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Anticancer evaluation of 2-aryl substituted benzimidazole derivatives bearing 1,3,4-oxadiazole nucleus

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ABSTRACT

*Anticancer evaluation was carried out for the derivatives of benzimidazole bearing 1, 3, 4-oxadiazole nucleus (**5a-5h**) by MTT assay method on human breast cancer cell line (MCF-7). Viable cells were counted after straining with Yellow MTT (3-(4,5-Dimethylthiazol-2-yl) 2,5-dimethyltetrazolium bromide) a tetrazole which is reduced to purple formazan in the mitochondria of living cells. Percentage viability of the cells was reported in co-relation to control. Compounds **(5e)** and **(5h)** had divulged good cytotoxic activity.*

Key words: Benzimidazole, 1, 3, 4-oxadiazole, anticancer activity, MTT assay

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INTRODUCION

In last few decades, various heterocyclic ring systems have been extensively exploited for the biological activities. Benzimidazole is one such ring system which possess wide spectrum of pharmacological activities *viz.*, antimicrobial, antiviral, antihelmintics, antihypertensive, analgesic, anti-inflammatory, anti-ulcer and antitumor activity [1, 2, 3]. It serves as a bioisoster of naturally occurring nucleotides (biomimetics of guanine), due to which the ring system can easily interact with the biopolymers of the living system [4]**.** To the present day, derivatives of benzimidazole attracted specific importance due to their potential anticancer activity [5]. The ring system has been explored particularly as topoisomerase I, PARP-1, kinase Chk2, Pgp and tyrosine kinase, serine protease inhibitors. Nocodazole, oncodazole, carbandazim, bendamustin, Hoechst-33258, Hoechst-33342, IMET-3393 are the classes of drugs carrying benzimidazole nucleus and are under clinical and pre-clinical trials [6, 7]. Further survey of literature shows the 2-substituted benzimidazoles are becoming potential area of research [8, 9].

1,3,4 oxadiazole is another heterocyclic ring which has a deep impact on multiple drug discovery programmes which includes diabetes, obesity, inflammation, cancer, and infection [10]. Precursors of mono- and di-substituted oxadiazole and their derivatives have been evaluated for cytotoxicity on macrophages and cancer cell lines. Carbomethyl derivatives of substituted 1,3,4-oxadiazole-2-thiones were found to have anticancer potential. A number of compounds containing oxadiazole moiety are in clinical trials including *Zibotentan* as an anticancer agent *Atalurenf*or the treatment of cystic fibrosis [11, 12, 13].

Inspired from the above facts, we planned to synthesize 2-substituted benzimidazole nucleus incorporated with 1, 3, 4 oxadiazole ring and to evaluate them for the anticancer potential.

MATERIALS AND METHHODS

Melting points were determined by capillary tube method and are uncorrected. All the chemicals, starting materials and reagents used were of analytical grade and used further without purification. Infrared spectra were recorded by Perkin Elmer spectrophotometer using KBr discs. Nuclear magnetic resonance spectra (1H NMR) were recorded on

Bruker Avance II 400 NMR spectrophotometer. Chemical shifts are expressed as parts per million (δ values) downfield using tetramethylsilane as internal standard. NMR data was expressed as multiplicity (s, singlet; d, doublet; triplet; m multiplet) and number of protons. All the reactions and purity of synthesized compounds was deduced by thin layer chromatography (TLC) using silica gel-G plate. The plates were developed by exposing to the iodine vapours.

General procedure

a) Synthesis of 2-substituted-1H-benzimdazole (1a, 1b, 1c) [14]

A mixture of *o*-phenylenediamne (0.1 mol, 10.8g) and substituted benzoic acid (0.1 mol) was taken and refluxed in *o*-phosphoric acid for 6 hrs. The reaction mixture was cooled and was poured onto crushed ice, after the completion of reaction. Concentrated ammonia solution was added to the cooled mixture dropwise, until got neutralized, and the resulting solid was filtered, washed with cold water, dried and recrystallized from ethanol.

b) Synthesis of ethyl-(2-substituted 1H-benzimidzol-1-yl) benzoate (2a, 2b, 2c) [15]

Anhydrous potassium carbonate (0.01mol, 1.5g) was added to a suspension of 2-substituted-1*H*-benzimidazole (0.01 mol) in dry acetone. To the reaction mixture added ethyl chloro benzoate (0.01 mol, 1.8 ml) dropwise at room temperature for 20-30 mins. The reaction mixture was stirred at room temperature for 10-12 hrs. The resultant solid was filtered off and the filtrate was concentrated under reduced pressure using rotary evaporator.

c) Synthesis of 2-(2-substituted 1*H*-benzimidazol-1-yl) benzohydrazide 3a, 3b, 3c [15]

In a round bottom flask, hydrazine hydrate (99%) (0.01 mol, 0.50 ml) was added to an ethanolic solution of Ethyl-(2-substituted-1*H*-benzimizol-1-yl) benzoate (0.01 mol) and the mixture was refluxed for 5 hrs. After completion of reaction, the reaction mixture was cooled and the product obtained was filtered and washed with cold water, dried and recrystallized from ethanol.

d) Synthesis of 2-[2-substituted-1*H*-benzimidazol-1-yl] *N*'-[substituted-phenyl methylidene]benzohydrazide (4a, 4b, 4c) [15]

Few drops of glacial acetic acid was added to the equimolar mixture of above synthesized benzohydrazide (0.0025 mol) and substituted benzaldehyde (0.0025) in ethyl alcohol (10 ml) and, and refluxed for 7 hrs. The reaction mixture was poured in ice cold water, filtered, dried and recrystallized from ethanol.

e) Synthesis of 2-substituted-1-(2-(5-substituted phenyl-1, 3, 4 oxadiazol-2yl)-phenyl)-*1H*-benzimidazole [15]

An equimolar mixture i.e. 0.0025 mol of 2-[2-substituted-1*H*-benzimidazol-1-yl] *N*- [substituted-phenylmethylidene] benzohydrazide and 0.0025 mol of chloramine T in ethanol was taken and refluxed for 5 hr. On completion reaction mixture was filtered off the sodium chloride which was separated out during the reaction. Excess of ethanol was completely removed from the reaction mixture by distillation under reduced pressure using rotary evaporator, leaving behind a solid mass which was recrystallized from ethanol.

Anticancer activity

The synthesized title compounds were screened for anticancer activity on human breast cancer cell line (MCF-7) at Animal Tissue Culture Laboratory, I.S.F College of Pharmacy, Moga. Screening of the compounds was done at different concentrations. Determination of anticancer activity was done by *MTT* assay and counting viable cells after straining with Yellow MTT (3-(4,5-Dimethylthiazol-2-yl) 2,5-dimethyltetrazolium bromide) a tetrazole which is reduced to purple formazan in the mitochondria of living cells. The absorbance of the coloured solution was measured at wavelength (500-600 nm) by *ELISA* automated plate reader.

MTT assay protocol [16- 21]

Stock solutions of synthesized compounds were prepared in DMSO with 1000 µg/ml concentration and serial dilution of 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml and 6.25 µg/ml were prepared by using DMEM media. Suspension of cells (in serum bovine medium) was seeded in the 92-well plate at 1×10^6 per ml. Plating of diluted cells was done in triplicate into wells of a microtiter plate with a cell density culture 5×10^3 cell/well in 96 well plate at 37º C in 5% CO_2 incubator for 24 hrs. For the blank absorbance readings, three control well of medium alone were provided. Under appropriate conditions cells were incubated for 6 to 48 hr. 20 µl of MTT aliquot was added to each well including control and plates were again incubated for 2 to 4 hr. The cells were examined periodically under the inverted microscope for intracellular punctate purple precipitate.

Detergent reagent (100µl) added to each well including control by swirling gently when the purple precipitate was clearly visible under microscope. The plate was left with cover in dark for 2 to 4 hr or overnight at room temperature. Absorbance for each well was recorded including the blank at 570/630 nm in a *ELISA*-microtiter plate

reader (BIORAD). The relative cell viability (%) related to control cell containing cell medium without the drug was calculated as:

[A] test/ [A] control x 100.

RESULTS AND DISCUSSION

Chemistry

o-phenylenediamine upon reaction with aromatic acids in presence of *o*-phosphoric acid gives 2-aryl benzimidazole **(1)** which reacted with ethyl chloro benzoate yielded 2-substituted-1*H*-benzimidazol-1-yl benzoate **(2)**. This on further reaction with hydrazine hydrate yielded benzohydrazide **(3)**. The benzohydrazide then reacts with different aromatic aldehydes to yield Schiff's bases **(4)**. The synthesised Schiff's bases undergone cyclization in presence of chloramine-T synthesising different compounds **(5)** shown in the general scheme (fig 1). The derivatives were obtained in appreciable yield which were purified by recrystallization from ethanol. The purity of derivatives was confirmed by single spot on TLC (silica gel) plates and spot was detected by exposing the silica gel plate to iodine vapours. The physicochemical analysis of the synthesized compounds is presented in table 1.

Spectral analysis

IR and ^{1}H-NMR spectra of selected compounds were recorded and interpreted thoroughly and are consistent with the assigned structure of the derivatives. The IR spectra of compound **1b**, **1c** appeared in the range of 3188-3057cm^{-1}(C-H strech aromatic), 1490-1600 cm^{-1} (C=C aromatic), 1685-1645cm^{-1} (C=N), 1334-111cm^{-1} (C-O) revealing completion of cyclization and preparation of 2-substituted benzimidazole pharmacophore. The IR peaks coming in the range of 1683-1653cm^{-1}(C=O), confirmed the presence of ester group substitution. Further (C-N stretch) in the range 1237-1018cm^{-1}, 1653-1597cm^{-1} (N-H bend) indicated the formation of benzohydrazide. Further derivatives (5a-5h) were prepared and we witnessed the appearance of peaks at 1685-1645cm^{-1} (C=N), 1543-1680cm^{-1}(N-H stretch),1334-1111cm^{-1} (CO),1334-1564cm^{-1}(N=O), 1018-1417cm^{-1} indicating the formation of 2-substituted-1-(2-(5-substituted phenyl-1,3,4-oxadiazol-2yl)-phenyl)-1*H*-benzimidazole.

The ^{1}H NMR spectrum of synthesised compounds revealed the appearance of chemical shift value for aromatic protons in the range of 7.69-7.24 δ(ppm) as doublet (d) confirming the presence of aromatic ring of benzimidazole in **1a**. The presence of N-H proton was observed at 9.83δ (ppm), appeared as a singlet (s) in **1a** and disappeared in further synthesised compounds. Peaks appeared in the range 3.90-3.88 δ(ppm) as singlet (s) confirmed the presence of protons for -OCH_3 groups at 2-aryl benzimidazole. Singlet (s) appeared in the range 2.30-2.12 δ(ppm) and at 3.87 δ(ppm) confirmed that -CH_3 and -OCH_3 groups substituted in 2-phenyl ring of 1,3,4-oxadiazole.

Table 1. Physicochemical characterization of the synthesised compounds

S.NO	COMP.	MOL. FORMULA	M. Wt	M. Pt	R_f	% YEILD
1.	5a	$C_{30}H_{21}N_5O_6$	519.52	341-349	0.74	60.93
2.	5b	$C_{31}H_{24}N_4O_5$	504.55	335-337	0.76	64.18
3.	5c	$C_{32}H_{27}N_5O_4$	517.59	342-350	0.82	65.93
4.	5d	$C_{29}H_{19}N_5O_5$	489.49	336-341	0.75	63.22
5.	5e	$C_{30}H_{22}N_4O_4$	474.52	319-326	0.81	64.90
6.	5f	$C_{28}H_{16}FN_5O_4$	477.76	321-329	0.72	71.80
7.	5g	$C_{29}H_{19}FN_4O_3$	462.49	309-314	0.78	68.71
8.	5h	$C_{30}H_{22}FN_5O_2$	477.52	328-336	0.84	68.59

TLC moblie phase: Hexane: Ethyl Acetate (5:5)

2-(4-methoxyphenyl)-1*H*-benzimidazole (1b)

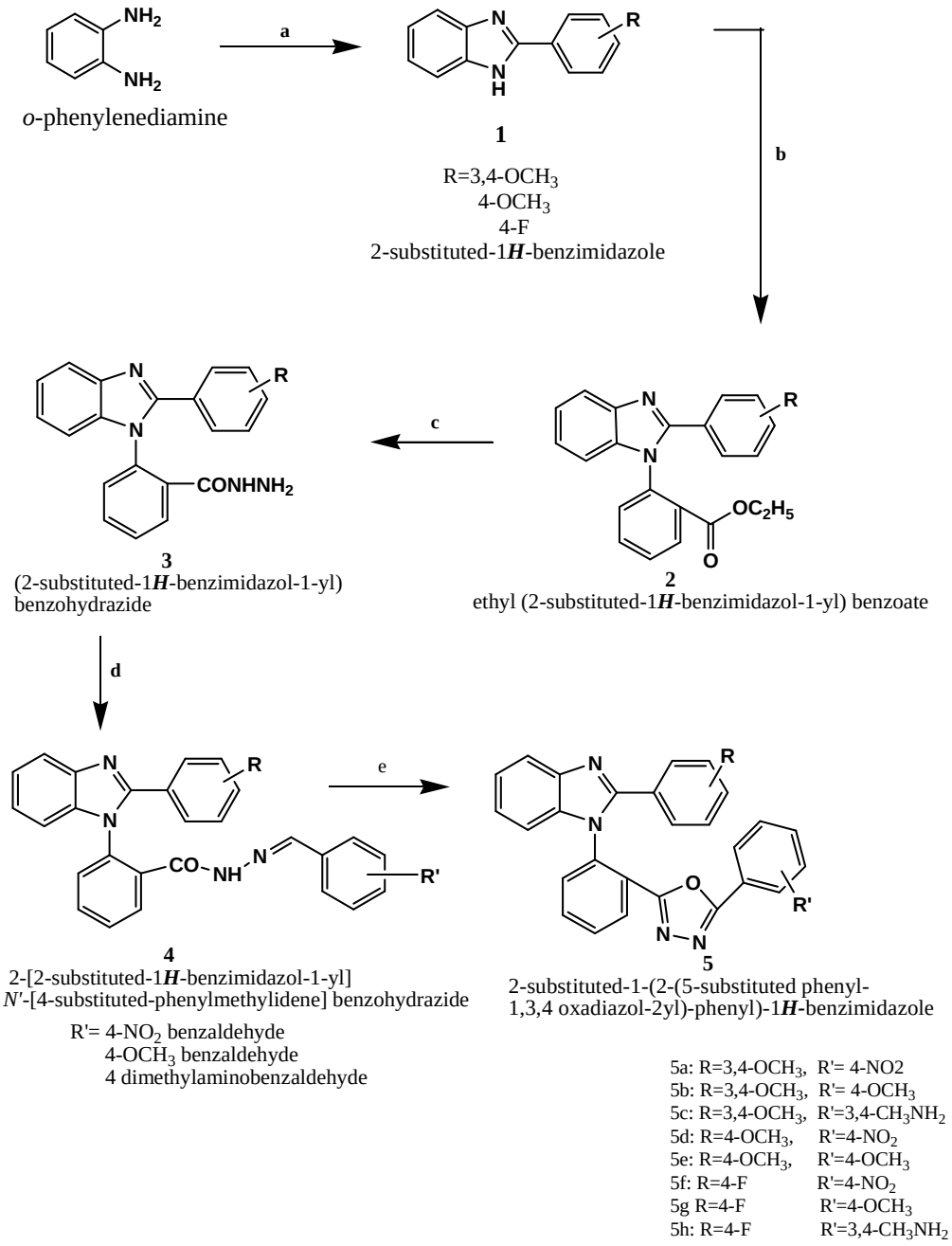
IR (KBr, cm^{-1}): 3080.32(C-H str., aromatic), 2980.02 (C-H str., aliphatic), 1685.79(C=N), 1653.00 (N-H bend), 1440.83(C=C str., aromatic), 1365.60 (C-N str.,), 1325.10 (C-O str.,). ^{1}H NMR DMSO, δ (ppm): 7.69-7.24 (4H, d, Ar-H, benzimidazole), 7.50-7.49 (2H, m, 2-aryl Ar-H), 3.88-3.90 (6H, s, OCH_3).

2-(4-flourophenyl)-1*H*-benzimidazole (1c)

IR (KBr, cm^{-1}): 3061.03(C-H str., aromatic), 1683.86(C=O), 1642.86(C=N str.,), 1597.06 (C=C str., aromatic), 1018.41(C-O).

2-[2-(3,4-dimethoxyphenyl)-1*H*-benzimidazol-1-yl]benzohydrazide (3b)

IR (KBr, cm^{-1}): 3059.10 (C-H str., aromatic), 1683.86 (C=O str.,), 1600.92(C=C aromatic), 1589.92 (N-H bend), 1321.24 (C-N), 1392.61(C-F).



***Reagents and conditions: (a)** RC_6H_5COOH, PPA refluxed for 6 hrs, **(b)** $C_6H_5COOC_2H_5Cl$, K_2CO_3, acetone stiring for 10-12 hrs, **(c)** hydrazine hydrate,C_2H_5OH refluxed for 5 hrs, **(d)** C_2H_5OH, R'CHO, glacial acetic acid refluxed for 7 hrs, **(e)** C_2H_5OH, chloramine T refluxed for 5 hrs.

Fig 1. General synthetic scheme for the synthesis of compounds 5a-5h

2-(3,4-dimethoxyphenyl)-1-{2-[5-(2-nitrophenyl-1, 3, 4-oxadiazole-2-yl]phenyl}-1*H*-benzimidazole (5a)

IR (KBr, cm^{-1}): 3057.17 (C-H str., aromatic), 2949.16 (C-H str., aliphatic), 1645.28(C=N), 1598.99 (C=C str., aromatic), 1016.49(C-O), 1490.27 (N=O) 1016.49(C-N),. ^{1}H NMR DMSO, δ (ppm): 8.37-8.22 (2H, d, 2-phenyl-1, 3, 4-oxadiazole), 7.90-7.89 (4H, m, Ar-H,benzimidazole), 7.78-7.46 (7H, m, Ar-H), 3.82 (6H, s, OCH_3).

2-(3, 4-dimethoxyphenyl)-1-{2-[5-(2-methoxyphenyl-1, 3, 4-oxadiazole-2-yl]phenyl}-1*H*-benzimidazole (5b)

IR (KBr, cm^{-1}): 3132.40(C-H str., aromatic), 2924.09 (C-H str., aliphatic), 1600.9 (C=C str., aromatic), 1645.28 (C=N), 1172.40 (C-O), 937.40(C-N),. ^{1}H NMR DMSO, δ (ppm): 7.78-7.30(4H, m, Ar-H, benzimidazole), 7.77-7.32 (6H, m, AR-H), 3.87 (2H, s, 2-phenyl-1,3,4-oxadiazole), 3.88 (6H, s, OCH_3).

(2-(aminomethyl)-5-(5-(2-(2-(3,4-dimethoxyphenyl)-1*H*-benzimidazol-1-yl)phenyl)-1,3,4-oxadiazol-2-yl) phenyl)methanamine (5c)
IR (KBr, cm^{-1}): 3062.96 (C-H str., aromatic), 2870.08 (C-H str., aliphatic), 1675.14 (C=O str.,), 1649.14 (N-H),1600.92 (C=C aromatic), 1020.24(C-N). ^{1}H NMR DMSO, δ (ppm): 7.88-7.31(4H, m, benzimidazole), 7.77-6.61 (6H, m, Ar-H), 2.30-2.12 (4H, s, 2-phenyl-1,3,4-oxadiazole), 3.90-3.88 (6H, s, OCH_3).

2-(4-dimethoxyphenyl)-1-{2-[5-(2-nitrophenyl-1,3,4-oxadiazole-2-yl]phenyl}-1*H*-benzimidazole (5d)
IR (KBr, cm^{-1}): 3188.33 (C-H str., aromatic), 2918.16 (C-H str., aliphatic), 1653.00 (C=N str.,), 1492.70 (C=C), 1334.74 (C-N), 1018.41 (C-O).

2-(4-dimethoxyphenyl)-1-{2-[5-(2-methoxyphenyl-1,3,4-oxadiazole-2-yl]phenyl}-1*H*-benzimidazole (5e)
IR (KBr, cm^{-1}): 3088.33 (C-H str., aromatic), 2958.16 (C-H str., aliphatic), 1650.00 (C=N), 1497.70 (C=C str., aromatic), 1650.00 (C=N), 1345.74 (C-N), 1018.41 (C-O).

2-(4-fluorophenyl)-1-{2-[5-(2-nitrophenyl-1,3,4-oxadiazole-2-yl]phenyl}-1*H*-benzimidazole (5f)
IR (KBr, cm^{-1}): 3030.17 (C-H str., aromatic), 2922.16 (C-H str., aliphatic), 1664.21(C=N), 1496.76 (C=C str., aromatic), 1020.34 (C-O).

2-(4-fluorophenyl)-1-{2-[5-(2-methoxyphenyl-1,3,4-oxadiazole-2-yl]phenyl}-1*H*-benzimidazole (5g)
IR (KBr, cm^{-1}): 3057.17(C-H str., aromatic, 2981.66(C-H str., aliphatic), 1674.21(C=N), 1591.27 (C=C str., aromatic), 1346.31(C-N), 1417.68(C-F), 1012.63(C-O).

(2-(aminomethyl)-5-(5-(2-(2-(4-fluorophenyl)-1H-benzo[d]imidazol-1-yl)phenyl)-1,3,4-oxadiazol-2-yl)phenyl)methanamine (5h)
IR (KBr, cm^{-1}): 3057.17(C-H str., aromatic), 2922.16 (C-H str., aliphatic), 1681.93 (C=N), 1598.99 (C=C str., aromatic), 1305.81 (C-N), 1396.46 (C-F), 1091.71 (C- O).

Table 4.4 In-vitro cytotoxicity activity of synthesised compounds against Breast cancer cell line (MCF-7)

S.No	Sample Code	% Viability				
		6.25µg/ml	12.5µg/ml	25µg/ml	50µg/ml	100µg/ml
1	1b	35.77	35.90	38.26	38.57	37.55
2	3b	36.48	35.59	37.86	38.70	37.42
3	5a	38.04	37.15	39.68	35.11	40.31
4	5b	38.26	42.70	37.90	38.84	43.24
5	5c	44.35	41.6	41.81	39.64	37.24
6	5d	42.70	39.46	40.48	37.61	37.37
7	5e	30.60	32.20	34.48	33.86	37.54
8	5f	32.57	33.09	30.88	30.75	24.87
9	5g	34.39	33.58	28.80	32.40	30.96
10	5h	32.03	35.40	31.25	33.69	34.45

Control % viability = 100

Evaluation of anticancer activity
The newly synthesised compounds **(5a-5h)** were screened for anti- proliferative activity on human breast cancer cell line (MCF-7) by MTT assay. Each compound was tested to calculate percentage viability of cell line against the said concentration which is presented in table 2.

The synthesised title compounds displayed good activity against tested cell line (MCF-7). The first intermediate **(1b)** was tested revealed the antineoplastic activity for 2-substituted aryl benzimidazole [22]. Further, substitution of benzene ring at the 1-*N* position in compound (**3b**) is not benefiting towards the cytotoxic activity as its cytotoxicity it equipotent to **(1b).** 2-(4-methoxyphenyl)-1*H*- benzimidazole derivative **(5e)** had shown good cytotoxic activity in contrast to the **(5a, 5b, 5c)** substituted with 3,4-OCH_3 at C-2 benzene ring of benzimidazole. Compounds **(5f, 5g, 5h)** had shown highest cytotoxic activity in contrast to the other derivatives which disclosed the significance of electron withdrawing group present at benzene ring of benzimidazole. Cytotoxic activity of compound **(5h)** is equipotent to that of **(5e)** that further bring out that activity is enhanced by electron donating group at the benzylidene benzene ring.

The percentage viability of the screened compounds is represented by following graphs where percentage viability is plotted against the respective concentrations for each compound. From the graphical representation of percentage viability for different compounds it can be observed that percentage viability of synthesised compounds is consistent.

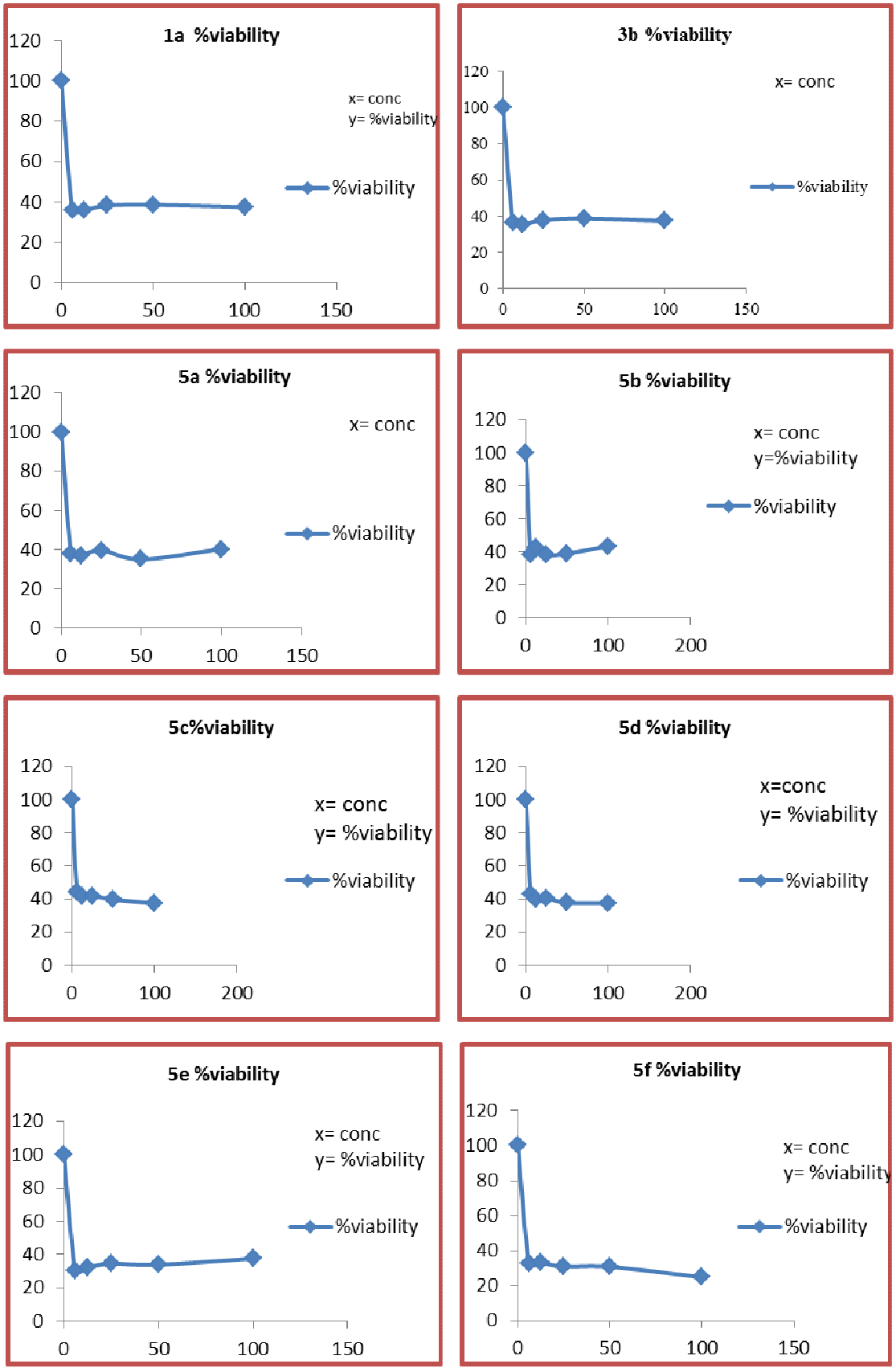


Fig 7. %viability of compounds 1a, 3b, 5a-5f

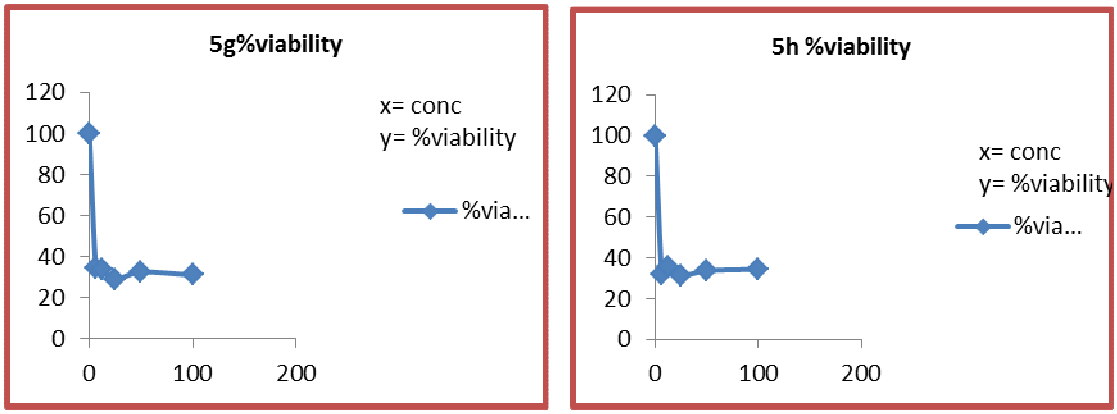


Fig 8. %viability of compounds 5g-5h

From the above discussion structure activity relationship can be deduced as follows

1. Intermediate **(1b)** had shown good cytotoxic activity, possibly due to the presence of substituted 2-aryl moiety at C-2 of benzimidazole. In contrast to **(1b)** intermediate **(3b)** had revealed equipotent cytotoxicity to that of **(1b)**, unfolding that substitution of 1-N with aromatic ring had not effected the activity.
2. Compound **(5e)** had shown excellent cytotoxic activity in comparison to its precursor **(1b)**, and which may be due to the presence of para -OCH_3 group present at benzylidene benzene ring.
3. Derivatives of 2-(4-fluorophenyl)-1*H*-benzimidazole **(5f, 5g, 5h)** had also displayed low percentage viability possibly due to the presence of electron withdrawing group present at the para position of benzene ring of C-2 of benzimidazole.

CONCLUSION

In concern to the cytotoxic activity of the synthesised compounds which were screened against MCF-7 (breast cancer cell line), compounds **5e** and **5h** flourished potent cytotoxic activity with minimum percentage viability. Intermediate **1b** also displayed good cytotoxic activity which revealed that 2-aryl substituted benzimidazole with free hydrogen at 1-N may be responsible for the cytotoxic activity.

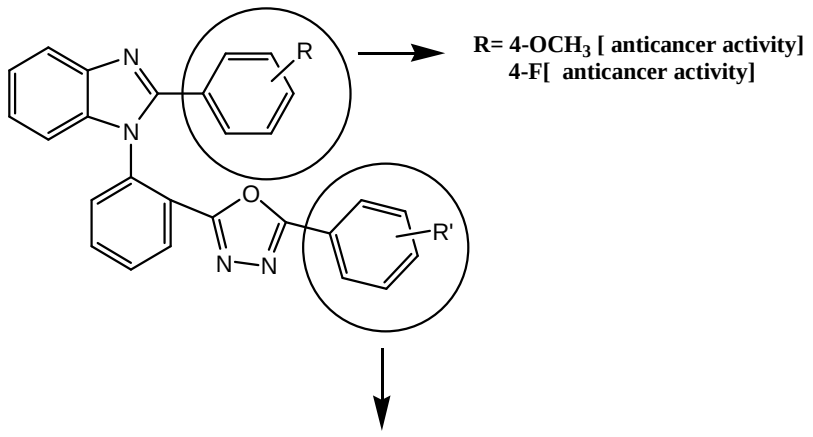


Fig 9. Various active sites of synthesised 2-aryl substituted benzimidazole compounds bearing 1,3,4-oxadiazole nucleus

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