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The discovery of new cytotoxic pyrazolopyridine derivatives

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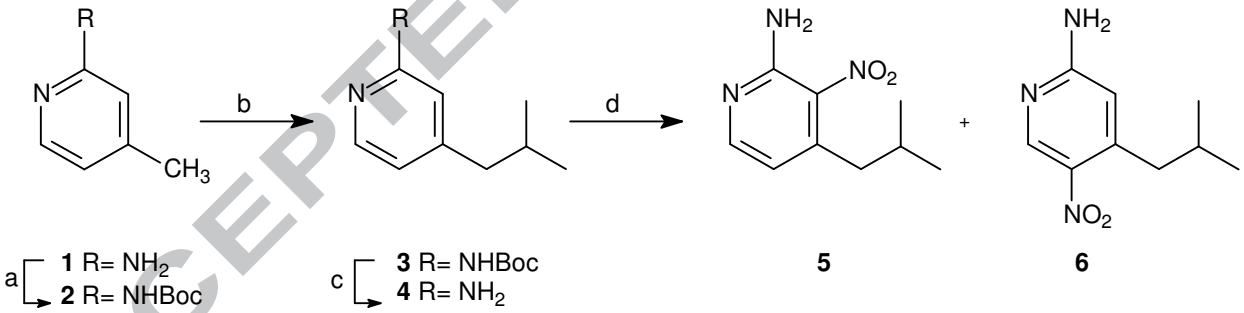
Abstract. A number of new 3,7-disubstituted pyrazolo[3,4-*c*]pyridines have been designed and synthesized from suitable 2-aminopyridines. The antiproliferative activity of the derivatives was determined against the pancreatic MIA PaCa-2 and ovarian SCOV3 cancer cell-lines. IC_{50} values of the most promising analogue **46** lie in the submicromolar or low micromolar range. Furthermore, compound **46** shows similar inhibitory activities against DU145, A2058 and PC-3 cancer cells, blocks the cell cycle at the G_0/G_1 phase and induce apoptosis, as determined by the appearance of apoptotic nuclei.

Keywords pyrazolo[3,4-*c*]pyridine; antiproliferative activity; Suzuki coupling; deazapurines; cell-cycle selectivity

In addition to their contribution in encoding of genetic information, the naturally occurring purines adenosine and guanosine and their nucleotide derivatives play crucial roles in a number of biological processes, mainly involved in cell metabolism and cell proliferation.[1] Many cellular proteins contain a purine recognition pocket for their interaction with their corresponding energy intermediates, substrates, allosteric modulators or cofactors, therefore, numerous purine derivatives have been designed and developed to selectively inhibit these enzymes and receptors.[2] Depending on their substitution pattern and the wide variety of the substituents, these compounds were found to exhibit a broad range of biological and pharmaceutical properties.[3-6] Besides serving as privileged medicinal chemistry scaffolds, these derivatives also inspired parallel development of alternative heterocyclic isosteres, in the attempt to explore the therapeutic potential or improve the physicochemical and pharmacokinetic properties of the corresponding purine derivatives.[7-10] However, all these compounds must closely mimic the overall shape of the endogenous natural products and retain certain hydrogen bond

donor/acceptor binding motifs, in order to maintain the ability to substitute for them in various biological processes. Changing the location of a nitrogen atom from position 9- to position 8- in the purine ring leads to pyrazolopyrimidines, which were initially reported as adenosine receptor antagonists, and were further found to exert potent anti-inflammatory,[11] anti-viral[12] and anticancer properties.[13-14] The closely related pyrazolopyridine core, which can be considered as the bioisosteric deazapyrazolopyrimidine scaffold, can also serve as pharmacophore for the discovery of biologically interesting compounds.[15-16] The anticancer potential of several pyrazolo[3,4-*d*]pyridines has been already studied,[13] as well as their investigated molecular mechanisms, such as inhibition of EGFR,[17] IGF-1R[18] and dual Src/Abl.[19]
In the course of our involvement in the synthesis and biological activity evaluation of pyrazolo[3,4-*c*]pyridine derivatives[20-22] we have initiated a project towards the study of the impact of certain substitution patterns on this interesting scaffold. We thus present herein the synthesis of some new 3,7-disubstituted pyrazolo[3,4-*c*]pyridines and their antiproliferative activity against cancer cell lines.

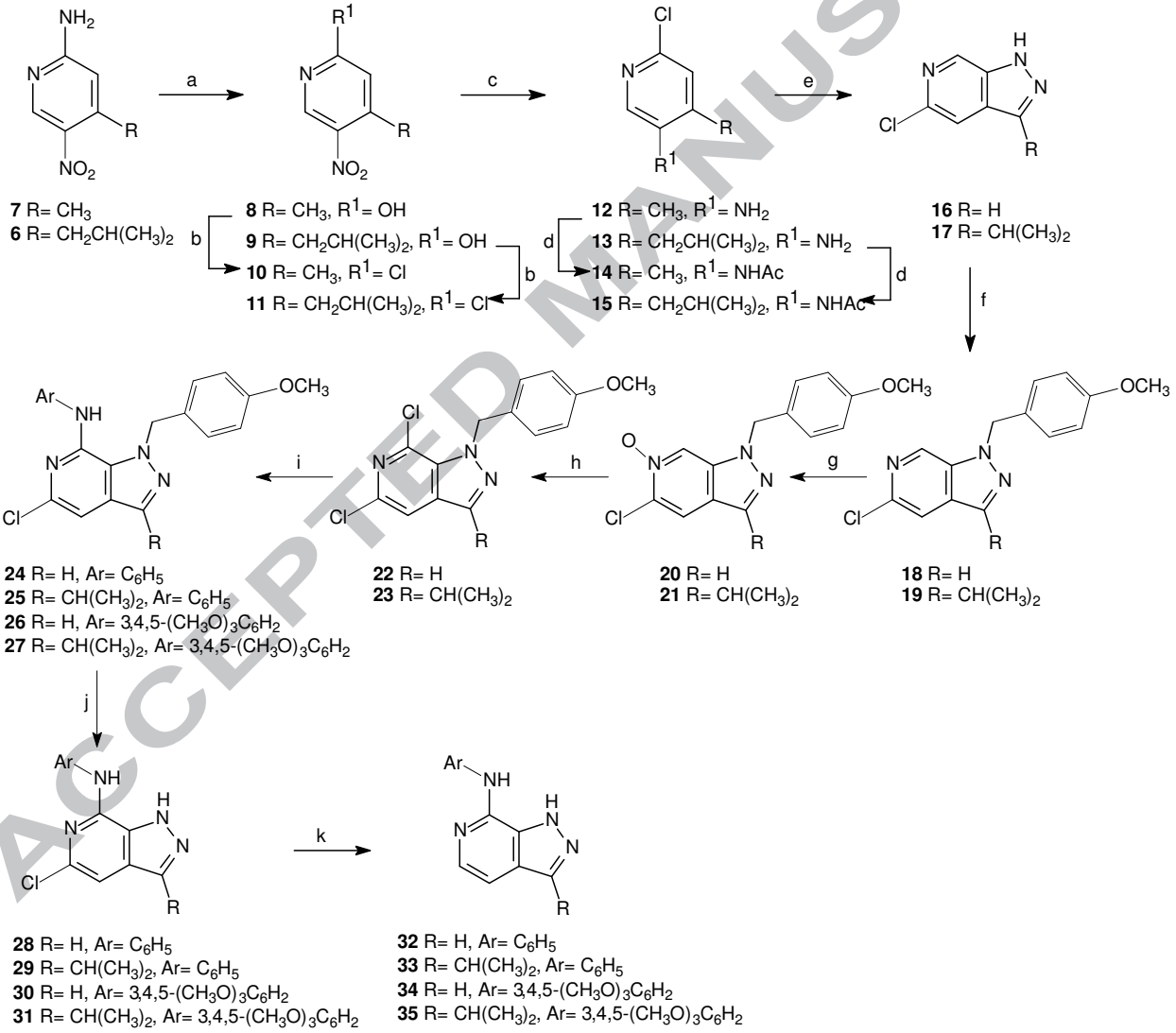
The new compounds were prepared using as starting materials the commercial aminopicoline **1**, as well as the substituted aminopyridine **6** (Scheme 1). The later compound has not been previously reported and was synthesized in four steps, according to the reactions depicted in Scheme 1. Thus, the picoline **1** was first protected through conversion to the corresponding carbamate **2**, which was lithiated using 2.5 equiv. of *n*-butyllithium, in THF at -80 ^{o}C and the resulting 4-methylene anion attacked isopropylbromide to provide the pyridine **3**. Compound **3** was then deprotected in acidic media and the resulting amine **4** was nitrated to give both nitroderivatives **5** and **6**,which were chromatographically separated and identified.



Scheme 1. Reagents and conditions. a) Boc_2O, *t*-BuOH, r.t.; b) i) *n*-BuLi (2.5 eq.), THF dry, -80 ˚C, then r.t., ii) isopropylbromide, -80 ˚C, then r.t.; c) HCl (5N), EtOH, r.t.; d) HNO_3, $H2SO_4$, 65 ˚C.

The synthesis of the target 7-substituted pyrazolo[3,4-*c*]pyridines as well as the corresponding 3-isopropyl analogues is depicted in Scheme 2. We used either 2-amino-5-nitro-4-picoline (**7**), which was prepared from the picoline **1** following a previously reported methodology[23], or the homologous picoline **6**. These aminopyridines were subjected to diazotization and the resulting

pyridinones were treated with phosphorous oxychloride to result in the chlorides **10**[24] and **11**. The 5-nitro group of the afore mentioned chlorides was reduced, using tin (II) chloride in HCl and the amines thus prepared, **12** and **13**, were acetylated to provide the acetamides **14** and **15**. Each acetamide was then treated with isoamyl nitrite in benzene at reflux in the presence of acetic anhydride and potassium acetate,[25-26] to result upon rearrangement of the intermediate *N*-nitroso compound in 1-acetyl-5-chloropyrazolo[3,4-*c*]pyridine or 1-acetyl-5-chloro-3-isopropylpyrazolo[3,4-*c*]pyridine. The acetyl group was cleaved upon treatment with methanolic ammonia to result in derivatives **16**[26] and **17**. The pyrazole NH of these derivatives was protected using the 4-methoxybenzyl group, upon reaction with 4-methoxybenzylchloride in the presence of NaH. In the case of **17**, we have obtained selectively the *N*1-substituted isomer, due to the presence of the bulky 3-isopropyl group. On the contrary, concerning compound **16**, we isolated both *N*1 and *N*2 regio-isomers which were separated and identified using NOE spectroscopic data.[27]

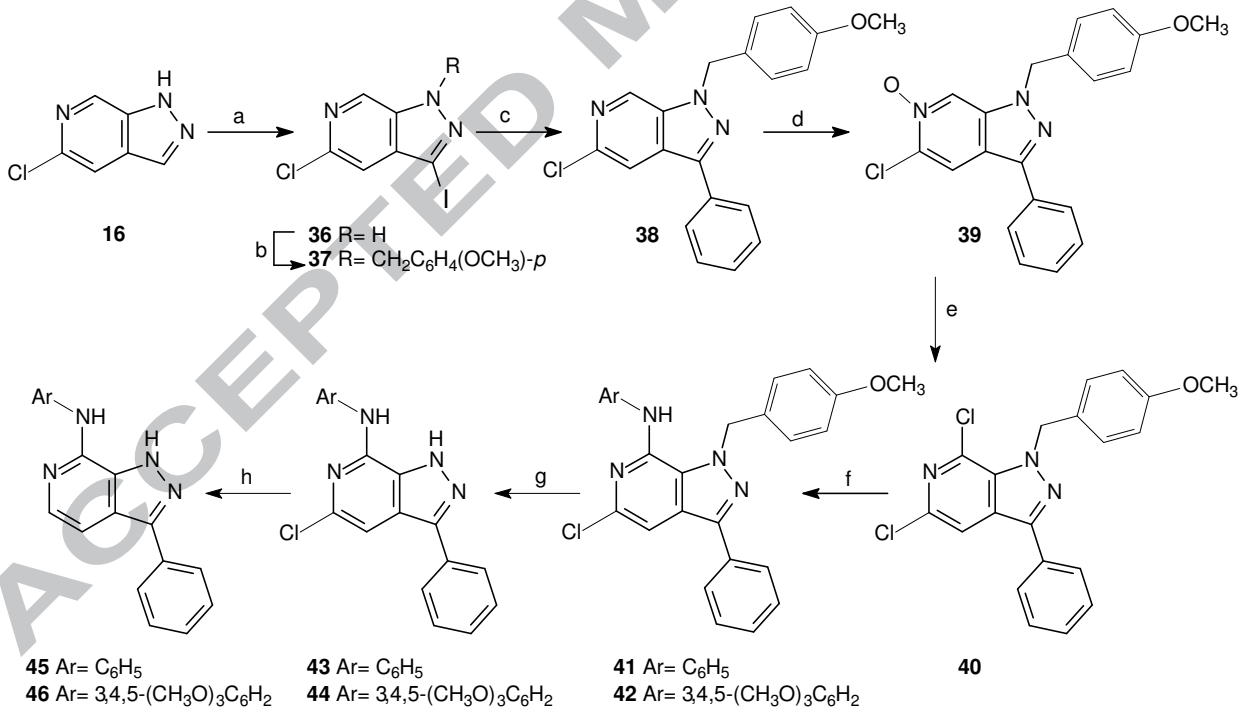


Scheme 2. Reagents and conditions. a) $NaNO_2$, H_2SO_4, H_2O; b) $POCl_3$, 100 °C; c) $SnCl_2{\cdot}2H_2O$, HCl(c.), 55 °C for **12** or r.t. for **13**; d) Ac_2O, CH_2Cl_2, r.t.; e) i) AcOK, Ac_2O, isoamyl nitrite,

benzene, reflux, ii) $NH_{3(g.)}$, MeOH, r.t.; f) i) NaH, DMF, r.t., ii) 4-methoxybenzyl chloride, DMF, r.t.; g) *m*-CPBA, $CHCl_3$, r.t.; h) $POCl_3$, THF, r.t.; i) NaH, aniline (for **24**,**25**) or 3,4,5-trimethoxyaniline (for **26**,**27**), DMF, 100 °C; j) trifluoroacetic acid, r.t.; k) Pd/C, H_2, AcOK, EtOH, 50 psi, r.t.

The protected derivatives **18** and **19** were converted to the corresponding *N*-oxides **20**[27] and **21**, using *m*-CPBA as oxidizing agent. The rearrangement of the *N*-oxides in the presence of phosphorous oxychloride, resulted in the 5,7-dichloropyrazolopyridines **22**[27] and **23**, which were used for the nucleophilic substitution of the 7-chloro group using aniline or 3,4,5-trimethoxyaniline, in order to provide compounds **24**-**27**. These derivatives were then subsequently deprotected to give the analogues **28**-**31**, which upon catalytic dehalogenation resulted in the target compounds **32**-**35**.

The preparation of the corresponding 3-phenyl derivatives is reported in Scheme 3. They resulted from the intermediate 5-chloropyrazolo[3,4-*c*]pyridine (**16**), which was easily iodinated upon treatment with NIS and the resulting 3-iodide **36** was selectively protected at *N*1, using 4-methoxybenzylchloride, providing **37**.[28] The iodide **37** was introduced to Suzuki coupling using phenylboronic acid in the presence of $Pd(PPh_3)_4$ and was converted to the 3-phenyl derivative **38**.[27] Compound **38** was introduced to a reaction sequence analogous to the one mentioned in Scheme 2 and provided in five steps the 3-phenyl analogues **45** and **46**.

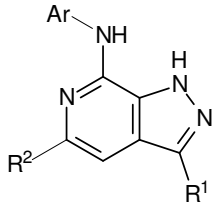


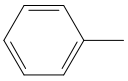
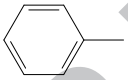
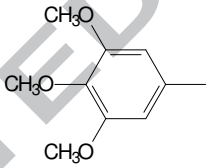
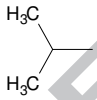
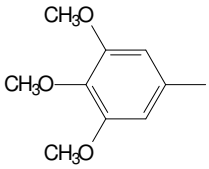
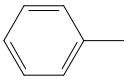
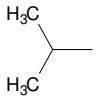
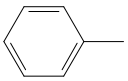
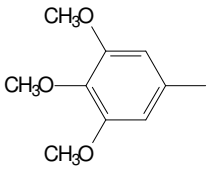
Scheme 3. Reagents and conditions. a) NIS, MeOH, r.t.; b) i) NaH, DMF, r.t., ii) 4-methoxybenzyl chloride, DMF, r.t.; c) phenylboronic acid, $Pd(PPh_3)_4$, $NaHCO_3$, toluene/ethanol/H_2O (10/1/0.2), reflux; d) *m*-CPBA, $CHCl_3$, r.t.; e) $POCl_3$, THF, r.t.; f) i) NaH,

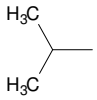
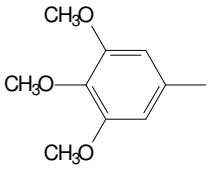
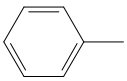
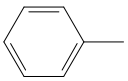
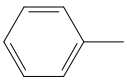
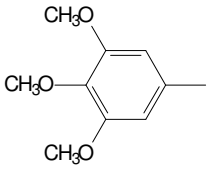
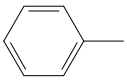
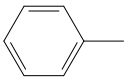
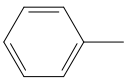
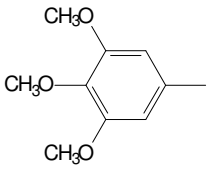
aniline (for **41**) or 3,4,5-trimethoxyaniline (for **42**), DMF, 100 °C; g) trifluoroacetic acid, r.t.; h) Pd/C, H_2, AcOK, EtOH, 50 psi, r.t.

The cytotoxic activity of the new derivatives was tested against two cancer cell lines, pancreatic (MIA PaCa-2) and ovarian (SCOV3), as well as against normal human fibroblasts (WI-38). The IC_{50} values of the derivatives are presented in Table 1.

Table 1. IC_{50} values of the derivatives. MTS assay for cell viability. Experiment in triplicate.



compd.	R^1	R^2	Ar	IC_{50} (μM) MIA PaCa-2	SCOV3	WI-38
28	H	Cl		36.2 ± 2.2	34.2 ± 2.9	>50
29		Cl		21.5 ± 2.4	>50	38.7 ± 1.0
30	H	Cl		16.2 ± 0.7	13.1 ± 0.7	>50
31		Cl		6.1 ± 1	5.6 ± 0.4	43.8 ± 15.0
32	H	H		>50	>50	>50
33		H		41.2 ± 3.5	48.3 ± 4.1	>50
34	H	H		>50	>50	>50

35		H		10.0 ± 0.6	22.9 ± 1.7	>50
43		Cl		4.3 ± 0.5	2.5 ± 0.3	23.4 ± 0.7
44		Cl		6.7 ± 0.6	5.9 ± 0.5	21.9 ± 12.0
45		H		9.0 ± 0.9	22.1 ± 1.4	24.7 ± 4.3
46		H		3.8 ± 0.3	0.73 ± 0.04	14.9 ± 0.7

A number of interesting structure-activity relationships can be extracted from the study of these series of derivatives. Compounds bearing only one substituent at position 7 (**32** and **34**) are inactive against both cancer cell lines. On the contrary, their counterparts, possessing a 5-chloro group (**28** and **30**) showed marginally interesting cytotoxicity with IC_{50} values ranging between 13 and 36 μM. This pattern is replicated in the case of the 3-isopropylpyrazolopyridine analogues, however, in this case, the compounds substituted at position 7 with the (3,4,5-trimethoxyphenyl)amino group (compounds **31** and **35**) possess quite interesting cytotoxic activity, mainly the 5-chloro analogue **31**, the IC_{50} value of which lies in the low micromolar level. Overall, the most important finding concerns the activity of the 3-phenylpyrazolopyridines **43**-**46,** which showed uniformly strong to good cytotoxicity. The most noticeable activity was displayed by compound **46**, which bears the 7-(3,4,5-trimethoxyphenyl)amino substituent. The high antiproliferative activity of compound **46** was also confirmed, when it was tested against DU145 prostate cancer cell line, where it was found to possess IC_{50} value 0.92 μM, against A2058 melanoma cell line, where it exhibited IC_{50} value 0.89 μM, as well as against PC-3 cells showing IC_{50} value 0.38 μM. It is of interest to notice that the precursor chloroderivative **44**, was found to be substantially less active when tested against PC-3 cell-line possessing an IC_{50} value 15 μM. Interestingly, all tested compounds (with the exception of **29** which was inactive against SCOV3) were selectively more active against cancer cells compared to normal human ones (WI-38). This is particularly evident regarding the most active compounds **46**, **43**, **31** and **44**.

Cell-cycle perturbations induced after incubation of exponentially growing PC-3 cells with compounds **44** and **46** for 48 h are given in Table 2 and in Figure 1. Both compounds inhibited DNA synthesis of PC-3 cells by blocking cells at the G_0/G_1 phase. Compound **46** was much more potent, since it led to the complete annulment of S phase, and, furthermore, it induced apoptosis, as determined by the appearance of a sub-diploid peak in the DNA-content histogram.

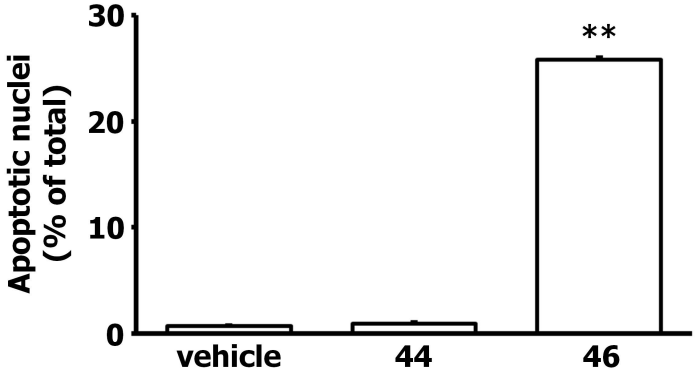
In particular, compound **46** induced the appearance of 25.8% (± 0.3) apoptotic nuclei compared to 0.7% (± 0.1) induced by the vehicle or 0.9% (± 0.2) by the chloroderivative **44** (Figure 1).

Table 2. Cell cycle phase distribution at 48 hours (%). [a] Exponentially growing PC-3 cells were treated with the compounds **44** and **46**, at concentration equal to their corresponding IC_{50} values, or the corresponding DMSO concentration (vehicle).

Compound	G_0/G_1	S	G_2/M
44	46.2 (±3.0)	30.3 (±0.4)	23.5 (±3.4)
46	80.8 (±4.4)	0.0 (±0.0)	19.2 (±4.4)
vehicle	38.3 (±0.7)	37.7 (±2.2)	24.0 (±2.9)

[a] Mean (± standard deviation) of two independent experiments

Figure 1. Induction of apoptosis by the test compounds. Percentage of apoptotic nuclei in PC-3 cells treated for 48 hours with the indicated compounds at concentration equal to their corresponding IC_{50} values, or with vehicle. Bars represent the mean of two independent experiments (error bars: SD); ** indicates $p<0.01$, * indicates $p<0.05$ (Student's *t*-test).



In conclusion, we have synthesized a number of new 7-arylaminosubstituted pyrazolo[3,4-*c*]pyridines and have determined their antiproliferative activity against pancreatic (MIA PaCa-2) and ovarian (SCOV3) cancer cell lines. Following the structure-activity relationships extracted by the target derivatives, we have further investigated the behavior of the most promising derivative **46**, against three additional cancer cell lines, where it exhibited potent antiproliferative

activity as well. This compound blocked the cell cycle at the G_0/G_1 phase and induced apoptosis when studied against prostate PC-3 cancer cells.

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Supplementary data concerning experimental procedures for the synthesis and biological evaluation can be found as supplementary file.

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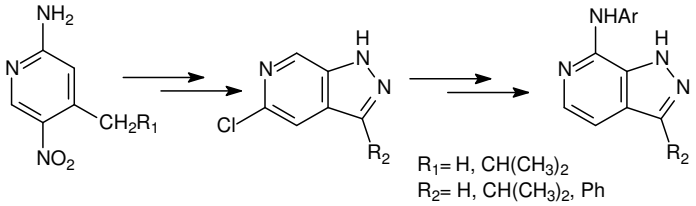
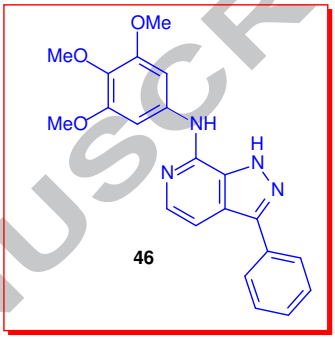
Graphical abstract





IC_{50} (μM): MIA PaCa-2: 3.8; SCOV3: 0.73; DU145: 0.92; A2058: 0.89; PC-3: 0.38