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ORIGINAL RESEARCH

Synthesis, cytotoxic, and DNA binding studies of novel fluorinated condensed pyrano pyrazoles

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Abstract One pot solvent-free synthetic method is developed for the synthesis of novel pyrano[2,3-c]pyrazole analogs. Cytotoxicity experiments conducted against a pair of cancerous, non-cancerous lung cell lines, and a cervical cell line is described. These compounds are selectively toxic against cancer cells and not normal cells. Molecular mechanism is established for the mode of DNA binding of these compounds using electrochemical and proton NMR methods.

Keywords DBU · Knoevenagel condensation · Pyrano[2,3-c]pyrazole · HeLa cells · Electrochemical · Intercalation

Introduction

Potential pharmacologically active compounds have been developed by exploring a wide variety of pyrazoles fused with different heterocycles that are known to contribute to various chemotherapeutic effects like antileukemic (Chou *et al*., 2007), antitumor (Li *et al*., 2006), antimicrobial (Holla *et al*., 2006), and antiviral (Yan *et al*., 2006) agents. Investigations in the chemistry and biology of 2-pyrones have been highly intensified with the recognition that they constitute an essential pharmacophore in many naturally occurring and biologically active agents (Dickinson, 1993). Some pyrones are reported to possess cytotoxic effect against a few human cancer cell lines (Marrison *et al*., 2002). The substituted pyrans can bind to DNA through an intercalative mode wherein the planar aromatic heterocyclic group inserts and stacks between the base pairs of DNA (Barton, 1986). In addition, fluorine is intentionally incorporated into inhibitors because of its lipophilicity and also its comparable size with hydrogen atom. Fluorine-containing molecules display better drug delivery properties.

The use of conventional organic solvents in the synthesis of 2-amino-3-cyano-4*H*-pyrans make the work up procedure complicated and lead to poor yields of products (Singh *et al*., 1996; Wang *et al*., 2003; Devi and Bhuyan, 2004). Lately, in addition to the usual synthetic methods, 2-amino-3-cyano-4*H*-pyrans have also been synthesized under microwave (Lin *et al*., 2002; Tu *et al*., 2002) with ultrasound irradiation (Li *et al*., 2004) in aqueous media (Tong-Shou *et al*., 2004; Jin *et al*., 2005) two-component (Kaupp *et al*., 2003) and three-component (Tong-Shou *et al*., 2004) condensations, etc. Each of these methods have some merits with at least one of the limitations of low yields, long reaction time, effluent pollutions, harsh reaction conditions, or tedious workup procedure.

These findings have motivated us to explore a novel method to synthesize fluorinated condensed pyrano[2,3-c]pyrazole derivatives. An efficient one pot synthesis using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as a base under solvent-free condition was developed to synthesize fluorinated condensed pyrano[2,3-c]pyrazole derivatives. These compounds were further tested for their cytotoxicity; few of them displayed cytotoxicity toward cancer cell lines.

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DNA binding studies (electrochemical) were carried out with the compound which showed maximum cytotoxicity. By finding interesting behavior of compound with DNA, we have further studied liquid-state proton NMR to delineate the mechanism of interaction of pyrazole derivatives and DNA nucleotides.

Results and discussion

Synthesis of pyrano[2,3-c]pyrazole derivatives

Substituted condensed pyrano[2,3-c]pyrazole derivatives (Table 1) were prepared by mechanical grinding of the three components—substituted aromatic aldehyde, malononitrile, and 1-phenyl-3-[trifluoromethyl]-1*H*-pyrazol-5-ol in one pot by using DBU base at room temperature. The reactions lead to completion in 5 min with good yields and easy work up. The synthetic protocol is shown as following in Scheme 1.

R: C_6H_5, 4-FC_6H_4, 3-ClC_6H_4.
R_1: C_6H_5, 4-$NO_2C_6H_4$, 3-$OCH_3C_6H_4$, 2-furyl, 2-thionyl, 4-$CH_3C_6H_4$, 4-$OCH_3C_6H_4$, 4-FC_6H_4.

According to the proposed mechanism, the first step of this reaction is the formation of Knoevenagel product by the condensation of an aldehyde with malononitrile (Goujon *et al*., 2002; Costa *et al*., 2008). Generally, base catalyzed three-component reaction is accompanied by the formation of many side products such as enaminonitrile, higher adducts, reduced products, and malononitrile which reduce the overall yield of the final product (Khurana *et al*., 2010). The better yield observed in Scheme 1 can be explained by the generation of stable DBU–H^+ species in the reaction, which suppresses the formation of these side products. The α-cyanocinnamonitrile formed initially by Knoevenagel condensation undergoes subsequent reaction with 5-hydroxy pyrazoles in the presence of DBU to give the desired products.

Table 1 Inhibitory efficiency of synthesized fluorinated condensed pyrano pyrazole derivatives against lung cancer (A549) and non-lung cancer cells (MRC-5) and cervical cancer (HeLa) cells

Compound	R	R_1	A549 (μM)	MRC-5 (μM)	HeLa (μM)
4a	C_6H_5	4-$NO_2C_6H_4$	>100	>100	>100
4b	C_6H_5	3-$OCH_3C_6H_4$	>100	>100	>100
4c	C_6H_5	2-furyl	>100	>100	>100
4d	C_6H_5	2-thionyl	75.5 (±1.90)	>100	74.07 (±1.40)
4e	4-FC_6H_4	4-$CH_3C_6H_4$	>100	>100	>100
4f	4-FC_6H_4	2-thionyl	>100	>100	>100
4g	4-FC_6H_4	4-$NO_2C_6H_4$	>100	>100	>100
4h	4-FC_6H_4	4-$OCH_3C_6H_4$	10.3 (±0.89)	>100	>100
4i	3-ClC_6H_4	4-$OCH_3C_6H_4$	89.4 (±1.05)	>100	>100
4j	3-ClC_6H_4	4-$NO_2C_6H_4$	>100	>100	>100
4k	3-ClC_6H_4	2-furyl	>100	>100	>100

Cytotoxicity

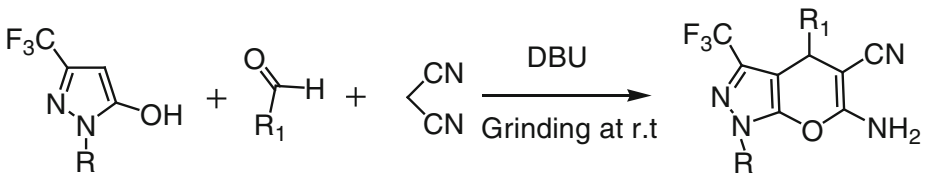
Inhibitory efficiency was tested for some of these fluorinated condensed pyrano pyrazoles against lung cancer (A549), non-lung cancer cells (MRC-5) and cervical cancer (HeLa) cells (Table 1). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed following the previously reported protocol in the 96-well plate (Myadaraboina *et al*., 2010). The IC_{50} values for the compounds **4a–4k** are summarized in the Table 1. Doxorubcin was used as the control. In general, A549 cells were sensitive against the compounds tested in the current study than HeLa cells. Compound **4h** was the best against A549 cells followed by **4d** and **4i**. Incidentally, compound **4d** also displayed equal activity against HeLa cells. Surprisingly, none of the compounds have displayed toxicity against the normal lung cells. Pyrano pyrazoles are known to bind to DNA (Barton, 1986). To understand the mechanism of inhibition displayed by compound **4h,** we have carried out DNA binding cyclic voltametry studies.

Cyclic voltammetry (CV) of compound **4h** and DNA interaction

The cyclic voltammogram of 1 mM DNA and 1 mM **4h** in sodium acetate buffer (**pH 4.6)** at graphite electrode at scan rate of 10 mV/s is shown in (Fig. 1). The CV of plain DNA in sodium acetate buffer is shown as inset in (Fig. 1a)—as seen from the figure, graphite electrode provides a stable background. The CV of **4h** alone in acetate buffer did not show any peaks at graphite electrode.

The CV of DNA mixed with **4h** shows two prominent peaks (Fig. 1b) at potential 0.45 V and 1.3 V versus Ag/AgCl. The peaks are due to the oxidation of guanine and adenine residues, respectively (Wang *et al*., 2001). Upon the interaction of **4h** with DNA, double stranded DNA separates partly into two single stranded DNA by the rupture of the hydrogen bonds. It means that adenine and guanine bases are exposed to electrode surface when the **4h** interacts with DNA so the oxidative reaction occurs easily. Successive CVs of the DNA and **4h** show gradual decrease in the two peak currents due to the adsorption of the DNA which forms a DNA multilayer on the graphite electrode. To understand the mechanism of DNA and **4h** interaction,

Scheme 1 Synthesis of pyrano[2,3-c]pyrazole derivatives



1 a-c **2 a-n** **3** **4 a-n**

R: C_6H_5, 4-FC_6H_4, 3-ClC_6H_4

R_1: C_6H_5, 4-$NO_2C_6H_4$, 3-$OCH_3C_6H_4$, 2-furyl, 2-thionyl, 4-$CH_3C_6H_4$, 4-$OCH_3C_6H_4$, 4-FC_6H_4.

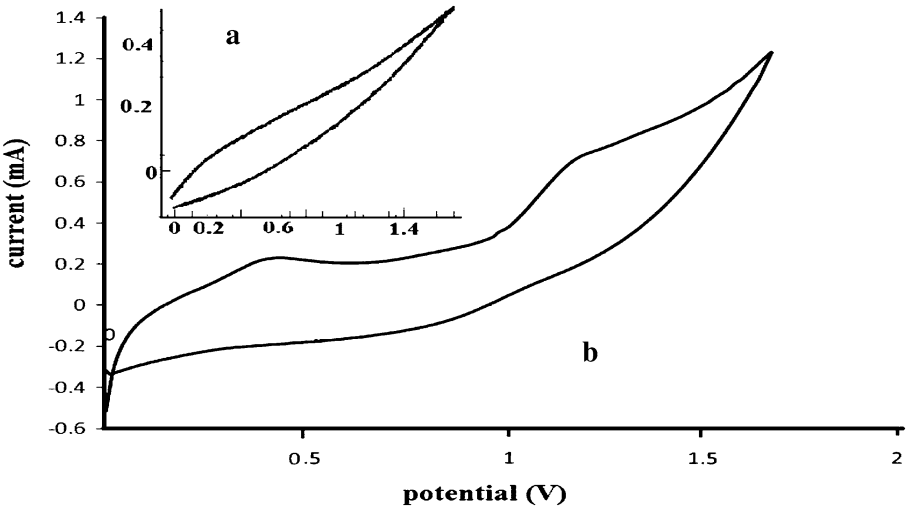


Fig. 1 Cyclic voltammogram of DNA mixed with compound "**4h**" in sodium acetate buffer at scan rate of 10 mV/s. *a* DNA alone in the buffer solution, *b* DNA mixed with 1 mM compound "**4h**" in the buffer solution

CV studies were further extended by adding cytosine to the mixture of DNA and **4h**.

Interaction of cytosine and compound **4h** with DNA

Cyclic voltammogram of 1 mM DNA and 1 mM **4h** in the presence of 1 mM cytosine in sodium acetate buffer at a scan rate of 10 mV/s is shown in (Fig. 2). The CV indicated only one peak at 0.45 V; the peak at 1.3 V was not observed immediately. However, it appeared after about 30 min. When the concentration of cytosine was increased to 3 mM, the peak at 0.45 V was persistent, but the peak at 1.3 V did not reappear even after 3 h.

^{1}H NMR spectra

To examine the compound **4h**'s interaction with cytosine, NMR studies of **4h**, cytosine, and cytosine mixed with **4h** were carried out (Fig. 3). The NMR spectra of **4h** mixed with cytosine shows the peak broadening (0.15 ppm) and field shift of the N–H protons of the **4h**. A similar NMR peak broadening was observed in DNA and indole derivative complexes (Sartorius and Schneider, 1995), which

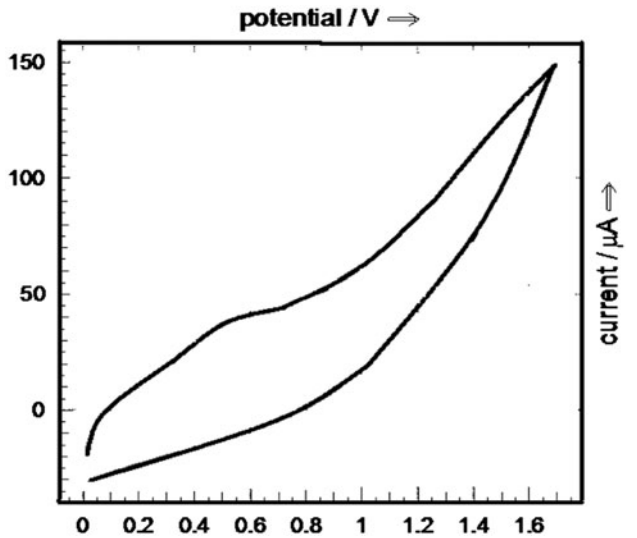


Fig. 2 Cyclic voltammogram of DNA mixed with 1 mM compound "**4h**" and 1 mM cytosine in sodium acetate buffer at scan rate of 10 mV/s

was suggested to be because of intercalation between DNA and indole molecule. Apart from DNA intercalation, we propose possible hydrogen bonding between **4h**-cytosine

and **4h**-adenine as shown in (Fig. 4) cytosine at equal concentrations. Note that broadening and also shift in peak position is observed in solution that contains the mixture of **4h** and cytosine suggesting their interaction.

Mechanism of interaction of compound **4h** with G–C and A–T base pairs

Drug bind to DNA both covalently as well as non-covalently (Chaires, 1998; Hurley, 2002; Neidle, 2001; Wemmer and Dervan, 1997). Covalent binding in DNA is irreversible and invariably leads to complete inhibition of DNA processes. Non-covalently bound drugs mostly fall under the following two class (a) Minor groove binders and (b) intercalators. Minor groove binding drugs are usually crescent shaped, which complement the shape of the groove and facilitates binding by promoting Van der Waals interactions. In addition, these drugs can form hydrogen bonds to bases, typically to N_3 of adenine and O_2 of thymine. Intercalators contain planar heterocyclic groups which stack between adjacent DNA base pairs.

It is clear that compound **4h** is able to induce unwinding of the dsDNA at both G–C and A–T centers. In the presence of equal concentrations of **4h** and cytosine, there could be three situations: interaction of **4h** with G–C and A–T pairs in DNA; interaction of **4h** with free cytosine; interaction of free cytosine with G–C and A–T in DNA. From the CV experimental observation oxidation of only G–C pair occurs readily when compared with A–T pair. This may be due to the preferential interaction of cytosine with only G–C pair and **4h** than with A–T pair. The slow interaction of cytosine with A–T pair may be due to narrow A/T regions than G/C groove regions and also because of the steric hindrance in the latter presented by the C_2 amino group of the guanine base. However, given enough time (about 30 min), a part of the **4h** becomes free from cytosine and promotes the A–T pair unwinding. In the presence of excess of cytosine, no free **4h** is available even in the extended time resulting in complete disappearance of peak

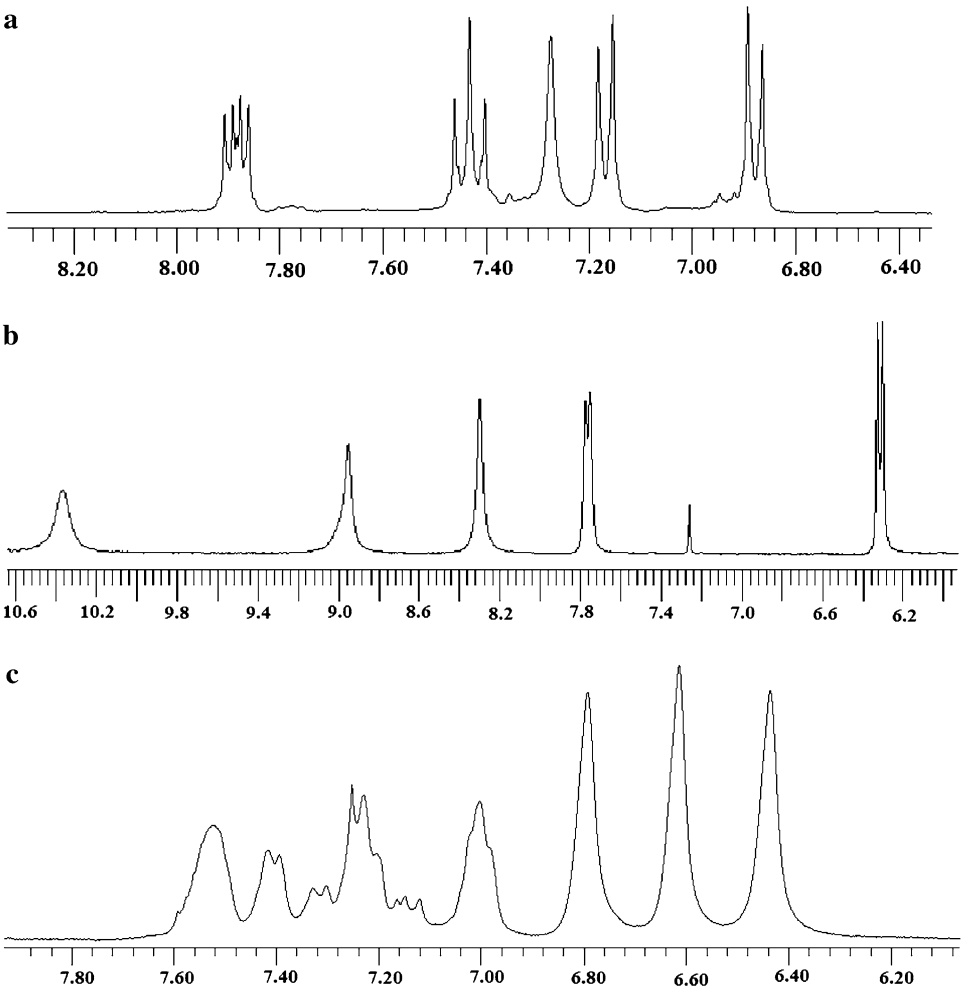


Fig. 3 Interaction of compound "**4h**" with cytosine in solution. **a** Proton NMR spectra of the compound "**4h**," **b** Proton NMR spectra of cytosine, **c** Proton NMR spectra of compound "**4h**" mixed with cytosine

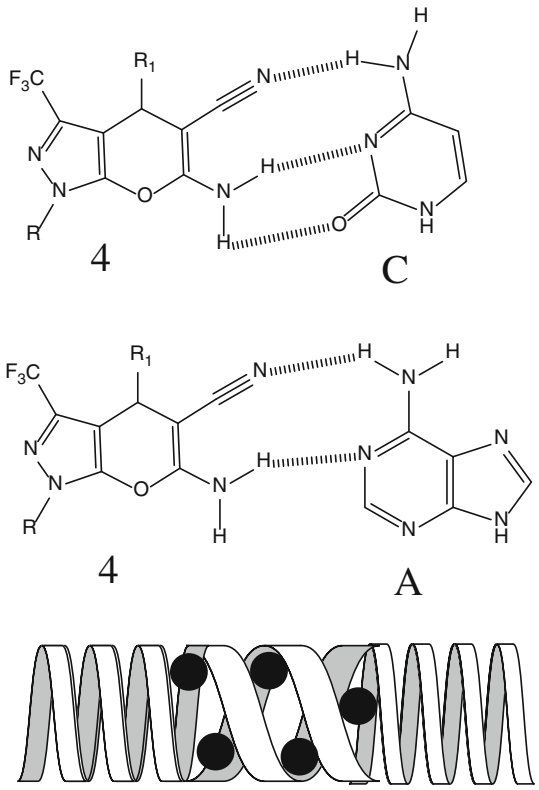


Fig. 4 Model of interaction of compound "**4h**" with DNA. The *top two panels* indicate possible hydrogen bonding between the basic skeleton of molecules and DNA bases. Unwinding of double stranded DNA in the presence of compound "**4h**" depicted as *spheres*

related to A–T pair. A model of interaction of **4h** with DNA and the unwinding of double stranded DNA in the presence of compound **4h** that leads to base pair oxidation during CV experiments is shown in (Fig. 4). Cyclic voltammetric results specify the interaction of the drug and DNA is non-covalent, and it is an intercalator. Intercalators contain planar heterocyclic groups which stack between adjacent DNA base pairs and it is thought to be stabilized by π–π stacking interactions between the drug and DNA bases. Non-covalent binding is reversible and is typically preferred over the covalent adduct formation keeping the drug metabolism and toxic side effects in mind.

Conclusion

In summary, we have reported an efficient method to synthesize novel pyrano[2,3-c]pyrazole molecules in one pot using DBU as a base under solvent free conditions. We also reported cytotoxic and DNA binding studies of novel fluorinated condensed pyrano pyrazoles cytotoxicity experiments were conducted against a pair of cancerous, non-cancerous lung cell lines, and a cervical cell line. Our compounds were selectively toxic against cancer cells and not normal cells. Molecular mechanism was established for the mode of DNA binding of these compounds by electrochemical and proton NMR methods. Based on these encouraging results, we are in the process of redesigning the pyrano[2,3-c]pyrazole moiety for a better efficacy.

Experimental

All the substituted pyrazoles were prepared by the known method (Liu *et al*., 2006) which involves reaction of the corresponding ethyltrifluoroaceto acetate with phenyl hydrazine in the presence of aqHCl catalyst using EtOH as the solvent. The chemicals used were of analytical grade and the solutions were prepared using double distilled water. 4-F-phenyhydrazine hydrochloride, DBU, and ethyl trifluoroacetoacetate (ETFAA) were purchased from M/s Sigma Aldrich (St. Louise, USA) and malanonitrile, para-methoxy benzaldehyde, and cytosine from M/s Lancaster (India). Miniprep kit was obtained from M/s Qaigen (USA). Melting points of all the pyrano[2,3-c]pyrazoles prepared were recorded on Casiae–Siamia (VMP-AM) melting point apparatus and are uncorrected. IR spectra were recorded on a Perkine-Elmer FT-IR 240-C spectrophotometer using KBr optics. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AV 300 MHz; ^{19}F NMR spectra were recorded on Inova 400 MHz spectrometer in DMSO using TMS as internal standard. Electron Spray Ionization (ESI) and high-resolution spectra was recorded on QSTARXL hybrid MS/MS system (Applied Biosystems, USA) under electrosprayionization. All the reactions were monitored by thin layer chromatography (TLC) on precoated silica gel 60 F254 (mesh); spots were visualized using UV light. Merck silica gel (60–120 mesh) was used for column chromatography.

General procedure for the preparation of pyrano[2,3-c]pyrazole derivatives (**4a–n**)

A mixture of aromatic aldehyde (2) (2 mmol), malononitrile (3) (132 mg, 2 mmol), 1-phenyl-3-(trifluoromethyl)-1*H*-pyrazol-5-ol (1) (2 mmol), and DBU (0.072 mg, 0.47 mmol) were taken in a mortar and ground thoroughly using pestle at room temperature (25 °C). The reaction was monitored by TLC. The product was isolated by passing through a column packed with silica gel (60–120 mesh) using n-hexane:ethylacetate (4:1) as eluents.

*6-Amino-4-(4-nitrophenyl)-1-phenyl-3-(trifluoromethyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carobonitrile (**4a**)*

Yield 0.76 g (87 %); m. p. 242–244 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.91 (s, 1H, CH), 7.28 (s, 2H, NH_2),

7.38–7.58 (m, 5H, Ar), 7.89 (d, $J = 7.7$ Hz, 2H), 8.19 (d, $J = 8.5$ Hz, 2H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 36.1, 57.1, 97.6, 119.0, 121.9, 123.6, 128.3, 129.2, 129.5, 136.5, 145.2, 146.7, 150.8, 158.8. ^{19}F NMR (DMSO-d_6, 376 MHz): δ −61.01 (s, 3F, CF_3). I.R (KBr, cm^{-1}): 3,424, 3,328 (NH_2), 2,203 (CN), 1667 (C=N), 1,519, 1,351 (NO_2), 1,143 (C–F). HRMS m/z calcd for $C_{20}H_{13}F_3N_5O_3$ ($M + H^+$): 428.097; found: 428.096.

*6-Amino-4-(3-methoxyphenyl)-1-phenyl-3-(trifluoromethyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carobonitrile (**4b**)*

Yield 0.67 g (81 %); m. p. 137–139 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.78 (s, 3H, OCH_3), 4.68 (s, 1H, CH), 6.69–6.79 (m, 3H, Ar), 6.91 (s, 2H, NH_2), 7.17–7.26 (m, 2H, Ar), 7.34–7.43 (m, 1H, Ar), 7.47–7.57 (m, 1H, Ar), 7.88 (d, $J = 7.7$, 2H, Ar). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 36.5, 54.9, 58.3, 98.7, 112.0, 113.7, 119.3, 119.8, 121.8, 123.0, 128.2, 129.2, 129.4, 133.3, 136.3, 144.9, 145.0, 158.6, 159.1. ^{19}F NMR (DMSO-d_6, 376 MHz): δ −59.97 to −60.07 (m, 3F, CF_3). I.R (KBr, cm^{-1}): 3,414, 3,327 (NH_2), 2,200 (CN), 1,661 (C=N), 1,146 (C–F). HRMS m/z calcd for $C_{21}H_{16}F_3N_4O_2$($M + H^+$): 413.11 found: 413.10.

*6-Amino-4-(furan-2-yl)-1-phenyl-3-(trifluoromethyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carobonitrile (**4c**)*

Yield 0.60 g (83 %); m. p. 180–182 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.89 (s, 1H, CH), 6.2 (d, $J = 3.2$ Hz, 1H, furan), 6.29–6.32 (m, 1H, furan), 6.87 (s, 2H, NH_2), 7.32–7.40 (m, 2H, furan & Ar), 7.45–7.53 (m, 2H, Ar); 7.84 (d, $J = 7.9$ Hz, 2H, Ar). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 30.5, 56.0, 95.6, 96.3, 106.0, 110.0, 118.7, 121.0, 127.4, 128.4, 128.9, 136.4, 141.7, 144.6, 153.5, 159.4. ^{19}F NMR (DMSO-d_6, 376 MHz): δ −61.26 (s, 3F, CF_3). I.R (KBr, cm^{-1}): 3,443, 3,323 (NH_2), 2,202 (CN), 1,661 (C=N), 1,145 (C–F). HRMS m/z calcd for $C_{18}H_{12}F_3N_4O_2$ ($[M + H]^+$): 373.091 found: 373.090.

*6-Amino-1-phenyl-4-(thiophen-2-yl)-3-(trifluoromethyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carobonitrile (**4d**)*

Yield 0.64 g (84 %); m. p. 182–184 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 5.09 (s, 1H, CH), 6.90–6.94 (m, 1H), 6.98 (d, $J = 3.2$ Hz, 1H), 7.02–714 (br, 2H, NH_2), 7.21–7.27 (m, 1H), 7.35–7.43 (m, 1H), 7.47–7.57 (t, $J = 7.4$, 2H), 7.87 (d, $J = 8.1$ Hz, 2H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 32.0, 58.7, 95.5, 98.8, 118.8, 120.9, 124.4, 124.7, 126.1, 127.5, 128.6, 129.0, 136.3, 144.1, 147.6, 158.5. ^{19}F NMR (DMSO-d_6, 376 MHz): δ −60.96 (s, 3F, CF_3). I.R (KBr, cm^{-1}): 3,462, 3,316 (NH_2), 2,198 (CN), 1,660 (C=N), 1,143 (C–F). HRMS m/z calcd for $C_{18}H_{12}F_3N_4OS$ ($[M + H]^+$): 389.068 found: 389.067.

*6-Amino-1-(4-fluorophenyl)-4-p-tolyl-3-(trifluoromethyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carobonitrile (**4e**)*

Yield 0.69 g (84 %); m. p. 177–179 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 2.33 (s, 3H, CH_3), 4.66 (s, 1H, CH), 6.90–6.97 (br, 2H, NH_2), 7.03–7.13 (m, 4H), 7.25 (d, $J = 8.9$ Hz, 2H), 7.88–7.94 (m, 2H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 20.5, 36.3, 59.0, 95.5, 98.8, 115.5, 115.8, 122.7, 122.8, 122.8, 126.9, 128.6, 135.9, 139.7, 144.4, 158.2. ^{19}F NMR (DMSO-d_6, 376 MHz): δ −60.8 (s, 3F, CF_3); −113.01 to −112.92 (m, 1F, Ar–F). I.R (KBr, cm^{-1}): 3,469, 3,320 (NH_2), 2,203 (CN), 1,660 (C=N), 1,141 (C–F). HRMS m/z calcd for $C_{21}H_{15}F_4N_4O$ ($[M + H]^+$): 415.118 found: 415.117.

*6-Amino-1-(4-fluorophenyl)-4-(thiophen-2-yl)-3-(trifluoromethyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carobonitrile (**4f**)*

Yield 0.66 g (82 %); m. p. 162–164 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 5.10 (s, 1H, CH), 6.90–7.02 (m, 2H), 7.23–7.36 (m, 5H, Ar & NH_2), 7.85–7.94 (m, 2H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 36.5, 57.4, 95.4, 115.5, 115.8, 118.2, 122.7, 122.8, 123.3, 128.1, 146.6, 149.5, 158.6, 162.6. ^{19}F NMR (DMSO-d_6, 376 MHz): δ −60.95 (s, 3F, CF_3); −112.85 to −112.95 (m, 1F, Ar–F). I.R (KBr, cm^{-1}): 3,451, 3,320 (NH_2), 2,200 (CN), 1,659 (C=N), 1,134 (C–F). HRMS m/z calcd for $C_{18}H_{11}F_4N_4OS$ ($[M + H]^+$): 407.048 found: 407.047.

*6-Amino-1-(4-fluorophenyl)-4-(4-nitrophenyl)-3-(trifluoromethyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carobonitrile (**4g**)*

Yield 0.75 g (85 %); m. p. 240–242 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.87 (s, 1H, CH), 7.04 (s, 2H, NH_2), 7.19–7.28 (m, 2H), 7.43 (d, $J = 8.7$ Hz, 2H), 7.87–7.96 (m, 2H), 8.18 (d, $J = 8.7$ Hz, 2H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 31.9, 58.6, 95.4, 98.8, 115.7, 116.0, 118.7, 123.1, 124.4, 124.8, 126.1, 132.5, 144.1, 147.5, 158.4, 162.6. ^{19}F NMR (DMSO-d_6, 376 MHz): δ −61.06 (s, 3F, CF_3); −112.79 to −112.89 (m, 1F, Ar–F). I.R (KBr, cm^{-1}): 3,423, 3,328 (NH_2), 2,203 (CN), 1,666 (C=N), 1,519, 1,349 (NO_2), 1,142 (C–F). HRMS m/z calcd for $C_{20}H_{12}F_4N_5O_3$ ($[M + H]^+$): 446.081 found: 446.080.

*6-Amino-1-(4-fluorophenyl)-4-(4-methoxyphenyl)-3-(trifluoromethyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carobonitrile (**4h**)*

Yield 0.72 g (85 %); m. p. 208–210 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.74 (s, 3H, OCH_3), 4.73 (s, 1H, CH), 6.88 (d, $J = 8.5$ Hz, 2H), 7.17 (d, $J = 8.5$ Hz, 2H), 7.27 (s, 2H, NH_2), 7.39–7.47 (m, 2H), 7.85–7.92 (m, 2H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 35.6, 54.7, 58.6, 98.8, 113.4, 115.8, 116.2, 119.1, 124.0, 124.1, 128.4, 132.4, 135.2, 144.6, 157.9, 158.1, 162.8. ^{19}F NMR (DMSO-d_6, 376 MHz): −60.61 (s, 3F, CF_3); −112.81 to −112.92 (m, 1F, Ar–F). I.R (KBr, cm^{-1}): 3,388, 3,324 (NH_2), 2,196 (CN), 1,661 (C=N), 1,143 (C–F). HRMS m/z calcd for $C_{21}H_{15}F_4N_4O_2$ ($[M + H]^+$): 431.113 found: 431.114.

*6-Amino-1-(3-chlorophenyl)-4-(4-methoxyphenyl)-3-(trifluoromethyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carobonitrile (**4i**)*

Yield: 0.72 g (82 %); mp 189–191 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.78 (s, 3H, OCH_3), 4.66 (s,1H, CH), 6.81 (d, $J = 8.687$ Hz, 2H), 6.86 (s, 2H, NH_2), 7.10 (d, $J = 8.687$ Hz, 2H), 7.32–7.40 (m, 1H), 7.44–7.52 (m, 1H), 7.84–7.92 (m, 2H). ^{13}C NMR (DMSO-d_6, 300 MHz): δ 30.5, 35.7, 54.9, 58.8, 99.3, 113.6, 119.3, 120.3, 121.4, 128.0, 128.7, 135.3, 131.1, 133.8, 137.4, 145.1, 158.2. ^{19}F NMR (DMSO-d_6, 400MHZ): −61.09 (s, 3F, CF_3). I.R (KBr, cm^{-1}): 3,392, 3,323 (NH_2), 2,193 (CN), 1,659 (C=N), 1,144 (C–F). HRMS m/z calcd for $C_{21}H_{15}F_3ClN_4O_2$ ($[M + H]^+$): 447.079 found: 447.078.

*6-Amino-1-(3-chlorophenyl)-4-(4-nitrophenyl)-3-(trifluoromethyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carobonitrile (**4j**)*

Yield 0.80 g (88 %); m. p. 224–226 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.89 (s, 1H, CH), 7.26–7.34 (br, 2H, NH_2), 7.37–7.55 (m, 4H), 7.86–7.95 (m, 2H), 8.19 (d, $J = 8.7$ Hz, 2H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 36.4, 57.2, 95.5, 97.6, 118.1, 118.4, 119.1, 120.7, 123.3, 127.4, 128.4, 130.3, 134.3, 137.4, 145.0, 146.6, 149.8, 158.6. ^{19}F NMR (DMSO-d_6, 376 MHz): δ −61.15 (s, 3F, CF_3). I.R (KBr, cm^{-1}): 3,414, 3,329 (NH_2), 2,205 (CN), 1,667 (C=N), 1,520, 1,350 (NO_2), 1,143 (C–F). HRMS m/z calcd for $C_{20}H_{12}F_3ClN_5O_3$ ($[M + H]^+$): 462.048 found: 462.047.

*6-Amino-1-(3-chlorophenyl)-4-(furan-2-yl)-3-(trifluoromethyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carobonitrile (**4k**)*

Yield: 0.64 g (82 %); m. p. 161–163 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.88 (s, 1H), 6.21 (d, $J = 2.644$ Hz, 1H), 6.32 (s, 1H), 7.14 (s, 2H, NH_2), 7.37 (d, $J = 7.554$ Hz, 2H), 7.44–7.53 (m, 1H), 7.81–7.92 (d, $J = 12.6$ Hz, 2H). ^{13}C NMR (DMSO-d_6, 300 MHz): δ 30.3, 55.6, 95.5, 106.1, 110.0, 118.9, 120.5, 122.0, 127.3, 129.8, 130.3, 134.3, 137.5, 141.8, 144.9, 151.3, 153.4, 159.3. ^{19}F NMR (DMSO-d_6, 376 MHz): δ −61.42(s, 3F, CF_3). I.R (KBr, cm^{-1}): 3,479, 3,331 (NH_2), 2,204 (CN), 1,664 (C=N), 1,142 (C–F). HRMS m/z calcd for $C_{18}H_{11}F_3ClN_4O_2$ ($[M + H]^+$): 407.0522 found: 407.0521.

*6-Amino-1-(3-chlorophenyl)-4-phenyl-3-(trifluoromethyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carobonitrile (**4l**)*

Yield 0.73 g (88 %); m. p. 181–183 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.71 (s, 1H, CH), 6.86 (br, 2H, NH_2), 7.16–7.39 (m, 5H), 7.45–7.51 (m, 2H), 7.80–7.93 (m, 2H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 36.6, 58.4, 95.4, 99.0, 118.9, 119.1, 120.6, 121.8, 122.2, 127.1, 128.0, 130.5, 133.4, 134.1, 137.5, 142.9, 158.4, 164.9. ^{19}F NMR (DMSO-d_6, 376 MHz): −61.69 (s, 3F, CF_3). I.R (KBr, cm^{-1}): 3,468, 3,326 (NH_2), 2,200 (CN), 1,664 (C=N), 1,136 (C–F). HRMS m/z calcd for $C_{20}H_{13}F_3Cl\ N_4O$ ($[M + H]^+$): 417.068 found: 417.067.

*6-Amino-1-(4-fluorophenyl)-4-phenyl-3-(trifluoromethyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carobonitrile (**4m**)*

Yield 0.66 g (83 %); m. p. 173–175 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.71 (s, 1H, CH), 7.10–7.63 (m, 9H, Ar & NH_2), 7.87–7.98 (m, 2H, Ar). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 36.7, 58.5, 95.4, 98.7, 115.7, 116.0, 118.9, 123.0, 123.1, 126.8, 127.1, 128.0, 132.7, 142.8, 144.6, 158.4. ^{19}F NMR (DMSO-d_6, 376 MHz): −61.25 (s, 3F, CF_3); −113.05 to −113.13 (m, 1F, Ar–F). I.R (KBr, cm^{-1}): 3,467, 3,325 (NH_2), 2,193 (CN), 1,659 (C=N), 1,142 (C–F). HRMS m/z calcd for $C_{20}H_{13}F_4N_4O$ ($[M + H]^+$): 401.102 found: 401.103.

*6-Amino-4-(4-fluorophenyl)-1-phenyl-3-(trifluoromethyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**4n**)*

Yield 0.61 g (85 %); m. p. 221–223 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.75 (s, 1H, CH), 7.06 (t, $J = 8.7$ Hz, 2H), 7.20–7.29 (m, 4H, Ar & NH2), 7.42 (t, $J = 7.4$ Hz, 1H), 7.51–7.58 (m, 2H), 7.87 (d, $J = 7.7$ Hz, 2H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 35.9, 58.2, 95.4, 98.5, 114.6, 114.9, 118.8, 121.0, 127.5, 129.0, 136.3, 139.0, 144.6, 158.3, 159.5, 162.7. ^{19}F NMR (DMSO-d_6, 376 MHz): δ −61.44 (s, 3F, CF_3); −112.95 to −113.03 (m, 1F, Ar–F). I.R (KBr, cm^{-1}): 3,414, 3,327 (NH_2), 2,200 (CN), 1,661 (C=N), 1,142 (C–F). HRMS m/z calcd for $C_{20}H_{13}F_4N_4O$ ($[M + H]^+$): 401.098 found: 401.097.

Cyclic voltammetry

Cyclic voltammetry measurements were performed using IM6ex Zahner-Elektrik (Germany). The three electrode system consisted of natural graphite sheet working electrode (50×50 mm^2), Ag/AgCl/KCl as reference electrode, and a platinum foil as counter electrode. The potential was scanned between 0 and 1.6 V at scan rate of 10 mV/s.

Preparation of DNA solution

pET15b vector with an insert with 2.7-Kb coding sequence for *Escherichia coli* aminopeptidase N (Gumpena *et al*., 2011) was purified from DH5α cells as per the Miniprep preparation method following the manufacturer's protocols.

Cytotoxicity studies

Cellular viability in the presence of test compounds was determined by the MTT microcultured tetrazolium assay following the reported protocol (Myadaraboina *et al*., 2010) MRC-5 (non cancer lung cells), A549 (lung cancer cells), HeLa cell lines are employed in the current study. All the three types of cancer cell lines and one normal cell line are seeded to flat bottom 96 (10,000 cells/100 ml)-well plate and cultured in the medium containing 10 % serum. Incubated for 24 h in a 5 % CO_2 humid chamber at 37 °C so that the cells adhere to the surface. MTT was dissolved in PBS at 5 mg/ml and sterile filtered.

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