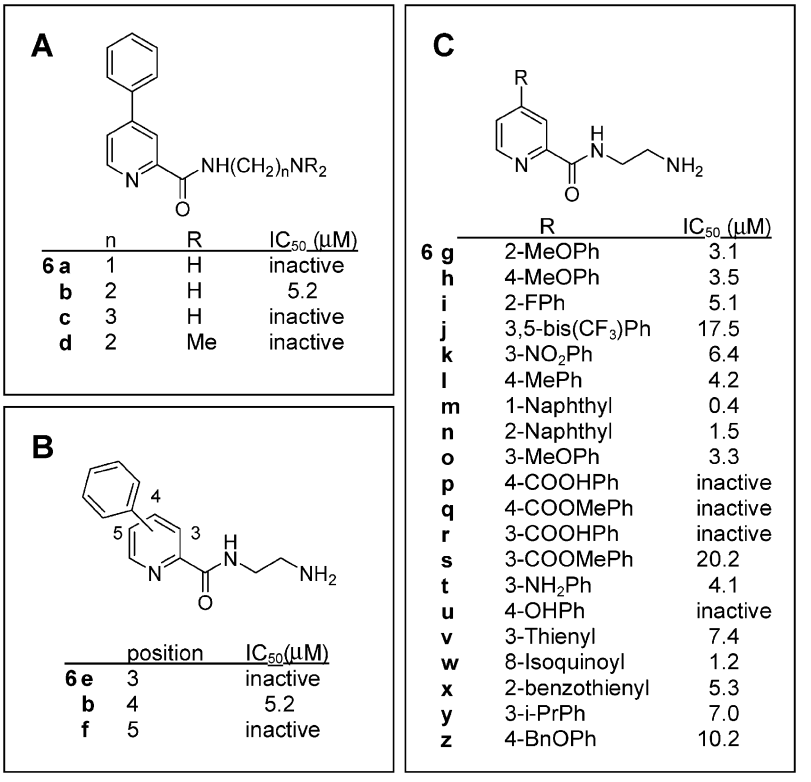
Structure–activity relationship and optimization of Ro 41-4439

After the identification of RO 41-4439 as a selective cell cycle inhibitor, a rapid exploration of the structural requirements for activity was undertaken using a small library of compounds that were either already available or prepared in parallel utilizing the methods depicted in Figure 1. In this report, only three types of modification of the lead structure will be discussed: pyridine side chain length and composition, and pyridine aryl substitution and pyridine aryl position. All analogs were assessed for antiproliferative activity by the MTT assay using DU145 cells (Figure 6).

Side chain modifications. With respect to the amide 'tail' attached to the 2 position of the pyridine, it was determined that the length of this chain was crucial. Both the 1-carbon or 3-carbon-linked side chain analogs (**6a** and **6c**) were completely inactive as compared to the unsubstituted parent (**6b**, Figure 6A). In addition, if the terminal amino group of the side chain was dimethylated (**6d**), this also resulted in a complete loss of activity.

Aryl ring position. The 4-aryl substitution of the pyridine ring appears to be a requirement for activity. Neither the 3 nor 5 phenyl analogs (**6e** and **6f**) retained any activity (Figure 6B).

Aryl ring substitution. A small library of 4-aryl-substituted derivatives were prepared in parallel according to the scheme in Figure 1. Although a wide range of activities was observed within this set, clear structure–activity relationships could not be established. In general, *ortho*, *meta* and *para* substitutions with both electron donating as well as electron withdrawing groups were tolerated. Larger



	n	R	IC_{50} (μM)
6 a	1	H	inactive
b	2	H	5.2
c	3	H	inactive
d	2	Me	inactive

	position	IC_{50}(μM)
6 e	3	inactive
b	4	5.2
f	5	inactive

	R	IC_{50} (μM)
6 g	2-MeOPh	3.1
h	4-MeOPh	3.5
i	2-FPh	5.1
j	3,5-bis(CF_3)Ph	17.5
k	3-NO_2Ph	6.4
l	4-MePh	4.2
m	1-Naphthyl	0.4
n	2-Naphthyl	1.5
o	3-MeOPh	3.3
p	4-COOHPh	inactive
q	4-COOMePh	inactive
r	3-COOHPh	inactive
s	3-COOMePh	20.2
t	3-NH_2Ph	4.1
u	4-OHPh	inactive
v	3-Thienyl	7.4
w	8-Isoquinoyl	1.2
x	2-benzothienyl	5.3
y	3-i-PrPh	7.0
z	4-BnOPh	10.2

Figure 6. Structure–activity relationship among phenyl-pyridine-2-carboxylic acid derivatives. IC_{50} was calculated from a 7-day MTT assay using DU145 cells.

groups in the *meta* or *para* positions (or both) generally resulted in a 2- to 5-fold decrease in activity (**6j**, **6y** and **6z**, Figure 6C). Acidic functionality in the meta or *para* positions (COOH, OH) were not tolerated (**6p**, **6r** and **6u**), nor were the corresponding esters (**6q** and **6s**). The aromatic ring could be heterocyclic (**6v**, **6w** and **6x**) and bicycles improved activity (**6m**, **6n** and **6w**). The 1-naphthyl and 2-naphthyl derivatives (**6m** and **6n**) are 2- and 7.5-fold more active then Ro 41-4439, respectively. Cell cycle analysis of SW480 and RKO cells treated with **6m** and **6n** confirmed that these compounds belong to the same mechanistic class of mitotic inhibitors as Ro 41-4439 (not shown).

Discussion

Ro 41-4439 represents a new class of cell cycle inhibitors, phenyl-pyridine-2-carboxylic acid derivatives with increased selectivity for cancer cells *in vitro*. They arrest cell cycle progression predominantly in metaphase and induce apoptosis similar to most known antimitotic cancer drugs.[2] However, Ro 41-4439 emerges as an agent with higher selectivity to proliferating cancer cells especially at IC_{50} values. It was shown previously that the selectivity detected by the panels of normal and cancer cells is not due to differences in the proliferation rate of the cells.[4] Presently, we do not know the molecular mechanism by which Ro 41-4439 causes mitotic arrest. Our preliminary studies did not show a measurable effect on tubulin polymerization *in vitro*, which is the most frequently observed cause of cell cycle arrest in metaphase. However, this observation does not exclude the possibility that microtubule dynamics can be affected by a weaker interaction that is difficult to detect using a cell-free tubulin polymerization assay.[10] Also, we cannot exclude the possibility that multiple cellular targets are responsible for the observed antimitotic activity.

It is reasonable to speculate that higher selectivity *in vitro* against cancer cells compared with normal proliferating cells will translate into an increased chance for tumor selectivity *in vivo*. In line with this expectation, Ro 41-4439 showed encouraging antitumor activity against established breast cancer

xenografts in nude mice with no signs of toxicity. Further evaluation of new analogs from this chemical class should address this possibility.

Phenyl-pyridine-2-carboxylic acid derivatives described here have not been reported previously as antitumor agents. The 4-phenyl pyridine carboxamide substructure can be found in some bruneomycin and streptonigirn derivatives; however, their cytotoxic activity has been attributed to the quinone moiety which is absent in our compounds.[11,12]

Cell cycle inhibitors which target mitosis (e.g. taxanes, vinca alkaloids) are among the most widely used cancer therapeutics today.[2] They are powerful inducers of apoptosis and highly potent antitumor agents.[3] However, their efficacy in the clinic has been hampered by the toxicity associated with suppression of proliferating non-tumor cells.[1] New antimitotic agents with better selectivity to cancer cells could offer a superior safety profile. Such improved selectivity may come from modulation of yet unknown molecular target (s) of the cancer cell cycle.[13] Further studies with the new class of mitotic inhibitors, phenyl-pyridine-2-carboxylic acid derivatives, may provide valuable clues to understanding the mechanism that accounts for their increased selectivity.

Conclusion

Ro 41-4439 and other phenyl-pyridine-2-carboxylic acid derivatives described in this report represent a novel class of mitotic inhibitors with increased selectivity to proliferating cancer cells *in vitro*. They inhibit cell cycle progression in metaphase by an unknown mechanism and the growth of human breast cancer xenografts in nude mice Further exploration of this chemical class may yield chemotherapeutic agents with improved safety profile.

Acknowledgments

We wish to thank Drs Grace Ju and Duncan Walker for critically reading the manuscript.

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(Received 12 January 2002; accepted 28 January 2002)