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Design, synthesis, *in silico* and biological evaluation of biotin-pyrazole derivatives as cytotoxic agent

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Abstract: Modified biotin derivatives are effective in killing cancer. Herein we describe the design and synthesis of conjugated biotin-pyrazole derivatives **3a-3h** to evaluate as potential anticancer agents. A set of compounds has been prepared by palladium catalysed aminocarbonylation using carbon monoxide in an autoclave. They were well characterized by various spectroscopic techniques and screened for anticancer activity. A cell viability assay (MTT assay) was performed on U251, A549, and HepG2 cell lines and determined their IC_{50} value. Among target agents, **3a** had a considerable activity against human brain cancer cell line U251 (IC_{50} 3.5 µM). **3a** could be a promising candidate for the development of new drugs to treat tumours, particularly for brain cancer.

Keywords: Anti-proliferative; aminocarbonylation; biotin; cancer; pyrazole; Swissadme.

1. Introduction

Cancer is a leading cause of death in the world wide and nearly 10 million deaths in 2021.[1] In some scenarios, it is stated that cancer cases increase due to various factors such as genetic influences, acquired unhealthy habits and environmental pollutants.[2] Chemotherapy is the first-line treatment for cancer, despite the fact that the drugs currently used in therapy have poor selectivity and high toxicity. Drugs used in cancer therapy have different mechanisms of action, such as antiproliferative activity, toxicity to specific cancer cells, or the ability to modify the cell cycle at specific stages.[3,4] In cancer

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research, the selectivity of anticancer drugs is challenge as a milestone. For this purpose, different approaches are applied like polymeric drug carriers with specific targeting agent like magnetic particles, vitamins, antibodies, hormones or peptides[5,6] besides drug targeting in which the target molecule is directly linked with drug.[7,8]

Biotin is a participant of the B complex group of vitamins and is an important nutrient for all mammals. Biotin is found during nutritional research that disclosed a factor in many foods.[9] Cancer cells require a huge amount of vitamins to keep growing at such a fast rate. However, there is a strong correlation between vitamins and the stage of tumour growth.[10] Some essential vitamins, such as biotin, B12, riboflavin, and folic acid, are chosen to target tumour cells.[11] Among all these vitamins, biotin appears to be as good potential targeting agent. Biotin derivatives were also found to be more effective at killing cancer cells, making biotin a highly potential vector.[12]

Biotin-conjugated drug targeting of anti-tumor drugs appears to be an interesting approach to improving effectiveness and potency and overcome resistance problem in cytotoxicity of anti-cancer therapy. Biotin mediated drugs means a biotin linked to a) drug, prodrugs or its derivative used in cancer therapy b) antitumor effective protein c) anticancer drug candidates. It is important to note that the use of biotin-anticancer drug conjugates in drug development is still in its early stages, and translation into clinical research is being done gradually. some chemical structure of biotin conjugated drug mentioned in (Figure 1).[13]

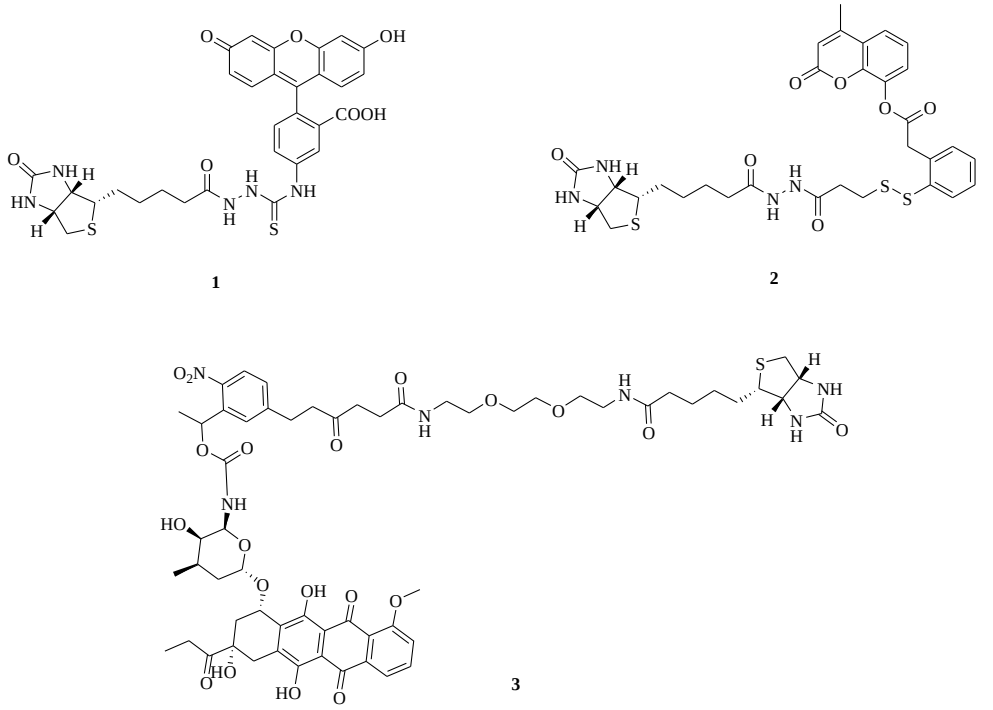


Figure 1. 1) Structure of biotin-fluorescein conjugate, **2)** biotin-coumarin conjugate, **3)** Chemical structure of biotin-doxorubicin conjugates

Morpholine is one of the most important intermediate in organic synthesis and it forms drugs are of high therapeutic value specifically in cancer. Oxygen atom in the morpholine ring rises the binding affinity by contributing in donor-acceptor type interactions with the corresponding receptor.[14] Many pyrazole derivative displayed wide range of biological activities like anti-cancer, anti-microbial, antiviral, antifungal, anti-inflammatory, analgesic, insecticidal etc.[15-18] In this paper we have selected pyrazole motif as anti-cancer drug derivative. Pyrazole play an important role in treatment of cancer[19]. Every year USFDA approved around three to four drugs contained pyrazole core.[20] Left hand side we have selected biotin core and right hand side choose pyrazole with substituted morpholine core to build new class of pyrazole biotin anti-cancer conjugated derivatives use for cytotoxic study by using MTT assay.

2. Experimental

2.1. Material and Methods

Commercial grade solvents and reagents were purchased from Sigma-Aldrich or Alfa Aesar or Spectrochem Mumbai and used as received. Reactions were monitored by thin layer chromatography on silica gel plates in ultraviolet light as well as in iodine stain. Separations by flash chromatography were performed on silica gel (40-60 µm, 230-400 mesh size). Melting points were measured using a melting point apparatus and are uncorrected. Infrared spectra were recorded on FTIR optics Bruker by KBR palate method. ^{1}H and ^{13}C NMR spectra were recorded on advance Bruker (400 MHz) NMR spectrometer in suitable deuterated solvents. ^{1}H NMR data were recorded as follows: chemical shift measured in parts per million (ppm) downfield from tetramethylsilyl (d), multiplicity, observed coupling constant (*J*) in hertz (Hz), and proton count. Multiplicities are reported as singlet (s), broad singlet (br s), doublet (d), triplet (t), quartet (q), and multiplet (m). ^{13}C NMR chemical shifts are reported in ppm downfield from tetramethylsilyl, and identifiable carbons are given. Mass spectra were determined by electrospray ionization (ESI)/mass spectrometry (MS), using a Shimadzu LCMS and elemental analyses were determined by the microanalysis.

2.2. Synthetic Procedure of Proposed Compounds

*(3aS,4S,6aR)-4-(5-(4-bromo-1H-pyrazol-1-yl)-5-oxopentyl)tetrahydro-1H-thieno[3,4-d]imidazol-2(3H)-one (**2**)*: In a round bottom flask, a mixture of 4-bromo-1*H*-pyrazole (2.15 g, 14.65 mmol) and N-hydroxy succinamide biotin (5.0 g, 14.65 mmol) was dissolved in dimethylformamide (50 mL). diisopropylethylamine (5.66 mL, 29.29 mmol) was added to the reaction and the resulting solution was stirred at rt for 4 h. After completion of the reaction, the reaction mixture was concentrated in vacuo. The crude residue was purified by silica gel chromatography, eluting with 1:9 methanol: ethyl acetate to yield compound **2** (4.5g, 82.31%). Off White Solid, melting point: 167-173^{0}C, ^{1}H NMR (400MHz, DMSO d6) δ 7.78 (s, 2H), 6.47 (s, 1H), 6.39(s, 1H), 4.31 (s, 1H), 4.14 (s, 1H), 3.11 (s, 1H), 2.84(t, *J* = 5.2 Hz, 1H), 2.60 - 2.57 (m, 2H), 2.17 (t, *J* = 6.0 Hz, 1H), 1.61-1.51 (m, 4H), 1.43 - 1.35(m, 2H). ^{13}C NMR (400MHz, DMSO) δ 174.91, 163.20, 145.21, 132.18, 92.19, 61.64, 59.66, 55.87, 40.41, 33.96, 28.58, 25.01. ESI-MS: *m/z* calcd for $C_{13}H_{17}BrN_4O_2S$ $[M + H]^+$ 374.02. calculation for Elemental Analysis: C, 41.83; H, 4.59; N, 15.01 Found: C, 42.02; H, 4.46; N, 14.93.

*General Procedure for Synthesis of Amides **3a-3h*** : A 100 mL autoclave charged with Aryl bromide (1 eq, 0.267 mmol), amine (3 eq, 0.803 mmol), Palladium acetate (0.1 eq, 0.0267 mmol), [1,1′-Bis(diphenylphosphino) ferrocene] dichloropalladium(II) (Sigma-697230) (0.1 eq, 0.0267mmol), triethylamine (1.5 eq, 0.401mmol) in acetonitrile (10 Vol) at room temperature. The reaction mixture flushed with N_2 gas and then again flushed with CO gas at 200 Psi. The reaction mixture was stirred at 110°C for 30min to 1h. After completion of reaction, the reaction mixture was cool, filtered through celiet bed, the solvent was removed under reduced pressure. The obtained crude compound was purified by using Prep HPLC purification (0.1% formic acid in water/acetonitrile) afford pure final compound. All the remaining reactions were performed following this general procedure. The spectral data of the synthesized compounds are provided below.

*(3aS,4S,6aR)-4-(5-(4-(3-methylmorpholine-4-carbonyl)-1H-pyrazol-1-yl)-5-oxopentyl) tetra hydro-1H-thieno[3,4-d]imidazol-2(3H)-one (**3a**)*: (70 mg, 70%). White Solid, melting point: 187-192^{0}C, ^{1}H NMR (400MHz, DMSO d6) δ 7.77 (s, 2H), 6.44 (s, 1H), 6.36 (s, 1H), 4.30 (s, 1H), 4.13 (s, 1H), 3.64 - 3.58 (m, 1H), 3. 28 (br s, 1H), 3.09 (s, 1H), 2.90 (t, *J* = 5.2 Hz, 1H), 2. 81 (t, J= 8.4 Hz, 1H), 2. 71 (s, 2H), 2.59 - 2.56 (m, 2H), 2. 33 (s, 3H), 1.60 - 1.33 (m, 9H). ^{13}C NMR (400MHz, DMSO) δ 170.01, 168.62, 164.77, 135.01, 134.55,111.41, 70.40, 66.51, 63.74, 62.44, 55.63, 45.28, 41.15, 40.56, 29.66, 28.35, 25.33, 24.49, 19.14. ESI-MS: *m/z* for $C_{19}H_{27}N_5O_4S$ $[M + H]^+$ 422.52. calculation for Elemental Analysis: C, 54.14, H, 6.46; N, 16.62 Found: C, 54.04; H, 6.38; N, 15.03.

*(3aS,4S,6aR)-4-(5-oxo-5-(4-(thiomorpholine-4-carbonyl)-1H-pyrazol-1-yl) pentyl) tetra hydro-1H-thieno[3,4-d]imidazol-2(3H)-one (**3b**)*: (58 mg, 51%). White Solid, melting point: 187-192^{0}C, ^{1}H NMR (400MHz, DMSO d6) δ 7.77 (s, 2H), 6.45 (s, 1H), 6.37 (s, 1H), 4.31 (s, 1H), 4.12 (s, 1H), 3.10 (s, 1H), 2.94 (s, 3H), 2.84 - 2.80 (m, 1H), 2.59 - 2.56 (m, 2H), 2. 20 - 2.17 (m, 3H), 1.60 - 1.33 (m, 9H). ^{13}C NMR (400MHz, DMSO) δ 170.02, 168.96, 164.75, 135.04, 134.58, 111.45, 63.74, 62.44, 55.65, 48.15, 41.11, 29.66, 28.36, 26.46, 25.36, 24.43. ESI-MS: *m/z* for $C_{18}H_{25}N_5O_3S_2$ [M + H]$^+$ 424.55, calculation for Elemental Analysis: C, 51.04, H, 5.95; N, 16.54 Found: C, 50.92; H, 6.02; N, 16.20.

*(3aS,4S,6aR)-4-(5-(4-(1,1-dioxidothiomorpholine-4-carbonyl)-1H-pyrazol-1-yl)-5-oxo pentyl)tetrahydro-1H-thieno[3,4-d]imidazol-2(3H)-one (**3c**)*: (85 mg, 70%). Off White Solid, melting point: 156-160^{0}C, ^{1}H NMR (400MHz, DMSO) δ 7.75 (s, 2H), 6.45 (s, 1H), 6.36 (s, 1H), 4.30 - 4.27 (m, 1H), 4.13 - 4.10 (m, 1H), 3.11 (s, 1H), 3. 05 (s, 4H), 2.95 (s, 4H), 2.79 - 2.76 (m, 1H), 2.59 - 2.56 (m, 1H), 2. 19 (t, J=7.2 Hz, 2H), 1.61 - 1.34 (m, 6H). ^{13}C NMR (400MHz, DMSO) δ 170.01, 168.96, 164.72, 135.01, 134.56, 111.41, 63.74, 62.44, 55.63, 53.35, 43.56, 41.15, 40.56, 29.65, 28.35, 25.33, 24.49. ESI-MS: *m/z* for $C_{18}H_{25}N_5O_5S_2$ [M + H]$^+$ 455.56, calculation for Elemental Analysis: C, 47.46, H, 5.53; N, 15.37 Found: C, 47.54; H, 5.64; N, 15.51.

*(3aS,4S,6aR)-4-(5-oxo-5-(4-(piperidine-1-carbonyl)-1H-pyrazol-1-yl)pentyl)tetrahydro-1H-thieno[3,4-d]imidazol-2(3H)-one (**3d**)*: (67 mg, 62%). White Solid, melting point: 151-153^{0}C, ^{1}H NMR (400MHz, DMSO d6) δ 7.76 (s, 2H), 6.50 (s, 1H), 6.36 (s, 1H), 4.30 (s, 1H), 4.13 (s, 1H), 3.09 (s, 1H), 2.84 - 2.74 (m, 6H), 2.59 - 2.50 (m, 2H), 2. 03 (t, J=6.8 Hz, 2H), 1.63 - 1.32 (m, 9H). ^{13}C NMR (400MHz, DMSO) δ 172.56, 170.00, 164.76, 135.01, 134.55, 111.41, 63.74, 62.44, 55.63, 49.96, 41.11, 29.65, 28.35, 25.43, 25.30, 24.43, 24.29. ESI-MS: *m/z* for $C_{19}H_{27}N_5O_3S$ [M + H]$^+$ 406.52, calculation for Elemental Analysis: C, 56.28, H, 6.71; N, 17.27 Found: C, 56.07; H, 6.56; N, 17.12.

*(3aS,4S,6aR)-4-(5-(4-(4-methyl-1-oxa-4,9-diazaspiro[5.5]undecane-9-carbonyl)-1H-pyrazol-1-yl)-5-oxopentyl)tetrahydro-1H-thieno[3,4-d]imidazol-2(3H)-one (**3e**)*: (55 mg, 42%). Off White Solid, melting point: 190-193^{0}C, ^{1}H NMR (400MHz, DMSO d6) δ 7.77 (s, 2H), 6.45 (s, 1H), 6.38 (s, 1H), 4.32 (s, 1H), 4.13 (s, 1H), 3. 87 (s, 1H), 3.10 (s, 3H), 2.90 – 2.87 (m, 1H), 2. 84 - 2.80 (m, 2H), 2. 73 (s, 2H), 2.59 - 2.56 (m, 2H), 2.20 (t, J=7.2 Hz, 2H), 2.18 (s, 3H), 1. 8 (br s, 1H), 1.61 - 1.57 (m, 2H), 1. 53 - 1.41 (m, 4H), 1. 33 - 1.28 (m, 4H). ^{13}C NMR (400MHz, DMSO) δ 172.56, 170.00, 164.76, 135.01, 134.55, 111.41, 75.76, 64.06, 63.74, 62.44, 61.45, 57.74, 55.63, 47.56, 41.11, 32.62, 29.65, 28.35, 25.30, 24.43. ESI-MS: *m/z* for $C_{23}H_{34}N_6O_4S$ [M + H]$^+$ 491.72. calculation for Elemental Analysis: C, 56.31, H, 6.99; N, 17.13 Found: C, 56.12; H, 7.08; N, 16.95.

*(3aS,4S,6aR)-4-(5-oxo-5-(4-(2-phenylmorpholine-4-carbonyl)-1H-pyrazol-1-yl)pentyl) tetra hydro-1H-thieno[3,4-d]imidazol-2(3H)-one (**3f**)*: (75 mg, 58%). Off White Solid, melting point: 186-189^{0}C. ^{1}H NMR (400MHz, DMSO d6) δ 7.77 (s, 2H), 7. 32 -7.26 (m, 5H), 6.43 (s, 1H), 6.37 (s, 1H), 4.38 (d, J=8.0 Hz, 1H), 4.30 (s, 1H), 4.13 (s, 1H), 3. 89 (s, 1H), 3.63 – 3.57 (m, 1H), 3.10 (s, 2H), 2.94 – 2.81 (m, 1H), 2.84 - 2.81 (m, 2H), 2. 76 (s, 2H), 2.59 - 2.56 (m, 2H), 2.21 (t, J=7.4 Hz, 2H), 1.62 - 1.57 (m, 2H), 1. 53 - 1.49 (m, 4H), 1. 33 - 1.29 (m, 4H). ^{13}C NMR (400MHz, DMSO) δ 170.01, 168.96, 164.72, 142.13, 135.01, 134.55, 128.63, 127.89, 127.41, 111.41, 81.22, 63.70, 63.66, 62.44, 59.41, 55.62, 46.02, 41.15, 29.65, 28.35, 25.33, 24.49. ESI-MS: *m/z* for $C_{24}H_{29}N_5O_4S$ [M + H]$^+$ 484.61. calculation for Elemental Analysis: C, 59.61, H, 6.04; N, 14.48 Found: C, 59.51; H, 5.93; N, 14.63.

*(3aS,4S,6aR)-4-(5-(4-(1,4-dioxa-8-azaspiro[4.5]decane-8-carbonyl)-1H-pyrazol-1-yl)-5-oxopentyl)tetrahydro-1H-thieno[3,4-d]imidazol-2(3H)-one (**3g**)*: (79 mg, 64%). Cream Solid, melting point: 161-163^{0}C. ^{1}H NMR (400MHz, DMSO d6) δ 7.75 (s, 2H), 6.47 (s, 1H), 6.37 (s, 1H), 4.27 (s, 1H), 4.12 (s, 1H), 3.86 (s, 2H), 3. 10 - 3.05 (m, 1H), 2.88 - 2.78 (m, 2H), 2.57 - 2.54 (m, 2H), 2.13 (t, J=7.8 Hz, 2H), 1.65 - 1.55 (m, 4H), 1.52 - 1.41 (m, 4H), 1.39 - 1.30 (m, 2H). ^{13}C NMR (400MHz, DMSO) δ 172.56, 170.00, 164.76, 135.01, 134.56, 116.82, 111.41, 64.35,63.74, 62.44, 55.63, 45.46, 41.11, 34.21, 29.65, 28.35, 25.30, 24.43. ESI-MS: *m/z* for $C_{21}H_{29}N_5O_5S$ [M + H]$^+$ 464.58. calculation for Elemental Analysis: C, 54.41, H, 6.31; N, 15.11 Found: C, 54.56; H, 6.43; N, 14.90.

(*3aS,4S,6aR)-4-(5-oxo-5-(4-(4-phenoxypiperidine-1-carbonyl)-1H-pyrazol-1-yl)pentyl)tetrahydro-1H-thieno[3,4-d]imidazol-2(3H)-one (**3h**)*: (47 mg, 35%). Off White Solid, melting point: 148-152^{0}C. ^{1}H NMR (400MHz, DMSO d6) δ 7.77 (s, 2H), 7. 28 -7.25 (m, 2H), 6.95 – 6.91 (m, 3H), 6.44 (s, 1H), 6.36 (s, 1H), 4.44 (s, 1H), 4.30 (s, 1H), 4.13 (s, 1H), 3.09 (s, 2H), 3.00 – 2.98 (m, 1H), 2.83 - 2.80 (m, 2H), 2.69 – 2.64 (s, 2H), 2.59 - 2.56 (m, 2H), 2.15 (t, J=7.4 Hz, 2H), 1.95 -1.92 (m, 2H), 1.61 - 1.58 (m, 2H), 1.51 - 1.49 (m, 4H), 1.33 - 1.29 (m, 2H). ^{13}C NMR (400MHz, DMSO) δ172.56, 170.01, 164.72, 157.42, 135.52,135.03, 129.36, 120.30, 114.42, 111.41, 76.85, 63.72, 62.42, 55.63, 41.10, 41.05, 30.71, 29.62, 28.31, 25.31, 24.42. ESI-MS: *m/z* for $C_{25}H_{31}N_5O_4S$ $[M + H]^+$ 498.61, calculation for Elemental Analysis: C, 60.34, H, 6.28; N, 14.07 Found: C, 60.54; H, 6.45; N, 14.12.

2.3.MTT Assay[21, 22]

The anti-cancer study of synthesized pyrazole-biotin hybride derivatives against the human brain cancer cell line (U251), lung cancer cell line (A549) and liver cancer cell line (HepG2) were selected for MTT assay.

All cell lines U251, A549, HepG2 and MB157 were purchased from ATCC, USA. Cell lines were cultured in respective medium are in accordance with the requirement of ATCC in humidified 5% CO_2 incubator at 37 °C. Standard drug Doxorubicin was purchased from TCI, India. MTT assay kit used for cell viability assay was purchased from Sigma India. All tissue culture treated, sterile 96 well plate, flask and pipette were purchased from Corning, India. Multichannel and single channel pipettes were purchased from Eppendorf, India.

The one-day old culture of cancer cells was seeded in 96 well plates (~2×10^4 cells per well) and incubated at 37 °C with 5% CO_2 pressure for 24 h. After incubation, the positive control cells were treated with pyrazole-biotin hybride in a culture medium at subsequent doses with various concentrations of DMSO. After incubation for 72 h, MTT solution was added to the wells and plates were incubated at 37 °C for 4 h. Later, the culture medium was discarded and formazan crystal was observed in the treated wells and followed by a dilute sample using 150 µL DMSO. The formation of color intensity wells was measured using a spectrophotometer (Shimadzu) at 540 nm. The cell viability assay was performed in triplicate. Based on triplicate values, the cell inhibition ratio was calculated by using the following formula,

Inhibition ratio = (1-A540compound/A540control)

EC_{50} (50% reduction in cell viability) values were determined using Graph Pad Prism software. Herein Doxorubicin regarded as positive control. The resulted EC_{50} value of the pyrazole-biotin hybride against HepG2, U251 and A549 cells was noted for further *in vitro* experiment.

*2.4. Cytotoxicity of **3a-3h** Against Normal Cell Line*

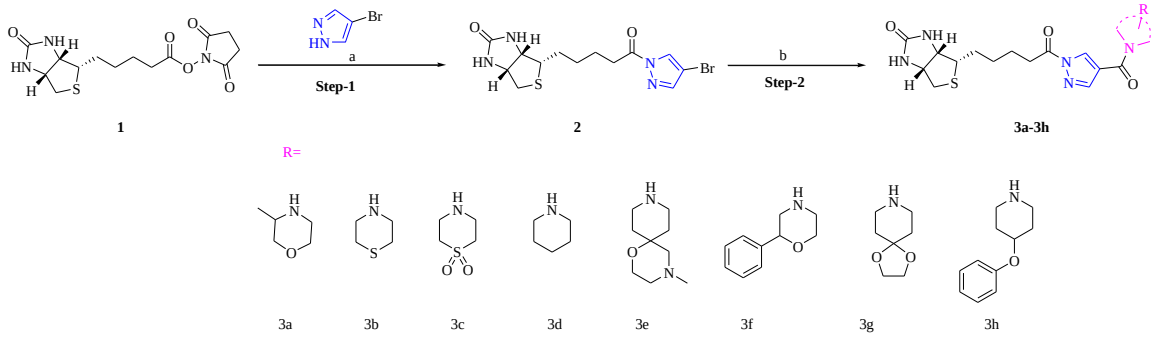
The toxicity of synthesized pyrazole-biotin hybrides was further checked on human normal breast cell line (MB157). The effect of all compound was studied in different doses on human breast cells (MB157) for 72 h and cell viability was measured by MTT assay. The obtained result mentioned in Table-1. All synthesized compounds are less cytotoxic against the normal cell line (MB157), thus they can be used for treatment of cancer cell (U251, HepG2 and A549). (Table 1).

3. Result and Discussion

3.1. Chemistry

The synthetic route of the target compounds **3a** to **3h** described is in Scheme 1. n-hydroxysuccinimide ester is easily reacted with amine nucleophile in presence of base for amide bond formation. (3a*S*,4*S*,6a*R*)-4-(5-(4-bromo-1*H*-pyrazol-1-yl)-5-oxopentyl)tetrahydro-1*H*-thieno[3,4-*d*]imidazol-2(3*H*)-one **(2)** was prepared by using biotin *N*-hydroxysuccinimide ester (1) with 4-bromo-1H-pyrazole in presence of diisopropyl amine in tetrahydrofuran with good yield (74%). Intermediate 2 was treated with different cyclic amine to afford target compounds **3a** to **3h** in the presence of CO(g) and palladium catalyst.[23, 24] The target compounds were obtained in 35-70% yields. All the structures of

newly synthesized compounds were elucidated on the basis of their elemental analysis and spectroscopic data.



Scheme 1. Reagents and condition: (a) DIPEA, THF, rt, 2h (b) CO(g), $Pd(OAc)_2$, $PdCl_2(dppf)$, MeCN, 200 psi, 60°C, 2-5 h

3.2. In Vitro Anti-proliferative Activity

The newly synthesized biotin pyrazole conjugated compounds were screened for their anti-cancer activity against human brain cancer cell line (U251), human lungs cancer cell line (A549), and human liver cancer cell line (HepG2) by using MTT assay. Doxorubicin was being used as a reference. The results were summarised in (Table 1). Synthesized compounds which showed a minimum of ~40% inhibition at higher concentration (10 µg/mL) were selected for dose-response studies (30 µM - 50 µM). The IC_{50} value of compound **3a** bearing 3-methylmorpholine showed prominent cytotoxic activity in U251 cell line and **3a** showed nearly equivalent potency as the positive control. Furthermore, the other compound having piperidine group **3d** cytotoxicity should not be ignored in U251 cell line with IC_{50} 8.41 µM. Given that compounds containing thiomorpholine core **3b** and **3c** showed moderate activity of U251 cells, the result implies that phenyl substituted morpholine derivatives **3f** and **3h** had no significant differences in activity. Compound having spiro substituted morpholine **3e** and **3g** contributes presumably to the minimal cytotoxicity against this cancer cell line. We can conclude that compound **3a** was the most potent one with IC_{50} 3.5 µM.

Result (Table 1 and Figure S19 in supoorting information) implies that compound **3b**, **3c**, **3d** exhibitedmoderate potency against lung cancer (A549). The IC_{50} value of these derivatives were in range of 25 to 40 µM.

Table 1. EC_{50} values of Compound **3a** to **3h** in U251, A549, HepG2 cell lines and normal cell line MB157[a]

		MB157	U251	A549	HepG2
No.	**Compound**	**EC_{50} (µM)**			
1	**3a**	41.2 ± 1.68	3.5 ± 1.35	19.4 ± 0.65	24.3 ± 0.82
2	**3b**	99.3 ± 2.87	21.4 ± 2.4	36.4 ± 0.98	51.2 ± 1.32
3	**3c**	71.7 ± 0.65	33.2 ± 0.78	41.7 ± 0.45	56.2± 0.23
4	**3d**	52.44 ± 0.98	8.4 ± 0.90	26.77 ± 1.26	43.2± 1.65
5	**3e**	134 ± 0.78	82.5 ± 0.87	57.2± 0.34	67.4± 0.87
6	**3f**	156 ± 1.89	59.8 ± 0.43	74.5 ± 1.54	143.1± 2.11
7	**3g**	89.1 ± 1.4	86.8 ± 0.67	54.01 ± 0.76	78.3± 1.75
8	**3h**	101.5 ± 0.8	42.14 ± 1.23	87.04 ± 0.67	187.3 ± 1.21
9	**Doxorubicin**	35.6± 0.65	1.47 ± 0.6	3.21 ± 0.19	5.41 ± 0.35

[a]Values were the means of three replicates ± standard deviation (SD)

While the substituted Spiro compounds **3e**, **3g** and thiomorpholine compound **3f, 3h** had minimal potency with IC_{50} value >50 µM against A549**.**

The IC_{50} values of synthesized compounds against HepG2 cells revealed that most of the compounds had minimal potency. Neither 3-methyl morpholine nor piperidine substituted derivatives

(**3a** and **3d**) had good antiproliferative activity against HepG2 and as (Table 1) implies, the IC_{50} values of these derivatives were 24.3 µM and 43.2 µM respectively. The IC_{50} value of rest of the other substituted morpholine derivatives were > 50 µM.

3.3. In silico ADMET analysis

The ADMET (Absorption, Distribution, Metabolism, Excretion/Elimination, and Toxicity) calculator is a computer programme that uses molecular structures to calculate the pharmacokinetic properties of drug-like compounds. To be qualified as a drug candidate, any target molecule must have a better pharmacokinetic profile in addition to being highly bioactive and low toxic. As a result, the ADMET properties of the designed compounds were computed using the Swiss ADME online software. Calculating such properties is critical in pharmaceutical chemistry in order to create highly efficient clinical candidates.[25-27]

According to a literature review, various properties such as number of rotatable bonds (NROTB), Topological Polar Surface Area (TPSA), Molecular weight (MW), number of hydrogen acceptors (HBA), number of hydrogen donors (HBD), number of aromatic heavy atoms (nAH), lipophilicity of the compound (Log P), and molar efractivity (MR) are used to define drug likeness properties for a molecule.[28] There are five different rules that are used as filters to determine a candidate's drug likeness as orally bioavailable based on the various physicochemical properties mentioned above. Lipinski's rule of five states that MW <500, miLogP ≤5, HBD ≤5 and HBA <10.[29] Ghose's rule for filtering derivatives as a lead is that the MW should be between 160 to 480, the MR should be between 40 to 130, the total number of atoms should be between 20 to 70, and the calculated WLogP should be between -0.4 to 5.6.[30]

The Veber rule states that NROTB should be ≤10 and TPSA should be ≤ 140 Å2 for good oral bioavailability.[31] According to the Egan rule, the derivative should have TPSA ≤ 131.6 Å2 and lipophilicity (WLOGP) ≤ 5.88.[32] According to Muegge's filter rule, MW should be between 200 to 600, TPSA should be 150 Å2, lipophilicity (XLOGP) should be between -2 to 5, number of carbon should be >4, number of rings should be ≤7, NROTB should be ≤15, number of heteroatoms should be >1, HBD should be ≤5 and HBA should be ≤10.[33]

Table 2. Physicochemical properties of **3a** to **3h**

Compound	MW	TPSA	NROT	HBD	NBA	nAH	MR	FCsp3
3a	421.51	130.86	8	2	5	29	119.76	0.68
3b	423.55	146.93	8	2	4	28	121.46	0.67
3c	455.55	164.15	8	2	6	30	122.84	0.67
3d	405.51	121.63	8	2	4	28	121.63	0.68
3e	490.62	134.1	8	2	6	34	143.73	0.74
3f	483.58	130.86	9	2	5	34	139.44	0.55
3g	463.65	140.09	8	2	6	32	128.39	0.71
3h	497.61	130.86	10	2	5	35	144.69	0.52

MW, molecular weight; TPSA, topological polar surface area; NROTB, number of rotatable bonds; HBD, number of hydrogen bond donors; HBA, number of hydrogen bond acceptors; nAH, number of aromatic heavy atoms; MR, Molar refractivity; FCsp3, Fraction Csp3 (number of sp3 hybridized carbons/total carbon count).

To investigate the physicochemical and pharmacokinetic properties of the synthesised **3a** to **3h**, the SwissADME free online webtool was used to perform *in silico* calculations of the physicochemical and ADME parameters of **3a** to **3h.** Structures of **3a - 3h** were initially converted to SMILES notations. When you visit *http://www.swissadme.ch* in a web browser, the submission page is displayed directly. The SMILES list was copied and pasted into the online server to compute all the properties.

Table 2 shows the calculated MW, TPSA, NROTB, HBD, HBA, nAH, MR, and saturation (Fraction Csp3) for all molecules. Based on the results, we can conclude that all are within the acceptable range with the exception of **3e**, **3f**, and **3h** (Ghose violation (MW>480, MR >130).

The drug likeness parameter is high because all target molecules follow the Lipinski rule, Ghose (except **3e, 3f, 3h**), Egan (except **3b, 3e**), Veber (except **3b, 3c, 3h**), and Muegge (except **3c**) rules, with all compounds having a bioavailability score of 0.55. The synthetic accessibility scores ranged from 4.23 to 5, indicating that they would be easier to synthesise. PAINS (pan assay interference compounds, also known as frequent hitters or promiscuous compounds) are molecules with substructures that exhibit potent response in assays regardless of the protein target. There is no alert for PAINS and one for Brenk, indicating that the compounds are highly specific in nature (Table 3)**.** As a result, these designed targets have a favourable pharmacokinetic profile to be considered as a drug candidate for orally bioavailable administration.[34]

Table 3. Drug-likeness evaluation of **3a** to **3h**

Compd.	Lipinski viol.	Veber viol.	Muegge viol.	Egan Viol.	Ghose viol.	PAINS alerts	Brenk alerts	Synthetic Accessibility	Bioavailability Score
3a	0	0	0	0	0	0	1	4.48	0.55
3b	0	1	0	1	0	0	1	4.23	0.55
3c	0	1	1	1	0	0	1	4.34	0.55
3d	0	0	0	0	0	0	1	4.25	0.55
3e	0	0	0	1	2	0	1	5.4	0.55
3f	0	0	0	0	2	0	1	4.68	0.55
3g	0	1	0	1	0	0	1	5	0.55
3h	0	0	0	0	1	0	1	4.76	0.55

PAINS: Pan Assay Interference Structures. viol:violations

A drug's aqueous solubility is important for its orally bioavailability and absorption. Three models predict water solubility: ESOL, (ALI) logS, and (SILICOS-IT) logS. ESOL is an acronym that stands for estimating aqueous solubility directly from molecular structure, followed by molecular weight, the proportion of heavy atoms in an aromatic system, and the number of rotatable bonds. Log S (Ali) is used to calculate the *in silico* prediction of aqueous solubility while accounting for the effect of TPSA. Log (SILICOS-IT) calculates the negative logarithm of a molecule's water solubility using a fragmental method. The log S scale value ranges from -10 (insoluble) to -6 (poorly soluble), -4 (soluble), -2 (very soluble), and 0 (insoluble) (highly soluble). According to (Table 4), all designed compounds are soluble (Except **3g, 3h** which is Moderately soluble).

Table 4. Solubility of **3a** to **3h**

Compound	ESOL LogS	ESOL Class	Ali LogS	Ali Class	Silicos-IT LogSw	Silicos-IT Class
3a	-2.17	Soluble	-2.49	Soluble	-2.96	Soluble
3b	-2.41	Soluble	-3.2	Soluble	-3.18	Soluble
3c	-1.8	Soluble	-2.24	Soluble	-2.96	Soluble
3d	-2.56	Soluble	-3.1	Soluble	-3.36	Soluble
3e	-2.56	Soluble	-2.52	Soluble	-3.56	Soluble
3f	-3.28	Soluble	-3.65	Soluble	-5.03	Moderately soluble
3g	-2.35	Soluble	-2.57	Soluble	-3.33	Soluble
3h	-3.97	Soluble	-4.73	Moderately soluble	-5.29	Moderately soluble

To cross the cell membrane and have biological activity, a drug must be lipophilic. As shown in (Table 5) lipophilicity can be calculated using various models such as iLogP, XLogP3, WLogP, miLogP, Silicos-IT LogP, and consensus LogP.[35]

Table 5. Lipophilicity of **3a** to **3h**

Compound	iLOGP	XLOGP3	WLOGP	miLOGP	Silicos-IT LogP	Consensus LogP (avg of 5)
3a	2.68	0.18	-0.03	1.06	0.68	0.91
3b	2.52	0.54	0.3	1.6	1.14	1.22
3c	1.97	-0.73	0.06	0.71	-0.1	0.38
3d	2.79	0.96	0.73	1.84	1.21	1.5
3e	3.55	0.15	-0.34	1.17	0.72	1.05
3f	3.2	1.27	1	1.92	1.71	1.82
3g	2.99	0.77	0.09	1.14	0.86	1.03
3h	3.47	2.34	1.76	2.39	1.89	2.38

Human intestinal absorption (HIA) was also predicted with no permeability to the Blood Brain Barrier (BBB). As a result, it is expected that the molecules will have a low incidence of CNS side effects, and compounds can be considered for GI absorption in (Table 6). They are predicted to inhibit the major five isoforms of CYP, such as CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4, which suggests that these molecules may cause pharmacokinetics-related drug-drug interactions, which can lead to toxic or other undesired adverse effects due to the drug's or its metabolites' lower clearance and accumulation (Table 6).[36,37]

Table 6. Distribution, Absorption and Elimination prediction of **3a** to **3h**

Compound	BBB	GIA	P-gP substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
3a	No	High	Yes	No	No	No	No	Yes
3b	No	Low	Yes	No	No	No	No	Yes
3c	No	Low	Yes	No	No	No	No	No
3d	No	High	Yes	No	No	No	No	Yes
3e	No	High	Yes	No	No	No	Yes	Yes
3f	No	High	Yes	No	No	No	Yes	Yes
3g	No	High	Yes	No	No	No	Yes	Yes
3h	No	High	Yes	No	No	Yes	Yes	Yes

4. Conclusion

In conclusion, we present synthesis and characterisation of a new series of pyrazole-containing biotin molecules and evaluated their anti-cancer activity using the MTT assay. The hybrid compounds were synthesised in adequate yields via palladium catalysed aminocarbonylation and demonstrated promising anti-cancer activity profiles. The IC_{50} value was determined using a cell viability assay (MTT assay) on U251, A549, and HepG2 cell lines. Amongst all synthesized molecules, **3a** showed significant activity against the human brain cancer cell line U251 (IC_{50} 3.5 µM). **3a** has the potential to be a promising candidate for the development of new drugs to treat tumours, particularly brain cancer.

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Supporting Information

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