Synthesis, molecular docking and *in vitro* cytotoxicity studies of 3,5-dimetyl arylazo pyrazole derivatives

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Abstract
A new series of 3,5-dimethyl-arylazopyrazoles (3a-h) was synthesized by reacting various oxobutyrates (2) with 4-methoxy benzhydrazide (1) in glacial acetic acid medium. The oxobutyrates were prepared by diazotization of substituted anilines and subsequent condensation with acetyl acetone in the alcohol medium.

The new compounds were elucidated on the basis of IR, ^{1}H-NMR and mass spectral data and the compounds were screened for their in vitro cytotoxic activity using MDA-MB-231 and MCF-7 cell lines. Some of the tested compounds showed weak to moderate cytotoxic effects.

Keywords: Arylazo pyrazoles, Oxobutyrates, Benzhydrazide, Cancer, Cytotoxic activity.

Introduction
The study of heterocyclic compounds is of great importance for the theoretical and practical point of view. They have been found a key structure in medicinal chemistry and also, they are frequently found in large percentage in biomolecules such as enzymes, vitamins, natural products and biologically active compounds.

Pyrazole belongs to well-known five-membered heterocyclic class of compounds and used in the synthesis of many pharmaceutical compounds and exhibit diversified biological and pharmacological activities[4]. Pyrazole ring has attracted much attention due to its presence in antipyrine, the later is one of the first drug which is composed of pyrazole moiety and it attracted many researchers. Many medicinally active compounds which are composed of pyrazole moiety are available in the market which include Kebuzone (antirheumatic), Butaglyon (antidiabetic), Dazopride (antiemetic), Muzolimine (diuretic) etc. Pyrazole derivatives are reported to possess various activities like anti-inflammatory[15], anticancer[8] analgesic[7], antimicrobial[11], antiviral[6], antitumour[10], anticonvulsant[9], antidepressant[3] etc.

Pyrazole derivatives play a very influential role among antitumor agents. Recently, two antitumor agents composed of pyrazole moiety are presently available in the market. They include Ruxolitinib -used in the management of high-risk myelofibrosis, a type of myeloproliferative disorder. Crizotinib is used in the treatment of some non-small cell lung carcinoma[9]. One of the latest pyrazole derivative attached with azo group at position-4, CAN508 was identified as novel anti-proliferative agent having selectivity for cancer cells.

Similarly, the incorporation of azo or hydrazano groups to the various heterocyclic moieties shows an increase in the biological activities[2]. The azopyrazoles also show various biological activities[16]. Nowadays, synthetic azo compounds possess diversified applications in the field of paints, foods, cosmetics, medicines and so on.

Cancer is a fatal disease that has posed serious threat to the human health. Over the years, these have been numerous advances in cancer diagnosis and treatment. Despite decades of research and clinical trials of promising new therapies, cancer remains a major cause of mortality[14]. Chemotherapy and surgery are the main therapeutic options, but chemo-preventive agents have narrow margin of safety. The emergence of drug resistance and cancer genotoxicity are the other complications of chemotherapy, by which the host normal cells may get affected severely[5].

Therefore, much efforts are needed to develop alternate therapeutic strategies, which could effectively treat the disease with no or minimum adverse effects. One of the treatment methods is chemotherapy wherein anticancer drugs are employed to treat cancer. However, due to the various side effects, limited efficacy and drug resistance, the need for new anticancer agents is burgeoning. Based on the above biological activities exhibited by azo compounds and pyrazoles, it was contemplated to synthesize a novel series of arylazopyrazole derivatives. The detailed reaction scheme leading to the formation of title compounds is discussed in the present investigation.

Material and Methods
Melting points were measured by Equiptronics digital melting point apparatus. The FT-IR spectra (cm^{-1}) were recorded with the help of Alpha Brucker IR Spectrometer. Bruker Avance-II NMR spectrometer was employed to record ^{1}H-NMR spectra operating at 400 MHz with DMSO/$CDCl_3$ as a solvent where TMS served as an internal standard.

The recording of the mass spectra was carried out by Perkin -Elmer GC-MS. The purity of the compounds was ascertained by TLC on precoated 0.2mm Merck silica gel 60 plates.

Table 1
Physical data of substituted arylazo pyrazoles (3a-h)

Comp	R-NH_2	Molecular formula	Molecular weight	MP (°C)	Yield (%)
3a	4-Cl	$C_{18}H_{17}N_4O_3ClS$	368.82	98-100	68
3b	4-F	$C_{18}H_{17}FN_4O_3S$	352.36	114-16	66
3c	4-Br	$C_{18}H_{17}BrN_4O_3S$	413.27	125-27	65
3d	4-NO_2	$C_{18}H_{17}N_5O_5S$	379.36	145-47	64
3e	2,4-$(NO_2)_2$	$C_{18}H_{16}N_6O_7S$	424.36	131-33	65
3f	2-NO_2-4-CH_3	$C_{19}H_{16}N_6O_7S$	393.39	178-80	63
3g	3,-4-$(Cl)_2$	$C_{18}H_{19}N_5O_5S$	403.26	159-61	62
3h	2-Cl-4-F	$C_{18}H_{16}N_4O_3ClFS$	386.80	140-42	61

General procedure for synthesis of 3,5-dimethyl-arylazo pyrazoles (3a-h): A mixture of 4- methoxy benzhydrazide (1) (0.01 mol) and oxobutyrates (2) (0.01 mol) was dissolved in glacial acetic acid (30ml) and the contents were refluxed for 16-24 hrs. After cooling the reaction contents, it was poured into ice cold water with stirring[12]. The solid which was formed is filtered, washed and dried. Recrystallization was carried out with ethanol. The physical data of compounds (3a-h) is given in table 1.

4-((4-chlorophenyl)diazenyl)-3,5-dimethyl-1-tosyl-1H-pyrazole (3a): IR(KBr,cm^1) 1501(C=C), 1590 (C=N), 1665(C=O), 3051 (C-H). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 2.47 (s, 2XCH_3,6H), 3.80(s, OCH_3, 3H), 7.00-7.88(m, Ar-H, 8H). MS (m/z): 368.82(M+).

4-((4-bromophenyl)diazenyl)-3,5-dimethyl-1-tosyl-1H-pyrazole (3b): IR(KBr,cm^{-1}): 1560(C=C), 1592 (C=N), 1645(C=O), 3082 (C-H). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 2.47 (s, 2XCH_3,6H), 3.81(s, OCH_3, 3H), 7.02-7.88(m, Ar-H, 8H). MS (m/z):413.26(M+).

4-((4-fluorophenyl)diazenyl)-3,5-dimethyl-1-tosyl-1Hpyrazole (3c): IR(KBr,cm^1): 1560(C=C), 1592(C=N), 1654(C=O), 3033 (C-H). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 2.51(s, 2XCH_3,6H), 3.80(s, OCH_3, 3H), 7.00-7.88(m, Ar-H, 8H). MS (m/z):352.36(M+).

3,5-dimethyl-4-((4-nitrophenyl)diazenyl)-1-tosyl-1H-pyrazole (3d): IR(KBr,cm^{-1}): 1559(C=C), 1593 (C=N), 1654(C=O), 3033 (C-H). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 2.46 (s, 2XCH_3,6H), 3.78(s, OCH_3, 3H), 7.00-8.33(m, Ar-H, 8H). MS (m/z): 379.36(M+).

4-((2,4-dinitrophenyl)diazenyl)-3,5-dimethyl-1-tosyl-1H-pyrazole (3e): IR(KBr,cm^{-1}): 1520(C=C), 1605(C=N), 1647(C=O), 300 (C-H). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 2.46(s, CH_3,3H), 3.27(s, CH_3,3H), 3.79(s, OCH_3, 3H), 7.00-8.14(m, Ar-H, 7H). MS (m/z):424.36(M+).

3,5-dimethyl-4-((4-methyl-2-nitrophenyl)diazenyl)-1-tosyl-1H-pyrazole(3f): IR(KBr,cm^1):1541(C=C), 1593(C=N), 1647(C=O), 2955(C-H). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 2.47(s, CH_3,3H), 2.50 (s, CH_3,3H), 2.63(s, CH_3,3H), 3.78(s, OCH_3, 3H), 7.00-8.23(m, Ar-H, 7H). MS (m/z): 393.39 (M+).

4-((3,4-dichlorophenyl)diazenyl)-3,5-dimethyl-1-tosyl-1H-pyrazole (3g): IR(KBr,cm^{-1}): 1490(C=C), 1593(C=N), 1681(C=O), 2961(C-H). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 2.43 (s, 2x CH_3,6H), 3.80(s, OCH_3, 3H), 7.00-7.88(m, Ar-H, 7H). MS (m/z):403.26 (M+).

4-((2-chloro-4-fluorophenyl)diazenyl)-3,5-dimethyl-1-tosyl-1H-pyrazole (3h):IR(KBr,cm^{-1}): 1499(C=C), 1576 (C=N), 1671(C=O), 2953(C-H). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 2.36 (s, CH_3,3H), 2.46 (s, CH_3,3H), 3.78(s, OCH_3, 3H), 7.52-7.83(m, Ar-H, 7H). MS (m/z): 386.80(M+).

***In silico* analysis:** Schrodinger 2018-3 suite device Maestro 11.7.012, (Ligprep, Glide XP docking, QikProp) was used for *in silico* analysis (Lipinski's RO5, molecular docking, ADMET properties). Docking of the synthesized compounds was carried out in the groove of binding site of 6B4D (the crystal structure of the complex of hcaII with a bioreductive antitumor derivative)[1].

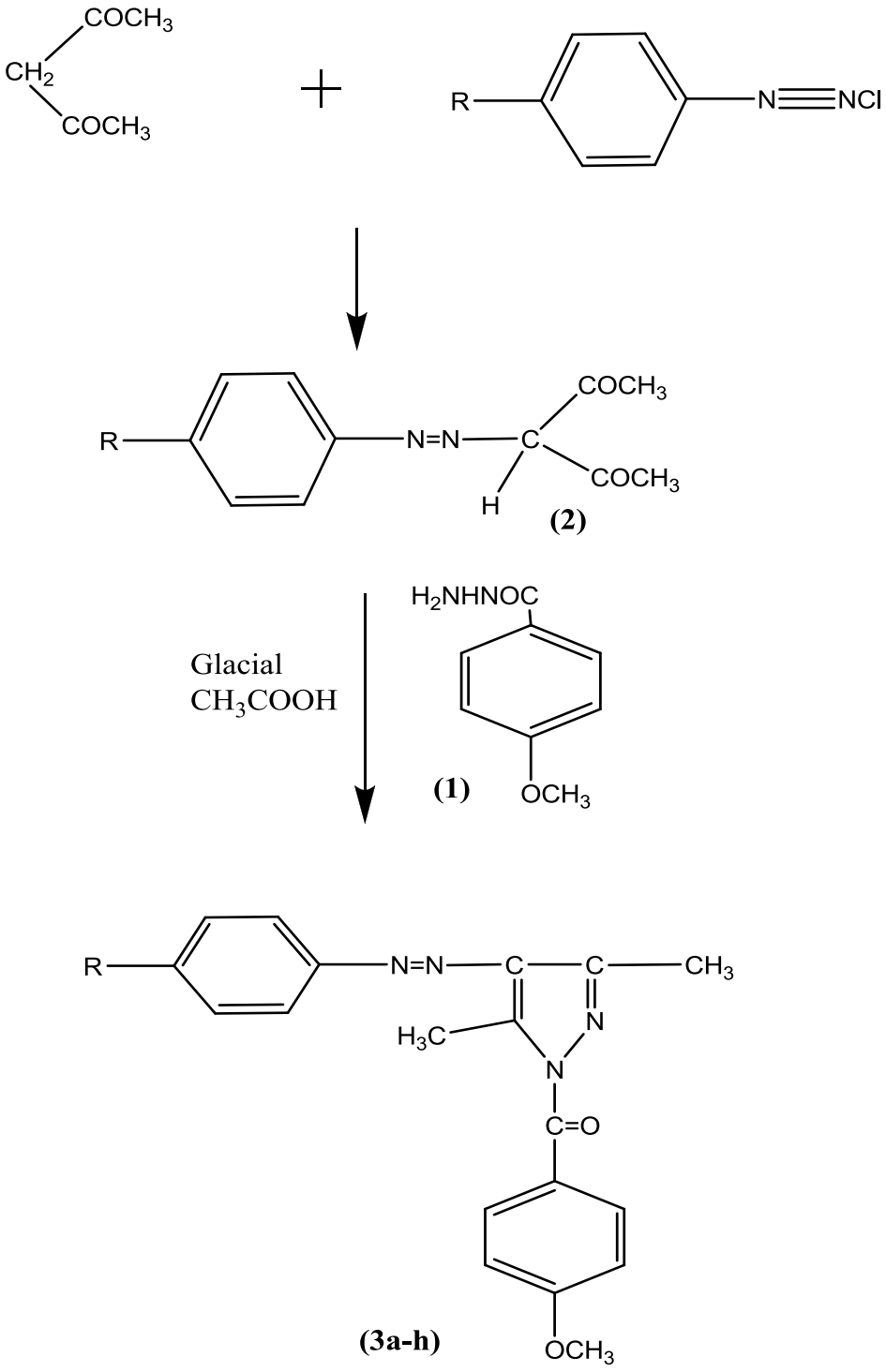
MTT Assay: The MDA-MB-231 and MCF-7 cell lines were cultured in DMEM. The medium is further supplemented with 10% fetal serum albumin (FBS) and 50 µg/mL of penicillin and streptomycin each. All the cell lines were maintained in an incubator at 37 °C containing 5% CO_2. Cells (1×10^6 / well) were plated in 1ml of medium/well in 96-well plates and grown overnight. The test compounds were treated with the cells at a concentration of 6.25, 12.5, 25, 50 and 100 µM and incubated for 48 hr. The assay was carried out using MTT cell proliferation kit. MTT reagent (100 µL) was added to each of the wells and again kept in the incubator for 4 hr. After the incubation, 0.04M HCl/ isopropanol were added. The absorbance was measured at 570 nm using UV-spectrophotometer and percentage cytotoxicity was calculated in comparison with control. All the reactions were carried out in triplicate[13]. The cytotoxicity results of the compounds are given in table 6.

Results and Discussion

Chemistry: The reported study demonstrates the synthesis of a new series of arylazo pyrazole derivatives (3a-h). The

route of synthesis followed is depicted in scheme 1. The key intermediates oxobutyrates were prepared as per the reported procedure[12]. The physicochemical features of the final synthesized compounds are shown in table 1. The purity of the compound was confirmed through melting points and TLC using silica gel G plates as stationary phase and ethyl acetate: methanol (95:5) in various proportions as mobile phase. They were further purified by recrystallization using appropriate solvents. The assignment of the new compounds was carried out on the basis of ^{1}H-NMR, FT-IR, GC-MS spectral data.

The IR spectra of the compound 3g depicted the absorption bands at 2961 for aromatic (C-H) and 1593 (C=N) respectively. The presence of carbonyl group at 1681 cm^{-1} further conforms the attachment of the pyrazole ring to the phenyl group. The mass spectrum of compound 3g showed a molecular ion peak at M/z=403.26(M+) which is in agreement with the assigned molecular formula. In the ^{1}H-NMR spectra of the compound, 3g revealed the singlet signals for CH_3, OCH_3, at δ 2.43 and 3.80 region respectively. The aromatic protons were observed as multiplets in the region δ 7.00-7.88.



Scheme 1: Synthesis of arylazo derivatives(3a-h)

Molecular Docking: In accordance to the Lipinski's RO5, the molecular weight of the molecule should be ≤500, partition coefficient ≤5, the number of hydrogen bond donors and acceptors should be ≤5 and ≤10 respectively. The rule also states that the polar surface area (PSA) ≤140 Å. All these properties along with molecular flexibility are regarded as essential determinants of oral bioavailability. Hence, an evaluation of the compounds ability to obey this rule was evaluated. Moreover, other physiological parameters such as QPPlogBB, % human oral absorption, QlogKp, QPlogKhsa and QPPCaCO-2 were predicted, studied and reported in table 4.

The compounds had the desired physicochemical characteristics (Table 2) having no deviations from the standard ranges. The number of rotatable bonds (nrobs) measuring the molecule's flexibility and a good indicator of drug absorption and bioavailability were within the acceptable limit of 10 for all the compounds.

tPSA is regarded as the capability of a molecule to undergo hydrogen bond formation. The tPSA values of all the compound were within the limit of 140 Å indicating good cell permeability and gave an insight into the hydrogen bonding potential of the compounds. In case the tPSA values were >140 Å, it would indicate that the drugs have poor absorption. The synthesized compounds obeyed Lipinski's rule of five as the molecular weight, Log P values and the number of hydrogen bond donors and acceptors, were all within the acceptable limits (Table 3). Compounds 3a-h possessed no hydrogen bond donors and hydrogen bond acceptors in the range of 6.25-8.25. The affinity of the compounds 3a-h with receptor 6B4D is given in (Table 4) in terms of dock score. The binding free energy of the compounds 3a-h ranges from -3.549 to -1.893 kcal/mol on docking with 6B4D.

Upon docking the compounds with 6B4D, the active residues were found to be PHE 348, GLY 351, ASN 352, ASP 353, VAL 354, ASP 356, TRP 357, ARG 303, GLY 304, HIE 305 and GLY 306.T LEU 57, ARG 58, ASN 67,GLU 69,PHE 70, ASP 71, ASP 72, ILE 91, GLN 92, ASP 130, PHE 131 and GLY 132. The highest affinity is demonstrated by compound 3f having a binding energy of -3.549 kcal/mol followed by compound 3a with a binding energy of -2.627 kcal/mol. The docking conformations of 3f and 3a with 6B4D are depicted in figures 1 and 2. Compound 3f participates in hydrogen bond interactions with ASN 67 and Pi-Pi stacking interaction with PHE 131 (Fig. 1). Hydrogen bond interactions are formed with ASN 67 in the case of 3a (Fig. 2).

The ADME studies are reported in table 4. The predicted Blood Barrier partition coefficient (QlogBB) gives an idea about the penetration of the drug into the CNS. Drugs with very high polarity cannot cross the BBB. The values for all the synthesized compounds fall within the recommended range of -3 to 1.2.

Table 2
Physicochemical properties of substituted arylazo pyrazoles (3a-h)

Comp	MR [cm^3/mol]	tPSA	Polarizability [Å3]	nrobs	Volume [cm^3]
3a	101.86±0.5	68.86	40.38±0.5 10^{-24}	4	318.36
3b	97.13±0.5	68.86	38.50±0.5 10^{-24}	4	309.76
3c	104.82±0.5	68.86	41.55±0.5 10^{-24}	4	322.71
3d	102.92±0.5	114.68	40.80±0.5 10^{-24}	5	328.16
3e	108.58±0.5	160.51	43.04±0.5 10^{-24}	6	351.50
3f	107.35±0.5	114.68	42.55±0.5 10^{-24}	5	344.72
3g	106.46±0.5	68.86	42.20±0.5 10^{-24}	4	331.90
3h	101.73±0.5	68.86	40.33±0.5 10^{-24}	4	323.29

Table 3
Lipinski's RO5 and dock scores of arylazo pyrazoles (3a-h)

Comp	Molecular weight	Log P	Donor HB	Acceptor HB	Dock score
3a	368.82	4.139	0.00	6.250	**-2.627***
3b	352.37	3.872	0.00	6.250	-2.354
3c	413.27	4.217	0.00	6.250	-2.022
3d	379.38	2.857	0.00	7.250	-2.623
3e	424.37	2.185	0.00	8.250	-2.009
3f	393.40	3.251	0.00	7.250	**-3.549****
3g	403.27	4.553	0.00	6.250	-2.479
3h	386.81	4.340	0.00	6.250	-1.893

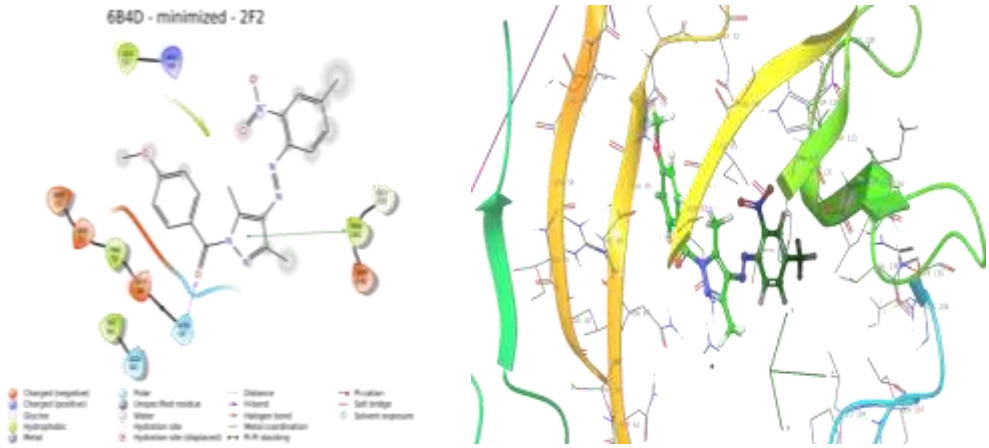


Fig. 1: 2D and 3D Interaction of compound 3f with 6B4D

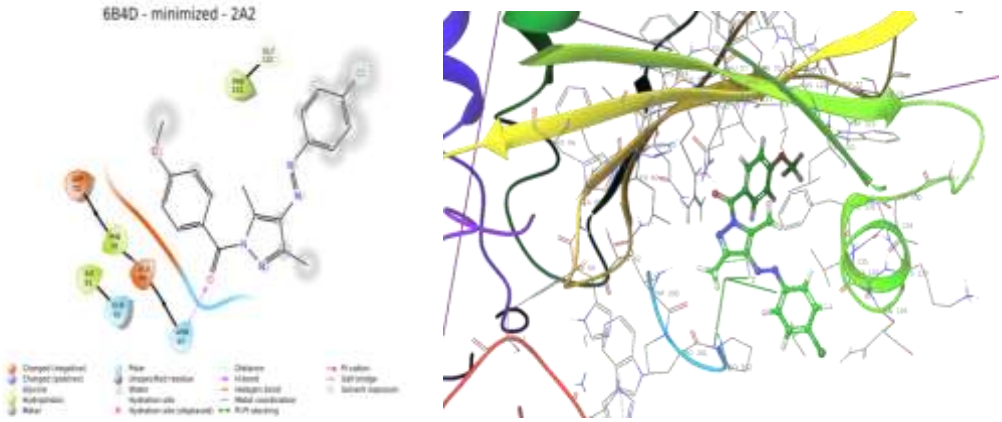


Fig. 2: 2D and 3D Interaction of compound 3a with 6B4D

Table 4
ADME properties of substituted arylazo pyrazoles (3a-h)

Compound	QlogBBB	QlogKp	QPPCaco-2	QPlogKhsa	Percent human oral absorption
3a	-0.068	-1.029	3075.1	0.168	100.00
3b	-0.122	-0.997	3069.9	0.086	100.00
3c	-0.058	-1.033	3068.7	0.193	100.00
3d	-1.131	-2.773	372.926	-0.068	89.702
3e	-2.226	-4.238	68.297	-0.202	59.614
3f	-1.096	-2.538	579.403	0.065	95.434
3g	0.082	-1.173	3107.7	0.275	100.00
3h	0.022	-1.139	3071.5	0.209	100.00

QPlogKp score is an indication of skin permeability. All the compounds have good skin permeability as the value of QPlogKp is within the recommended range of -8 to -10. Caco-2 cells are a great model to depict gut-blood barrier and non-active transport. QPPCaco-2 predicts the apparent Caco-2 cell permeability in nm/sec. A value above 500 indicates great intestinal permeability whereas a value below 25 indicates very poor permeability. The predicted values of QPPCaco-2 indicated that the synthesized compounds have good intestinal permeability.

QPlogKhsa score is used to predict the binding of the compounds to the human serum albumin. A higher value indicates that the compound is strongly bound to albumin and hence is not available for activity. Compounds 3a-h fall within the recommended range indicating that the binding to the human serum albumin is not strong and hence the compounds are free for activity. Except compound 3e, all the compounds showed very good % human oral absorption property.

***In vitro* Cytotoxic activity:** All the newly synthesized compounds (3a-h) were evaluated for the short-term *in vitro* cytotoxicity using MDA-MB-231 and MCF-7 cells by employing MTT assay. As per the IC_{50} data (Table 5), the compounds 3b, 3g and 3h have shown moderate effect on both MDA-MB231 and MCF-7 cancer cell lines.

Table 5
Cytotoxicity IC_{50} (µM) of 3a-h

Comp	MDA-MB-231	MCF-7
3a	33.22 ± 1.22	44.18± 1.02
3b	**23.75 ± 0.44**	33.11± 2.33
3c	45.12± 1.26	37.26± 0.28
3d	45.19± 1.38	54.11± 0.85
3e	52.14± 1.66	32.64± 1.26
3f	64.21± 2.05	28.68± 1.74
3g	32.15± 2.36	**22.14± 0.64**
3h	**24.15± 1.08**	**25.28± 0.88**

Considering the IC_{50} values for 3a-h, we tried to link a correlation between the cytotoxicity and molecular structure by looking at the position and nature of the functional groups on the pyrazole-hydrazide derivatives. The compound (3h) showed moderate activity against both the cell lines. The presence of 3,4-dichloro (3g), chloro/fluoro groups (3h) attached to the pyrazole moiety exhibited highest antiproliferative activity. On the other hand, the presence of fluoro groups (3b) decreased the antiproliferative efficiency. All the other tested compounds showed weak activity against both the cell lines.

Conclusion

In conclusion, a new series of arylazo pyrazole derivatives was synthesized by reacting oxobutyrates and 4- methoxy benzhydrazide in glacial acetic acid medium and the new compounds were established by spectral data. All the compounds were subjected to *in silico* analysis and the compounds exhibited good pharmacokinetic properties.

The new compounds were evaluated for *in vitro* cytotoxicity effect and some of the tested compounds displayed weak to moderate cytotoxicity effect against MDA-MB-231 and MCF-7 cell lines. The presence of electron withdrawing groups was found beneficial in exhibiting the moderate activity.

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