Synthetic modulation including structure establishment, antiproliferative activity of some *p*-aryl substituted (*Z*)-2-cyano-ethylideneacetohydrazides, and their structure activity relationship

Deepak Chowrasia[1,3,*], Nisha Sharma[1], Ajay Kumar[1], Md Arshad[2], Sahabjada Siddiqui[2,4], Asif Jafri[2] and Juhi Rahis[2]

[1]University Institute of Pharmacy,
Chhatrapati Shahu ji Maharaj (CSJM) University, Kalyanpur,
Kanpur 208 024, India
[2]Department of Zoology, Lucknow University,
Lucknow 226 007, India
[3]School of Pharmaceutical Sciences,
Rajiv Gandhi Technical University, Airport Bypass Road,
Gandhi Nagar, Bhopal 462 036, India
[4]Department of Biotechnology, ERA's Lucknow Medical College and Hospital, Era University, Lucknow 226 003, India

A series of *p*-substituted aryl-2-cyanoethylidene-acetohydrazides derivatives (2a-j) were successfully synthesized in the laboratory (yield 60–80%). The synthesized compounds were screened for their anti-proliferative activity against MCF-7 (estrogen dependent human breast cancer cell line), SaOS-2 (osteosarcoma cell line), and K562 (myeloid leukemia cell line) by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. They showed moderate to mild antiproliferative activity, (2j) being the most potent in the series with an IC_{50} 55, 64 and 35 μM against MCF-7, SaOS-2 and K562 cell lines, depict *p*-nitro as a better antiproliferative substituent comparatively. We have also tested the hypothesis – 'Electron withdrawing phenomenon affects antiproliferative activity'.

Keywords: Cancer, cyanoacetohydrazide, electron withdrawing ring substituent, MTT assay.

CANCER is the second leading cause of mortality responsible for 8.8 million deaths (2015). WHO estimates around 9.6 million deaths in 2018 (ref. 1). Being genomic in origin and having direct impact on cellular proliferation as well as differentiation machinery, cancer in earlier and/or in-later stages causes mass-lumps, exposing living tissue to an unnecessary metabolic burden rendering them to perform normal physiological task[2,3]. Though global cancer research has come a long way, the need for novel anticancer agent/s is still unfulfilled and is the need of the present era. Rapid progression of the disease, higher incidence rate, and uncontrolled mortality have alarmed researchers worldwide to search for better cancer chemotherapeutics. However, excessive distal metastasis, multi-organ involvement, delayed detection, uneconomical and longer treatment plan, drug resistance, therapeutic side effects are some protuberants rendering for its cure and management. Other facets such as severe side-effects of cancer regime promote poor patient compliance leading to progression of the disease and causing death. In the past few years, heterocyclic[4–8] and non-heterocyclic moieties, especially nitrogen containing motifs endowed with anti-proliferative activity, were extensively targeted, among which hydrazide-hydrazone including their structurally modified derivatives were proved to be prominent, versatile, and potent core template for development of novel anticancer agent/s[9–16].

Hydrazones ($R_1R_2C{=}NNH_2$) are related aldehyde or ketone derivatives in which oxygen is replaced covalently with $–NNH_2$ functionality consecrating the core structure with two active centres (viz. carbon and nitrogen) responsible for wide biological activities. Likewise, hydrazide (E(O=)–NR–NR; R=H), the acylated derivative of hydrazine, is another distinct class possessing covalently bonded dual nitrogen system configured for formation of nitrogen assisted inter-chemical bonds with host-targeted protein conferring the molecule diversified pharmacological activities[17–20].

2-Cyano-*N*′-(1-(pyridine-3-yl)ethylidene)acetohydazide was selected as lead molecule for derivatization and yield newer synthetic daughter molecules. The main criteria that were considered while selecting this molecule include ease of laboratory derivatization for structure activity relationship (SAR) development, availability of synthetic building block, fewer level reaction, handling procedure, product purity including yield (60–80%), optimum molecular weight (less than 300) of end product, Lipinski rule of five, novelty of chemical structure, and *in vitro* reported sub-micromolar activity of lead template against estrogen dependent breast cancer cell line (MCF-7)[21].

All commercial chemicals and solvents used are reagent grade and were used without further treatment unless otherwise noted. ^{1}H-NMR spectra were recorded by Bruker Avance-II NMR spectrometer. Chemical shift was recorded in parts per million (ppm) and reported relative to the TMS. Mass spectra were recorded on an applied Biosystem Qtrap 3200 LC-MS/MS system in ESI mode. The FT-IR spectra of the synthesized compounds were recorded on Brukers FTIR. Melting points of all compounds were determined using Veego digital melting point apparatus and reported uncorrected. The purity of compounds was confirmed by thin layer chromatography (TLC) using Merck silica gel 60-F 254 coated alumina plates both at near and far UV.

Synthesis of ethylcyanoacetyl hydrazide (**1a**) (Scheme 1)[22]: A mixture of ethyl cyanoacetate (1 M) and hydrazine hydrate (1 M) in ethanol (5 ml) was stirred at 0°C until reddish brown mass of cynoacetylhydrazine was

*For correspondence. (e-mail: chowrasia.deepak@gmail.com)

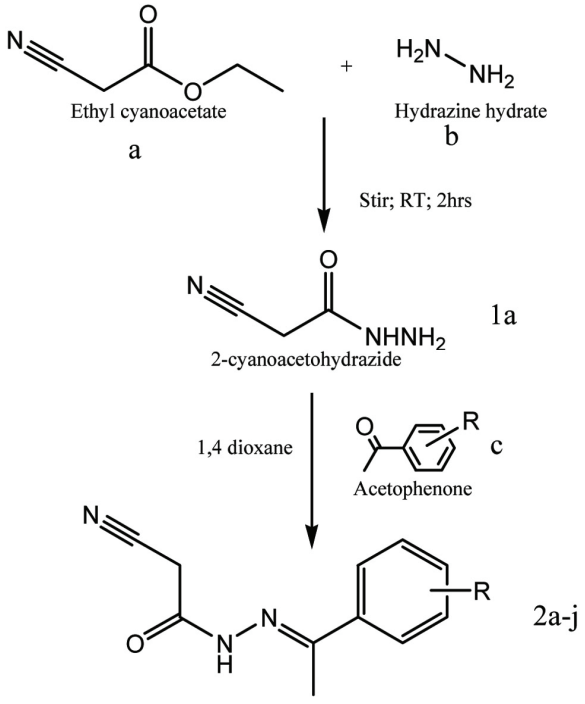
obtained; this was washed thrice from diethyl ether and dried at room temperature before further use.

Synthesis of *p*-substituted aryl cyanoacetylhydrazide derivatives (**2a-j**) (Scheme 1)[22]: A mixture of cynoacetylhydrazine (0.1 M) and substituted acetophenone/s (0.1 M) in 1,4-dioxane was refluxed for 2 h. The reaction mixture was cooled to room temperature; subsequently cold water was added and the resulting crystals were washed thoroughly with chilled water, dried, and recrystallized from ethanol. Completion of the reaction was monitored on pre-coated silica gel-G TLC plates using ethyl acetate and petroleum ether (3 : 7) as a binary eluent. Spots were visualized under both far and near UV light.

(*E*)-2-cyano-*N*′-(1-phenylethylidene)acetohydrazide (**2a**): Yield: 83(%), m.p. 197–198°C, IR (KBr) ν cm^{-1}: 2959.84 (Ar-CH stretching, 2920.06 (CH_3 stretching), 2260.47 (CN stretching), 1689.79 (C=O stretching), 3197.60 (NH stretching); MS (ESI): *m*/*z* 201.1 (M^+H).

(*E*)-2-cyano-N′-(1-(pyridin-3-yl)ethylidene)acetohydrazide (**2g**): Yield: 75.23(%), m.p. 201°C, IR (KBr) ν cm^{-1}: 3067.78 (Ar-CH stretching), 2850.64 (CH_3 stretching), 2259.13 (CN stretching), 1707.67 (C=O stretching), 3386.23 (NH stretching); MS (ESI): *m*/*z* 200.8 (M^+H). ^{1}H NMR (500 MHz) δ 2.28 (s, 2H, CH_2), 7.43–8.89 (m, 4H, pyridine H), 10.81 (s, 1H, NH).

(*E*)-2-cyano-*N*′-(1-(3-nitrophenyl)ethylidene)acetohydrazide (**2j**): Yield: 81.45(%), m.p. 201°C, IR (KBr) ν cm^{-1}: 3199.86 (Ar-CH stretching), 2920.98 (CH3 stretching), 2261.67 (CN stretching), 1683.29 (C=O stretching), 3104.22 (NH stretching); MS (ESI): m/z 245.0 (M^+H).



Scheme 1. Synthetic pathway to yield **2a-j**.

The human cancerous cell lines, viz. MCF-7, SaOS-2 and K562 were obtained from cell repository – NCCS, Pune, India. The cells were maintained in Eagle's minimal essential medium (MEM, Himedia), McCoy's 5a medium (Himedia) and RPMI-1640 (Himedia) respectively, supplemented with $NaHCO_3$, sodium pyruvate and 10% fetal calf serum (Himedia). Cells were maintained at 37°C, 5% CO_2 in humidified air.

The anti-proliferative activities of the compounds were determined using MTT (3-(4,5-dimethlythiazol-2yl)-2,5-diphenyltetrazolium bromide) assay[23]. Approximately, 1×10^4 cells/well were seeded in 100 µl complete culture media in each well of 96-well culture plates and incubated for 24 h at 37°C in carbon dioxide incubator. The test compounds diluted to the desired concentration in culture media were added to the well with respective vehicle control (DMSO). After 21 h (incubation period), media were removed successfully and to each well 10 µl of MTT (5 mg/ml of media without phenol red and serum) was added; plates were further incubated for the next 4 h at optimum temperature (37°C). Supernatant from each well was carefully removed and formazan crystal thus formed was solublilized by mixing in 100 µl of DMSO. Subsequently for suspended cell line K562 plates were centrifuged at 1500 rpm for 10 min after addition of MTT and absorbance was recorded at 540 nm by microplate reader (BIO RAD Model 680).

The percentage cytotoxicity (CT) was determined by the following equation

$$\%CT = \frac{(OD_c - OD_t)}{OD_c},$$

where % CT is the percentage cell toxicity, OD_c the OD of control and OD_t is the OD of test sample.

The inhibitory concentration at 50% (IC_{50}) value is calculated by plotting the percentage cell toxicity (% CT) with test concentration.

The *p*-aryl substituted-2-cyanoethylideneacetohydrazides derivatives (**2a-j**) were synthesized in satisfactory yield (60–80%) and their structure was confirmed by suitable spectrometric methods, viz. IR, ^{H}NMR and mass spectroscopy. To elucidate *in vitro* biological activity the aforesaid synthesized compounds (**1a-j**) were subjected to MTT assay as per the standard protocol.

The synthesized *p*-aryl substituted-2-cyanoethylideneacetohydrazides derivatives (**2a-j**) were screened for *in vitro* antiproliferative activity against MCF-7 (human breast cancer cell line estrogen dependent), SaOS-2 (osteosarcoma cell line) and K562 (myeloid leukemia cell line) by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. Tamoxifen, a potent anticancer drug was used as a reference standard. Synthesized compounds show moderate to mild antiproliferative activity ranging from 35 to 81 µM (Table 1)

Table 1. Anti-proliferative activity of *p*-substituted aryl-2-cyanoacetylhydrazide derivatives

Compound code	*R*	MCF-7 (IC_{50})	SaOs-2 (IC_{50})	K562 (IC_{50})
2a	H	78.10	81	48
2b	4-OCH_3	75	72	43.5
2c	4-Br	73	73.2	45
2d	4-Cl	70	70.5	42.5
2e	3-NO_2	77	77	45.5
2f	4-CH_3	68.5	65.5	41
2g	Pyridine	64.5	69.5	39
2h	3,4-OCH_3	–	–	–
2i	4-F	60	64.5	37
2j	4-NO_2	55	64	35

Table 2. Comparative study between electron withdrawing ring substituents and their *in vitro* antiproliferative activity

Potency (in-series)	MCF-7; 	SaoS-2; 	K652; 
Most potent	**2j**; *p*-NO_2 **2i**; *p*-F **2g**; Pyridine	**2j**; *p*-NO_2 **2i**; *p*-F **2f**; *p*-CH_3	**2j**; *p*-NO_2 **2i**; *p*-F **2g**; Pyridine
Moderate potent	**2f**; *p*-CH_3 **2d**; *p*-Cl **2c**; *p*-Br	**2g**; Pyridine **2d**; *p*-Cl **2b**; *p*-OCH_3	**2f**; *p*-CH_3 **2d**; *p*-Cl **2b**; *p*-OCH_3
Least potent	**2b**; *p*-OCH_3 **2e**; *o*-NO_2 **2a**; H	**2c**; *p*-Br **2e**; *o*-NO_2 **2a**; H	**2c**; *p*-Br **2e**; *o*-NO_2 **2a**; H
No activity	**2h**; *o*, *p*-OCH_3	**2h**; *o*, *p*-OCH_3	**2h**; *o*, *p*-OCH_3

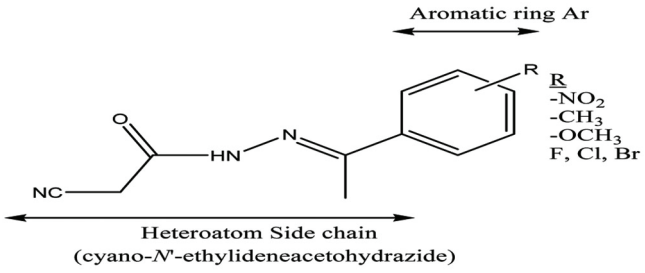


Figure 1. Lead antiproliferative template derivatize to yield **2a**-**j**.

against the three cell lines, viz. MCF-7, SaOS-2 and K562. The compound **2j** was most potent in series with an IC_{50} of 55, 64 and 35 μM against MCF-7, SaOS-2 and K562 cell lines respectively. On other hand **1a** was the least active with an IC_{50} of 78, 81, and 48 μM against MCF-7, SaOS-2 and K562.

All synthesized *p*-aryl substituted-2-cyanoethylideneacetohydrazides derivatives (**2a–j**) share a common core structure, an aromatic ring linked directly to a side chain enveloping cyano-ethylideneacetohydrazide. Presence of heteroatom (N and CN) along with carbon in the side chain imparts unique chemical characteristic to the molecule making it a versatile template for electro- as well as nucleophilic bonding. For SAR development, we consider derivatization of aromatic ring especially at *para*-position characteristically with electron withdrawing groups (EWG) and correlate their antiproliferative activity.

Though similar (Figure 1) in core structure, the compounds differ substantially in physico-chemical characteristics as well as in spectrometric data due to presence of different functionalities at *para*-position on aromatic ring. The *p*-nitro (**1j**) group was the most potent not only in series, but among the screened tumour cell lines, contrary to other *para*-substituted derivatives (**2i**, **2g**, **2f**, **2d**, **2c**, **2b**, **2e**). Furthermore, among different cell lines and within the series, the antiproliferative activity of screened compound in descending (most to least potent) order includes **2j** > **2i** > **2g** > **2f** > **2d** > **2c** > **2b** > **2e** > **2a** > **2h** for MCF-7, **2j** > **2i** > **2f** > **2g** > **2d** > **2b** > **2c** > **2e** > **2a** > **2h** for SaOS-2 and **2j** > **2i** > **2g** > **2f** > **2d** > **2b** > **2c** > **2e** > **2a** > **2h** against K652, indicating essentially of *p*-nitro functionality for antiproliferative activity, while on the other hand *p*-fluorine (**2i**), pyridine ring (**2g**) and *p*-methyl (**2f**) are other functionality principally imparting moderate *in vitro* antiproliferative activity to synthesized compounds. Substituted analogues posses comparatively better antiproliferative activity with unsubstituted

molecules (Table 2). In order to correlate antiproliferative activity depending upon the chemical behaviour of substituents, it has been found that substituents with greater electron withdrawing effect pose better antiproliferative activity compared to their counterpart which exhibits lesser electron withdrawing effect except **2f**.

Series of *p*-substituted aryl-cyanoacetohydrazide derivatives (**2a-j**) were synthesized and *in vitro* targeted successfully against three cancer cell lines, viz. MCF-7 (estrogen receptor positive human breast cancer cell line), SaOS-2 (osteosarcoma cell line) and K652 (myelogenous leukaemia cell line) to find new anticancer template. Among the synthesized compounds, **2j** was found to be the most potent not only in series, but also against all the three screened tumour cell lines; if derivatized suitably at other positions (*ortho*, *meta*, *ortho-para*, *ortho-meta*) on the aromatic ring especially with electron withdrawing functionality, it may yield a more potent and effective future antitumour molecule.

Conflict of interest: The authors declare they had no conflict of interest.

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ACKNOWLEDGEMENTS. We acknowledge the sophisticated analytical instrumental laboratory (*SAIL*) of RPGV, Bhopal for mass spectroscopy and IIT Kanpur for IR and ^{1}H-NMR spectra used in this study.

Received 18 January 2018; accepted 11 May 2018

doi: 10.18520/cs/v115/i12/2287-2290