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Original Research Article

Synthesis, crystal structure studies, characterization and *in vitro* study of novel heterocyclic thiadiazoles as caspase 3 inhibitors for anticancer activity

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ABSTRACT

In the present study we have reported the synthesis of some novel heterocyclic derivatives comprising imidazole and 1,3,4-thiadiazole containing cyclopropyl moiety. Imidazothiadiazoles are of interest because of their diverse biological activities and clinical applications. Primarily docking studies are carried out with reference structure Pdb code:2DKO. We have reported the new series of 5,6 diaryl substituted with imidazo[2,1- b] [1–3]thiadiazoles analogs to target caspase family cysteine proteases. The reaction was monitored by Thin-layer chromatography using suitable mobile phase. The Rf values were compared and determined the melting point of synthesized compounds. Further these derivatives were characterized and confirmed by IR, 1H-NMR, 13C-NMR, and Mass spectral (MS) studies. For anticancer activity, all the selected compounds submitted to National Cancer Institute (NCI) for in vitro anticancer assay were evaluated for their anticancer activity. Primary *in vitro* one dose anticancer assay was performed in full NCI 60 cell panel in accordance with the protocol of the NCI, USA. The compounds *Va* and *Vc* showed good activity against all cancer cell lines. The synthesized drugs were monitored with Caspase 3 inhibitor kit.(CASP3C-1KT)using colorimeteric assay.

1. Introduction

Many of the more recently approved cancer treatments target either the cell surface receptors at the head of these signaling pathways, or the intermediate phosphoproteins and kinases in the pathway signaling cascades. Understanding the phosphoprotein activation state of key signaling molecules in tumor cells can yield critical information on the type, stage and status of those cells, aiding in the diagnosis, prognosis and treatment of an individual's disease. [1]

Caspases are aspartic acid-specific cysteine proteases, which become activated in most forms of apoptosis. In cells, they localise in nucleus, cytoplasm, and mitochondrial intermembrane space, and can also be translocated to the plasma membrane receptors via adapter proteins. [4]

Caspases are expressed in most tissues in an inactive pro-form, which has an amino- terminal prodomain, a large subunit (~20 kDa) and a small subunit (~10 kDa) (Figure

1A). Upon activation, a procaspase is proteolytically cleaved to remove the prodomain and to release the large and the small subunit. Two small and two large subunits are assembled together to yield an active caspase enzyme with two active sites. [2]

DED = Death Effector Domain (dotted), QACQG= consensus amino acid sequence of the

active site of caspases (black ellipses) in large subunit,

arrows = sites of processing to release large subunit (light grey) and small subunit

(dark grey) from prodomain (white).

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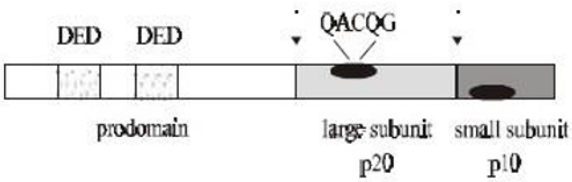
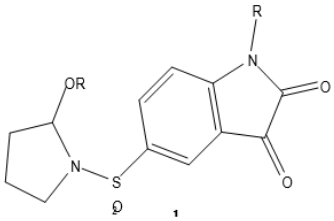


Figure 1: Schematic presentation of the general structure of caspases.[2]



5-(2-aryloxypyrrolidin-1-ylsulfonyl)-1-arylindoline-2,3-dione

Figure 2: Active caspase tetramer.[5]

1.1. Role of caspase in cancer

Dying cells in the tumor mass provide the initial signals to promote tumor repopu- lation. Specifically, dying cells release growth-promoting signals to stimulate the proliferation of surviving cells.Caspases play a crucial role for intratumoral dying cells in promoting the rapid repopulation of tumors from a small number of live tumor cells. In addition, caspase 3, a cysteine protease involved in the 'execution' phase of cellular apoptosis, is a key regulator of growth-promoting signals generated from the dying cells. Caspase-mediated tumor repopulation mechanism has key roles in cytotoxic cancer therapy.[3]

1.2. Caspase inhibitors:[6,7]

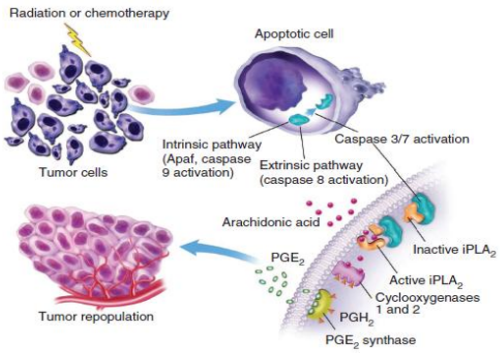


Figure 3: Role of caspase in cancer[3]

1.3. Chemistry of imidazo [2, 1-b][1–3] *thiadiazoles*

The bicyclic molecule many be viewed as 2-vinylimino-3H-3- alkyl-1, 3, 4- thiadiazole.

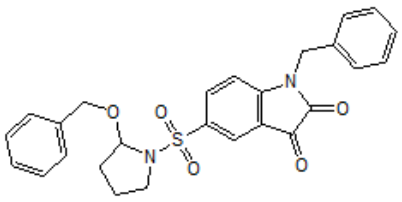
Important canonical structure of imidazo[2, 1-b][1–3] thiadiazole are given. They indicate greater delocalisation of π electrone in the imidazole ring, while the double bond of the thiadiazole ring is almost localized in structure is maximum contributing structure. It is pseudo-aromatic in behavior containing imidazole moiety as electron rich center. Chlorine or bromine dose not add to the double bond at 2, 3-position on the other hand, the electrophillic substitution reactions like bromination, nitration etc take place at 5-position. His bicyclic ring system with desired substituents at 2, 5, and 6-position can be built by starting with appropriate synthons, for introduction of substitutents at 5 and 6-position, the synthons R1COCHR2Br is employed for subtituents at 2-position, 5-substituted 2-aminothiadiazole is required. The ring nitrogen in imino form of the starting thiadiazole attacks the carbon carrying bromine in the synthon.[8,9] Imidazo[2,1 –b][1–3] thiadiazoles contain nitrogen as a bridge head atom at 4th position electron charge density measurement indicated that the imidazole ring is rich in electron density having at N-7 position and then at C-5 position. This accounts for the preferable electrophilic substitution reaction at C-5 position. Of the three nitrogen atoms, N-7 is the most basic center. The order of basicity is N-7> 3> N-4. Therefore, protonation occurs first at N-7 then at N-3 position.

Two types of bicyclic imidazo[2,1b][1–3]thiadiazole ring systems are possible.

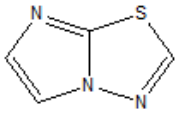
2. Materials and Methods

2.1. Docking studies

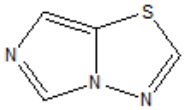
In our present study, we used computational approach to identify the potent and selective Caspase-3 inhibitors. Three-dimensional (3D) pharmacophore models were generated using the known set of Caspase-3 inhibitors, to reveal the chemical features required for its activity. Best pharmacophore is validated with docking and structure based pharmacophore studies. These models were used to rapidly screen compounds from database, for the



5-(2-benzyloxypyrrolidin-1-ylsulfonyl)-1-benzylindoline-2,3-dione



IMIDAZO[2,1-b][1,3,4]THIADIAZOLE



IMIDAZO[5,1-b][1,3,4]THIADIAZOLE

Figure 4: showing imidazolering system

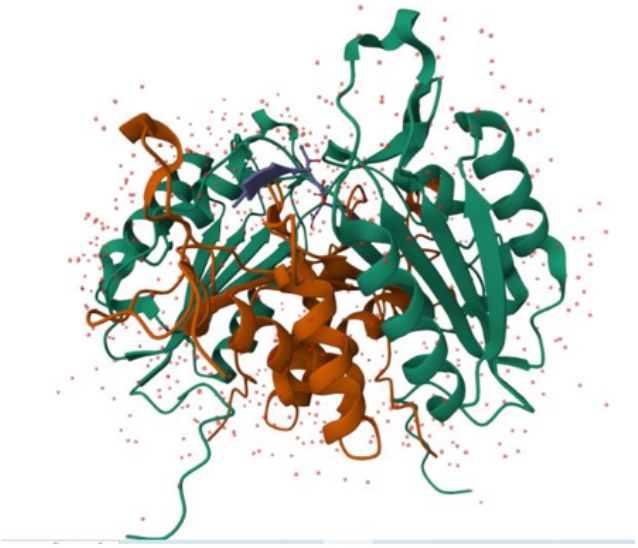


Figure 5: Docking structure of caspase 3 inhibitor (PDB structure 2DKO)

identification of a series of novel and highly potent Caspase-3 inhibitors. Molecules were selected from virtual screening using pharmacophore as query and these molecules are selected for synthesis and in vitro screening studies based on the docking scores, predicted binding location and their drug like properties. [10,11]

The identification and characterization of the compound were carried out by the following procedure to ascertain that all prepared compounds were of different chemical nature, than the respective parent compound.

1. Melting point
2. Solubility
3. Thin layer chromatography
4. Infrared spectroscopy
5. Proton nuclear magnetic resonance
6. Mass spectroscopy

The chemicals employed in the synthetic work i.e thiosemicarbazide and trifuoroaceticanhydride were purchased from Sigma-Aldrich while all other chemicals i.e. cyclopropanecarboxylic acid, Bromine, various acetophenones, DMF and POCl3 etc. were purchased from Spectrochem. All the solvents were used after distillation. Most of the solvents and chemicals used were of LR grade. The purity of the compounds was confirmed by thin layer chromatography using precoated TLC plates and solvent systems of Benzene: Acetone (9:1), (7:3); T:E:F (5:4:1), and Chloroform: Methanol (9:1). The spots were visualized under ultraviolet lamp. Melting points were determined in one end open capillary tubes on a liquid paraffin bath and are uncorrected.

Infrared (IR) and 1H nuclear magnetic resonance (1H NMR) spectra were recorded for the compounds on Perkin Elmer IR 4000-400 (ν max in cm-1) Spectrophotometer in KBr pellets and Bruker Model Advance II 400 (400 MHz, 1H NMR) instrument, respectively. Chemical shifts are reported as δ parts per million (ppm) using tetramethylsilane (TMS) as an internal standard.

3. Reaction Scheme

3.1. Synthesis of 5-cyclopropyl-1,3,4-thiadiazol-2-amine (III)

Mixture of cyclopropanecarboxylic acid (I (0 05 mol , thiosemicarbazide (II (0.05 mol) and POCl3 (13 ml) was heated at 75 °C for 0.75 h. After cooling down to room temperature, water was added. The reaction mixture was refluxed for 4 h. After cooling, the mixture was neutralized to pH 7 by the drop wise addition of 50% NaOH solution under stirring. The precipitate was filtered and crystallized from ethanol.

3.2. Synthesis of 2-bromo-1,2-(substituted-aryl)ethanone (IVa-i)

To a mixture of phenyl acetic acid/p-substituted phenyl acetic acid (10a/b, 7.3 mmol), substituted aromatic hydrocarbon (one of *VIII a–i,* 8.8 mmol), and 88–93% orthophosphoric acid (8.8 mmol) was added trifluoroacetic anhydride (29.5 mmol) rapidly with vigorous stirring at 250 C. The mixture turned into a dark colored solution with vigorous exothermic reaction. The reaction mixture was stirred for 1 min at the same temperature and poured into ice-cold water (50 mL) with stirring. Then it was washed with cold hexane (2· 10 mL) to obtain (*IV a–i)* as solid.

3.3. Synthesis of 2-cyclopropyl-5,6-diarylsubstituted imidazo[2,1-b]-1,3,4- thiadiazole(Va-i)

A mixture of 2-amino-5-substituted-1,3,4-thiadiazole(III, 10 mmol) and an appropriate a-bromo-1-(4"-substituted)phenyl-2-(4' substituted)phenyl-1-ethanone (one of IVa–i, 10 mmol) in dry ethanol (150 mL) was heated to reflux on a water bath for 6–8 h, phosphorus pentoxide (3 mmol) was added, and refluxing was continued for another 4–6 h. The reaction mixture was cooled overnight at room temperature. Excess of solvent was removed under reduced pressure and the solid hydrobromide separated was filtered, washed with cold ethanol, and dried. Neutralization of hydrobromide salts with cold aqueous solution of Na2CO3 yielded the corresponding free bases *(Va–i)*, which were purified by recrystallization from dry ethanol. Further, the compounds were purified by column chromatography using 200–400 mesh silica gel and eluted either with ethyl acetate/ hexane (2:8) or chloroform/hexane (1:9) as mobile phase.

3.4. Caspase colorimetric kit

The Caspase 3 Colorimetric Assay Kit is based on the hydrolysis of acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in the release of the p-nitroaniline (pNA) moiety. p-Nitroaniline is detected at 405 nm (ε_{mM}=10.5). The concentration of the pNA released from the substrate is calculated from either the absorbance values at 405 nm or from a calibration curve prepared with pNA standards (pNA standard included with the kit).

The assay can be performed (a) in 1 mL volume and measured using a spectrophotometer, or (b) in 100 μL volume in a 96-well plate using an ELISA reader.[12,13]

4. Result and Discussion

We have tried to synthesized a series of 9derivativesof imidazo [2,1,b][1–3] thiadiazole using cyclopropanecarboxylic acid as starting material. Synthesis was carried according to reaction shown in Reaction Scheme. The compounds *Va-Vi* containing substituted aryl group at 5th and 6th position by reacting 2-amino 5-substituted 1, 3, 4-thiadiazole of general formula *III* with substituted a- haloaryl/heteroaryl ketones as depicited in scheme. The reaction was monitored by Thin-layer chromatography using suitable mobile phase such as Benzene: Acetone (7:3) n-hexane: Ethyl acetate: Formic acid (5:4:1); Chloroform: Methanol (9.5:0.5). The Rf values were compared and found that they were different from each others. The melting point of the derivatives was determined.

The supplementary material is attached of characterization of compounds.

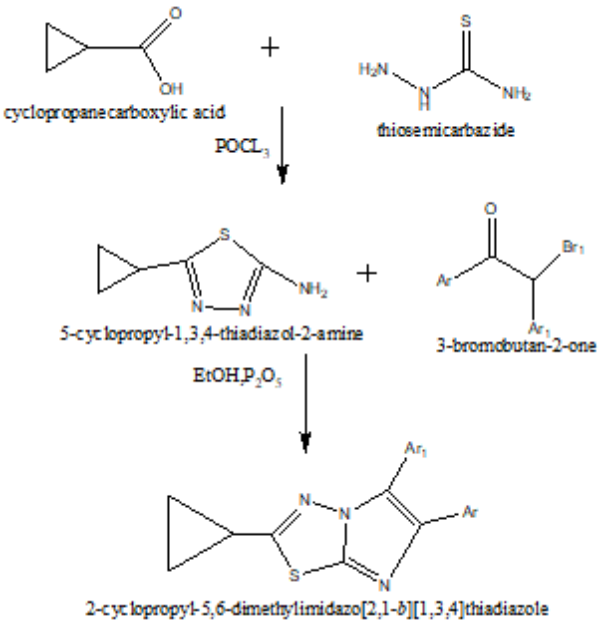


Table 1: The docking scorewith various details

Sr. No.	Structure code	Docking Score	HBD	HBA
a.	Va	-79.2	2	6
b.	Vb	-21.9	3	7
c.	Vc	-82.1	3	7
d.	Vd	-49.6	2	6
e.	Ve	-13.6	2	6
f.	Vf	-69.4	3	7
g.	Vg	-46.3	2	7
h.	Vh	-19.4	3	6
i.	Vi	-64.3	2	7
	2DKO	-88.32	3	7

5. NCI-60 DTP Human Tumor Cell Line Screen

The screening is a two stage process, beginning with the evaluation of all compounds against the 60 cell lines at

Table 2: List of proposed derivatives.

Compound	Ar	Ar 1
IV/Va	4-(OCH3)C6H4	C6H5
IV/Vb	C6H5 C6H5	C6H5
IV/Vc	4-(CH3)C6H4	C6H5
IV/Vd	4-(SCH3)C6H4	C6H5
IV/Ve	4-ClC6H4	C6H5
IV/Vf	4-BrC6H4	4-OCH3C6H4
IV/Vg	4-(OCH3)C6H4	4-OCH3C6H4
IV/Vh	4-(SCH3)C6H4	4-OCH3C6H4
IV/Vi	4-(Cl)C6H4	

a single dose of 10μM. The output from the single dose screen is reported as a mean graph and is available for analysis by the compare program. Compounds which exhibit significant growt inhibition are further evaluated against the 60 cell panel at five concentration level.[8,14]

a) Methodology of the in vitro cancer screen:[5] The human cancer cell lines of the cancer screening panel are grown in RPMI 1640 medium containing 5% fetal bovine serum at 2 mM L-glutamine. For a typical screening experiment, cells are inoculated into 96 well microtiter plates in 100μL at plate densities ranging from 5,000 to 40,000 cells/well depending upon the doubling time of individed cell lines. After cell inoculation, the microtiter plates are incubated at 37 °C, 5% CO2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. After 24 h, two plates of each cell lines are fixed *in situ* with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs are solubilized in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate is thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50μg/ml gentamicin. Additional four, 10-fold or $\frac{1}{2}$ log serial dilutions are made to provide a total of five drug concentrations plus control. Aliquots of 100μl of these different drug dilutions are added to the appropriate microtiter wells already containing 100μl of medium, resulting in the required final drug concentration.

Following drug addition, the plates are incubated for an additional 48 h at 37 °C, 5% CO2, 95% air and 100% relative humidity. For adherent cells, the assay is terminated by the addition of cold TCA. Cells are fixed in situ by the gentle addition of 50μl of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at

4 °C. The supernatant is discarded and the plates are washed five times with tap water and air dried. Sulforhodamine (SRB) solution (100μl) at 0.4% (w/v) in 1% acetic acid is added to each well and plates are incubated for 10 minutes at room temperature. After staining, unbound dye is removed by washing five times with 1% acetic acid and the plates are air dried. Bound stain is subsiquently solubilized with 10mM trizma base, and the absorbance is read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology is the same except that the assay is terminated by fixing settled cells at the bottom by the wells by gentle adding 50μl of

80% TCA (final concentration 16% TCA). Using the seven absorbance measurements

[time zero, (Tz), control growth, (C) and the test growth in the presence of drug at five concentration levels (Ti), the percentage growth is calculated at each of the drug concentrations levels. Percentage growth inhibition is calculated as:

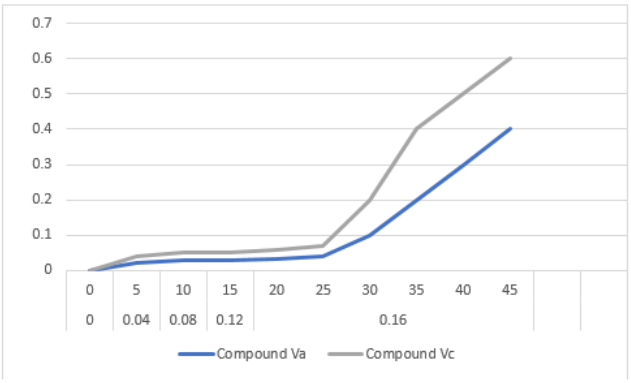


Figure 6: Showing the result with caspase 3 inhibitor
Xaxis Concentration(μg/mL)
Yaxis Absorbance(Au)

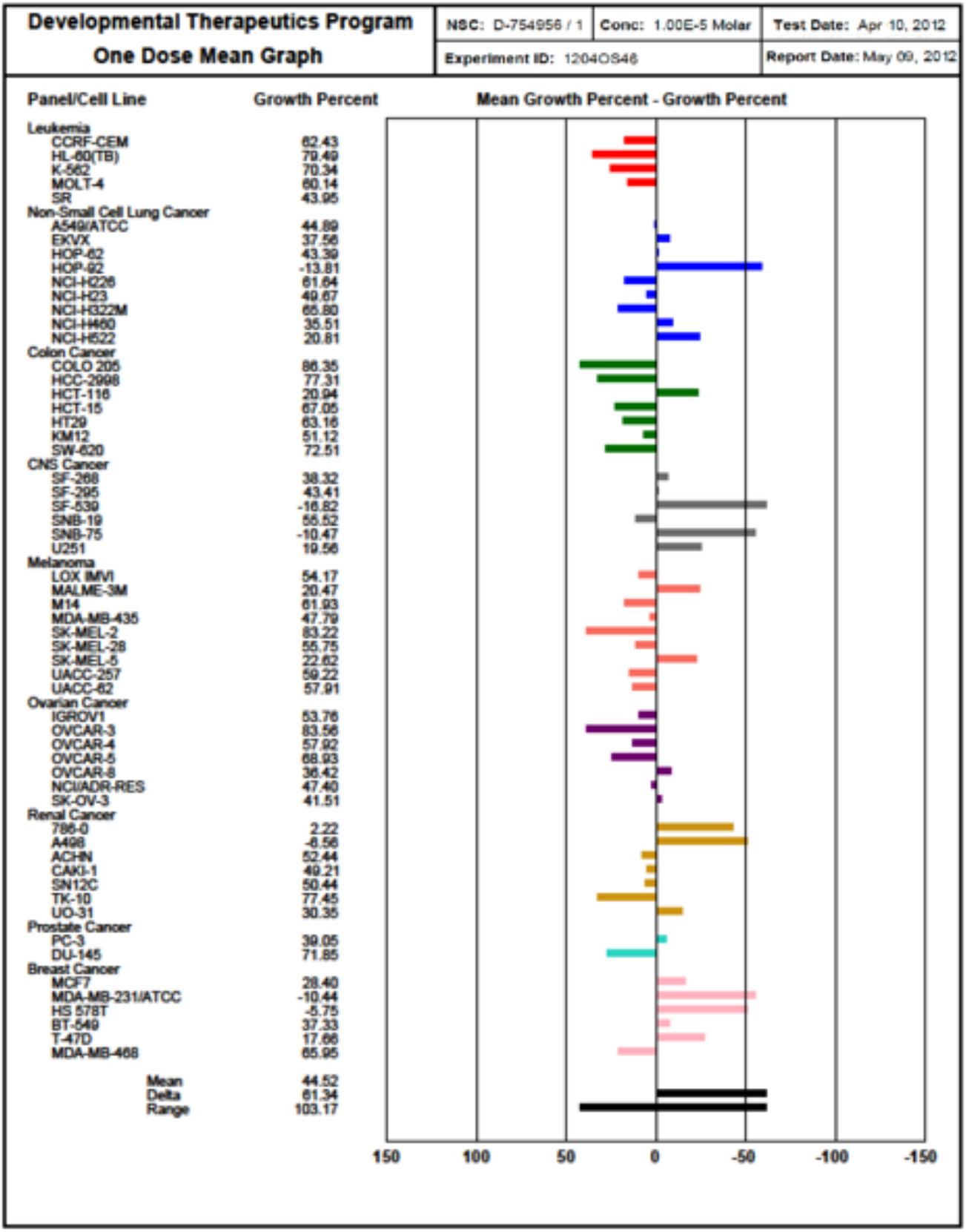
[(Ti-Tz)/(C-Tz)] X 100 for concentrations for which Ti ≥Tz [(Ti-Tz)/Tz] X 100 for concentrations for which Ti<Tz

Three dose response parameters are calculated for each experimental agent. Growth inhibition of 50% (GI50) is calculated from [(Ti-Tz)/(C-Tz)] X 100 = 50 which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) is calculated from Ti=Tz. The LC50 (concentration of the drug resulting in 50% reduction in the measured protein at the end of the drug treatment as compared from.

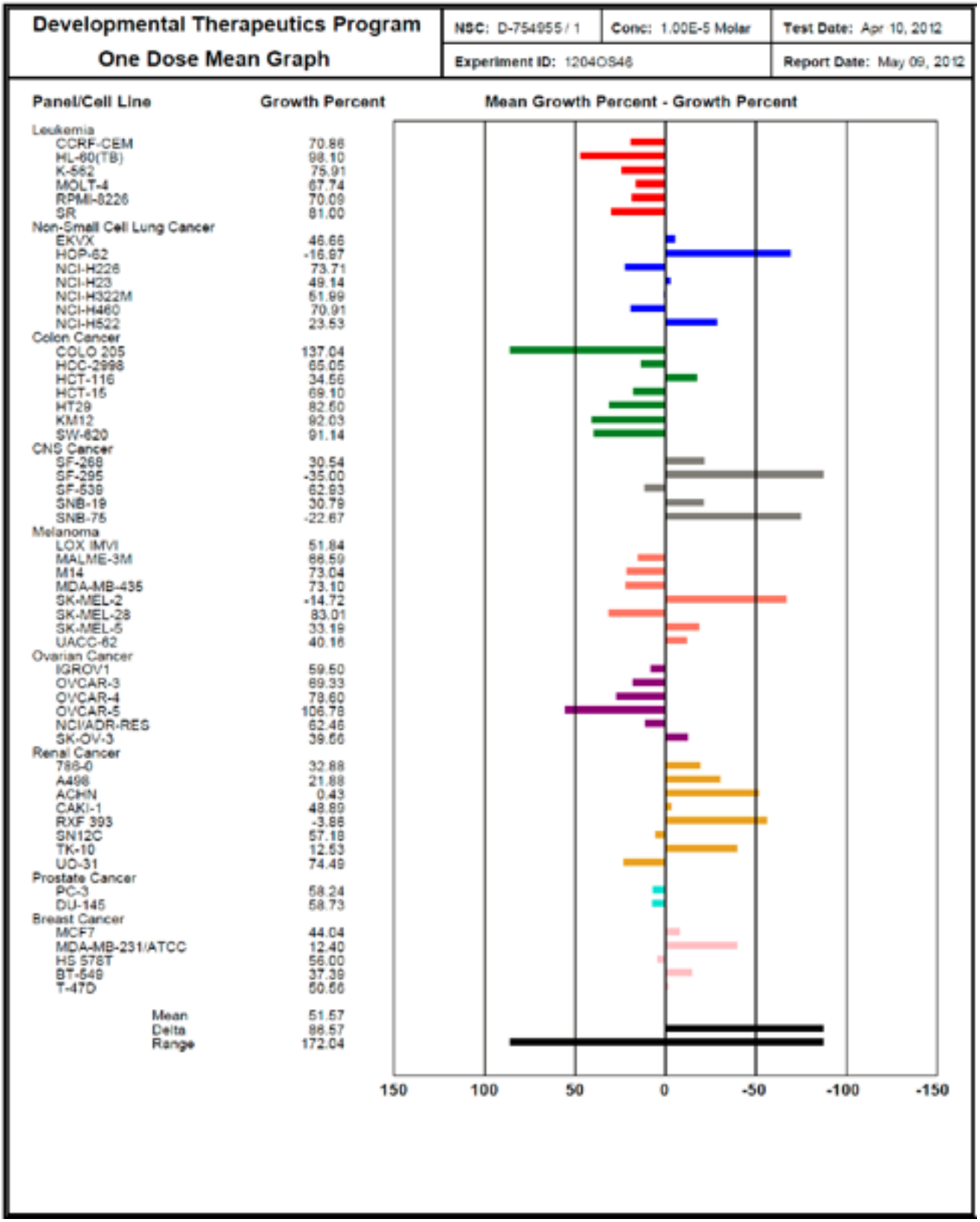
[(Ti-Tz)/Tz] X
100 = -50.[15]

Values are calculated for each of these parameters if the level of activity is reached; however, if the effect is not reached or is exceeded, the value for that parameters isexpressed as greater or less than the maximum or minimum concentration tested.

Further structures of the synthesized compounds were established on the basis of spectral data. (IR, 1H-NMR, 13C-NMR, and Mass) Compounds III showed peaks at Cm-1 for NH2, compound Va-Vi showed absorption bands ranging from1600-Cm-1 for C=N stretch, in their respective



Graph 1: Mean graph of the one dose screen for the compound (Va)



Graph 2: Mean graph of the one dose screen for the compound (VC)

spectra.

In particular, it must be seemed that in 1H NMR the disappearance of a singlet between δ- 6.0-6.5ppm indicates the formation of bridgehead nitrogen heterocycle *(Va-i)* by cyclodehydration process via *III*. Compounds showed singlet peaks at around 3.8ppm indicating presence of methoxy group of the compound *Va,Vg-i* and the characteristics peaks at δ1.16-2.39, 3.73, 2.47 ppm indicated the presence of cyclopropane group, methoxy and methyl groups in their respective structure. The compounds *Va-i* showed prominent signals of aaromatic protons around δ 7.09- 7.8ppm In[8] C-NMR spectrum it was observed most characteristic signals appear around δ 123.961, 159.137, 162.905ppm for the C-N of imidazo, and C-S of the thiadiazole carbon in their respective structure. The carbon signals of phenyl group at δaround128.0-132ppm were seemed. Also (M+1) peak of compound *Va* appeared at 348.0.

The synthesized compounds were evaluated for their *in-vitro* anticancer activity at NCI, USA. The result of anticancer activity is presented in Graphs 1 and 2. We studied the effects of various substituents at 4' and 4" position of aromatic ring. Among them compound *Va* and *Vc* showed encouraging anticancer activity.

6. Conclusion

The present work, which has undertaken is bonafied, for the synthesis of Imidazo[2,1- b]-1,3,4-thiadiazole derivatives. A novel series of derivatives were synthesized comprising Imidazo[2,1-b]-1,3,4- thiadiazole containing cyclopropyl moiety by refluxing substituted 2-amino-1,3,4- thiadiazole with various substituted 2-bromo-1,2-diarylethanone in dry ethanol. The yield of the synthesized compounds was found to be in range from 70-80 %. The Phenyl derivatives were obtained in good yield as compared to substituted phenyl derivatives. All the newly synthesized compounds were characterized on the basis of their physical, spectral and analytical data.

The IR spectra, NMR spectra and Mass spectra of the representative compounds were analyzed.

7. Source of Funding

None.

8. Conflict of Interest

None.

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