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Design, synthesis and docking study of novel coumarin based 1,3-oxazine derivatives as probable anti cancer drugs

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ABSTRACT

Both oxazines and coumarins are the important class of heterocyclic compounds by virtue of its medicinal, pharmaceutical and therapeutic properties. Coumarin which is an important chemical entity with fused form of benzene and pyrone, 1,3 oxazine has its own significance when it is in individual aliphatic ring form or fused with other chemical entity. This work describes the formation of oxazine bridge between coumarin moiety and a pyridine ring followed by its docking study against two cancer related target proteins viz.rho associated protein kinase 1(PDBID : 3TWJ) for breast cancer and aryl amine N-transferase 2(PDBID : 2PFR) for colon cancer. All the synthesized compound sexhibited good resistance to the target enzymes as indicated by better glide score as compared to standard reference drugs. The compounds were able to interact strongly with active sites pocket residues resulting in the formation of strong favorable molecular interactions. 8-methyl amino-7-bromo coumarin was prepared by reducing 8-formyl -7-bromo coumarin in methanolic ammonia under hydrogen pressure. Condensing ethyl esters of α, β and γ picolinic acids with 8-methyl amino-7-bromocoumarin followed by cyclization in presence of P_2O_5 resulted a new series of 1,3 oxazines.

Key words: NAT 2, 3TWJ, Oxazines, Coumarins, Anti cancer, Docking

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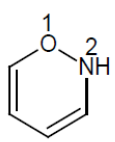
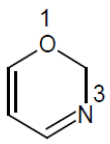
INTRODUCTION

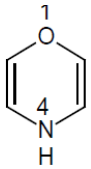
Oxazines are very important class of heterocyclic compounds, they are classified into three isomeric forms like 1,2Oxazines, 1,3 Oxazine and 1,4 Oxazines. Similarly coumarins possess several biological properties[**1,2,3**] especially novobiocin[**4**],coumermycin A1[**5**] and Clorobiocin[**6**] are potent inhibitors of DNA gyrase. Keeping the anti cancer properties of coumarin derivatives



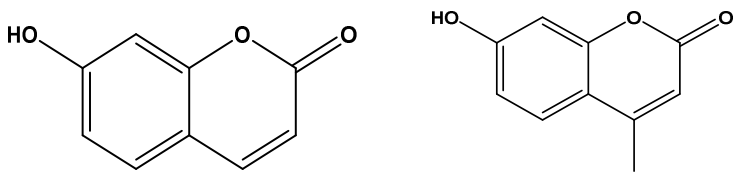
1,2- oxazine



1,3- oxazine

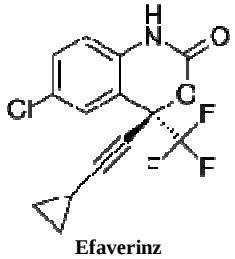


1,4- oxazine



Umbelliferone and 4-Methyl Umbelliferone

Oxazines were first synthesized Holley and Cope [**7**]by under Mannich reaction conditions. 1,3oxazines have drawn more attention as they exists as natural and synthetic compounds with wide range of biological activities[**8**]. Efaverinz[**9**]is an anti-HIV drug considered as an essential medicine by WHO , which has been actively used in the treatment of HIV in combination with other drugs like Tenofovir and Emtricitabine. Efaverinz belongs to 1,3-oxazinone family. The structural features of Efaverinz, led us to design a new series of 1,3-oxazine derivatives which are expected to be good anti cancer agents



Efaverinz

Different synthetic methods were adopted to prepare different 1,3oxazine derivatives. The most common method was through three component system by mixing a β-naphthol, an aldehyde and ammonia.

Betti *et al*[**10**]found that an organic base was formed when 2-Naphthol and benzaldehyde in presence of an amine like aniline. During the reaction an imine was formed initially between aldehyde and aminewhich was then substituted on 2-naphthol to get the base called *Betti Base*.

With the concept of the Bettibase , a large number of 1,3-oxazine derivatives were synthesized by various scientists.

Sunil Dhanya *et al*[11]have synthesized a new series of 1,3-Oxazine derivatives of 4-(4-substituitedphenyl)-6-substituited-6H-1,3-oxazines have been synthesized from acid catalysed reaction between chalcones and urea. All the synthesized compounds were screened for anti bacterial and anti fungal activity.

Girly Tony *et al*[12]synthesized a novel 1,3-oxazine and 1,3-thiazine compounds by the reaction between chalconesand thiobenzamide/benzamide. All the compounds were subjected to docking studies against cytochrome p450 14 alpha sterol demethylase from mycobacterium tuberculosis(M Tb).1,3-thiazine derivatives have shown more activity than the corresponding 1,3-oxazines.

Beena K. P *et al* [13]by using Claisen-Schmidt reaction conditions prepared a new class of 1,3-oxazine derivatives. [6-(p-substituted aminophenyl)-4-(p-substituted phenyl)—6H-1,3oxazin-yl]-acetamides were synthesized and anti microbial activity was carried out.

Mohamed J. Elarfi *et al*[14] prepared 1,3-oxazines , thiazines and isoxazole derivatives by reacting chalcones with urea, thiourea and hydroxyl amines . All these compounds were tested for microbial activity , which revealed good potent anti microbial agents.
A new series of Schiff Bases Of 1,3-Oxazines were prepared by Ramesh l. Sawant *et al* [15],1,3-oxazines were synthesized by reaction between substituted chalcones and urea, which were treated with substituted benzaldehyde , all these schiff bases of 1,3-oxazines were screened for anti coagulant activity.

Substituted Benzoxazine-2,4-diones were prepared by Alan R. Katritzky *et al*[16] , he developed different processes by reactions of 2-(methoxycarbonyloxy)benzoyl chloride with substituted amines.phenyl salicylates with

isocyanates , silver trifluoroacetate mediated reaction of salicylic acid with isocyanates, reaction of salicylamides with $ClCO_2Et$and palladium catalyzed cyclocarbonylation of *o*-iodophenols.

By using silica supported Preysslerheteropolyacid, $H_{14}[NaP_5W_{30}O_{110}]/SiO_2$ (50%) as a heterogeneous catalyst, Ali Gharib *et al*[17]prepared 1,2-dihydro-1-aryl-3*H*-naphth[1,2-*e*][1,3]oxazin-3-one derivatives by condensation of *β*–naphtol, aromatic aldehydes, urea under ethanol reflux. This is a green chemistry approach where in the heterogeneous could be recycled.

Xiaoyan Zhu *et al* [18]has introduced a novel method by introducing a catalyst for the preparation of 1,3 oxazine-3-ones . It has been reported a one pot synthesis of 1,2-Dihydro-1-arylnaphtho[1,2-e] [1,3]oxazine-3-ones and 1,2-Dihydro-1-arylnaphtho[1,2-e][1,3]oxazine-3-thiones by using a catalyst Triphenyl phosphine Ruthenium dichloride $[RuCl_2(PPh_3)_3]$, the formation of undesired un-cyclised product was minimized and a cyslised compound was achieved by one pot synthesis of 2-naphthol with benzaldehyde and urea in toluene medium 2-amino benzimidazole and 2-amino benzthiozole compounds were condensed with 2-naphthol and aqueous formaldehyde to yield 1,3-oxazine derivatives. Their anti microbial activity was performed[19].

ZuhalTurgut *et al* [20]prepared 1,3-Disubstituted-2,3-dihydro-1*H*-naphth[1,2-e][1,3]oxazines by the reaction between aminobenzylnaphthols with substituted aryl- and heteroarylaldehydes absolute MeOH at ambient temperature through ring closure.Phosphomolybdic acid was used as catalyst for the synthesis of 1,2-dihydro-1-aryl-3*H*-naphth[1,2-*e*][1,3]oxazin-3-one derivatives by a one-pot, three-component reaction of *β*-naphthol, aromatic aldehydes and urea in excellent yields.

Hanan.F .Mehssen *et al*[21] reported two new 1,3-oxazine derivatives by adopting different chemical strategies. m-aminoacetophenone was treated with two different aromatic aldehydes viz.p-chlorobenzaldehyde and p-nitro benzaldehyde to form respective chalcones. These chalcones were treated with 2,4-dichloro benzaldehyde which yielded corresponding Schiff's bases, which were treated with Urea to get the 1,3-oxazine derivatives.

M.S. AI-Ajely*et al*[22]has reported the preparation of new series of 7-chloro-4,5-dioxopyrano[3,4-e]-l,3-oxazine derivatives. Initially melanoyl chloride treated with benzyl thiocyanide to get thio derivatives of pyrano -1,3 -oxazine, which was then treated with either amines or peptide derivatives giving new amino or peptide derivatives of pyrano 1,3- oxazines. Among them few compounds were exhibited tremendous anti bacterial activity.

With the help of all the synthetic methods adopted by different scientist to synthesize a variety of 1,3 oxazine derivatives , a new route of synthesis was chosen, by which an oxazine bridge is introduced between coumarin and pyridine moieties

Introduction aboutTarget Proteins

Rho kinases (ROCK1 and ROCK2) is a serine/threonine (Ser/Thr) kinase belonging to the AGC family of protein kinases[**23**], and play acentral roles in the organization and regulation actomyosin cytoskeleton[**24**].ROCK protein exists in two isoforms known as ROCK-I and ROCK-II or Rho-kinase β and α. This protein is composed of N terminal region, a kinase catalytic domain, a coiled-coil domain and a pleckstrin homology domain(Figure 1). Crystal structure have revealed that ROCK-1 is a dimer and possess a head to head arrangement[25]. ROCK protein regulates actin-myosin contractibility and controls cell shape, cell invasion, migration and motility. Thus given its role in motility and migration, over expression of ROCK's protein have been reported to induce migration and invasion in various tumor types especially towards breast cancer[26]. Experimental studies also have revealed that the expression of ROCK's protein is 10 fold higher in breast tumor biopsies[28,29]and henceforth Rho kinases have become attractive targets for the treatment of breast cancers.

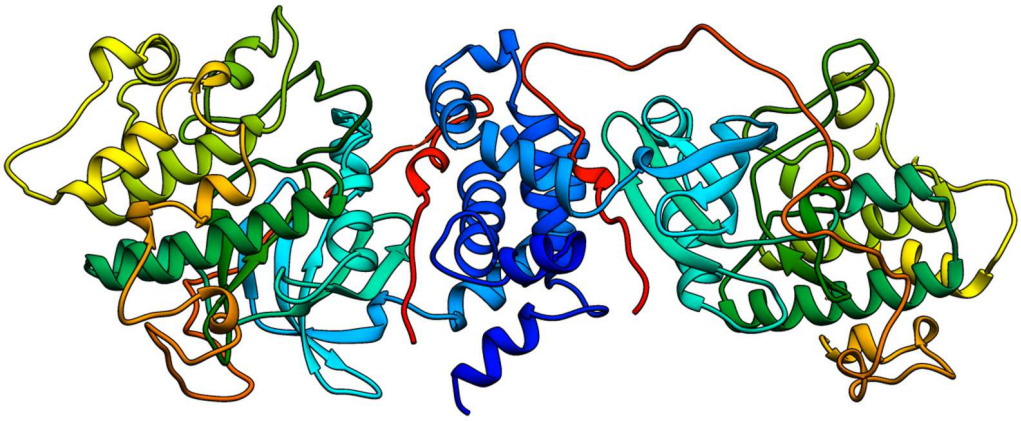


Figure 1: Three dimensional structure of Rho associated protein kinase 1 protein (PDB ID: 3TWJ)

Human aryl amine N-acetyltransferases viz. NAT1 and NAT2 are the cytosolic enzymes which catalases the acetyl coenzyme of A dependent and O-acetylation of primary arylamine and hydrazine drug metabolism and their hydroxylamine metabolites. NAT proteins are located in chromosome 8(pter-q11). NAT1 and NAT2 proteins are composed of 290 amino acids which are of molecular mass of 33,898 daltons and 33,542 daltons respectively. Both NAT1 and NAT2 share 81% identity sequence of amino acid structurally and differ in 55 amino acids.

Crystal structure of NAT-1 and NAT-2 elucidates high similarity three dimensional structure with an r.m.s.d of 0.7Å when superimposed upon one another [30]. The protein is composed of three domains, the amino terminal domain consisting of five helices and one short β strands between α2 and α3 (Figure 2). The second domain consists of nine β strands whereas the third domain has four anti-parallel β strands and a helix α11 respectively. There were reports where acetylator proteins predispose drug-induced toxicities and cancer risks, such as bladder, colon and lung cancer. Colon cancer is the fourth most common disease causing deaths in human society. This is chiefly due to hereditary back ground of the family , but a recent data reports showed that there are some other non-hereditary factors. Dietary heterocyclic aromatic amines (HAAs) are the chemicals considered as mutagenic compounds related to colon cancer. The polymorphic NAT2 enzyme exhibits an important role in the transformation of HAAs to carcinogens. NAT2 enzyme activity is expressed in a genotype-dependent manner in colon epithelium. Precisely activation of HAAs in colon leads to a risk to develop colon cancerwhich is directly proportional to high NAT2 enzyme activity.

Consequently, both ROCK-1 and NAT-2 protein played a significant role in development of cancers especially towards breast and colon cancers. Moreover, various researches are undergoing to develop small molecules inhibitors against these proteins. Thus with this objective, a molecular docking studies will be carried out with the synthesized coumarin based 1, 3-oxazine derivatives to investigate the importance of these compounds and study binding affinities against ROCK-1 and NAT-1 protein to find more potent small molecule antagonist against them.

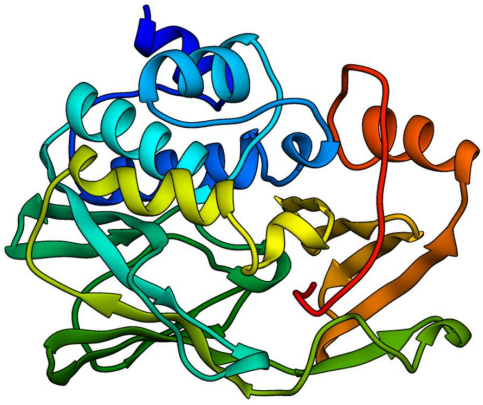


Figure 2: Three dimensional structure of N-human acetyltransferase 2 protein (PDB ID: 2PFR)

MATERIALS AND METHODS

The key starting materials like 7-bromo coumarin and 7-bromo-4-methyl coumarin were purchased from MolBase Chemicals. Other chemicals like ethyl esters of α,β,γ picolinic acids were procured from Chandak Labs .India. Laboratory grade chemical were used for the entire synthetic work. All the chemicals were purchased from Sigma Aldrich and SD fine Chem, India. Melting points were determined on a Creative digital Melting point apparatus and are uncorrected. Thin layer chromatography for completion of reaction and column purification was performed on silicagel coated plates from Macherey-Nagel-Germany , which were visualized by UV light and ninhydrin spray.^{1}H and ^{13}C NMR (proton decoupled) spectra were recorded on a Varian 400 MHz spectrometer using DMSO-d^{6} and $CDCl_3$ as solvent .Mass spectra was recorded on an Agilent triple quadrapole mass spectrometer equipped with a turbo ion spray interface at 360 ^{o}C. Elemental analyses were performed using EA 1112 Thermo Finnigan instrument. FT-IR spectra were recorded on Bucker Alpha-T .Molecular docking studies were performed at Centre for Bioinformatics, Pondycherry University, Pondycherry.

2.0. Experimental procedure;

2.1.Chemical synthesis :

This procedure consists of three stages , preparation of 8-amino methyl derivatives of 7-bromocoumarin and 7-bromo-4-methyl coumarin followed by an amide derivative by condensing with different picolinic esters. These three amides of three picolinic esters were treated with phosphorous pentoxide in xylene to get achromeno-oxazinespyridine derivatives. The schematic path was shown below.

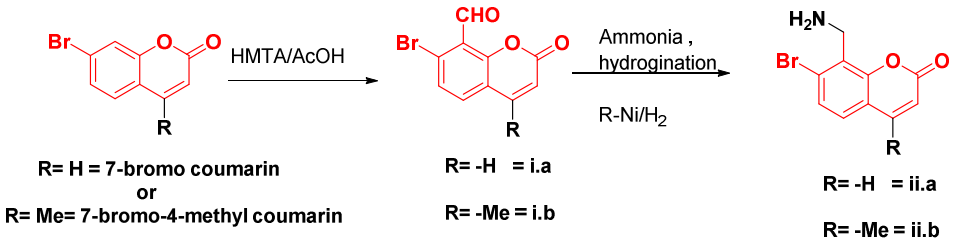
2.1.1. Preparation of 8-(aminomethyl)-7-bromo-4-methyl-2H-chromen-2-one and 8-(aminomethyl)-7-bromo-2H-chromen-2-one.

7-bromo coumarin (20.0 g, 0.0888 mol) was dissolved in glacial acetic acid(150 ml), and hexamethylenetetramine(31 g,0.2222 mol) was added in single portion. The reaction mixture was maintained at 90-95 ^{o}C for 6 h followed by addition of dilute hydrochloric acid and maintained for 30min. Reaction mass was cooled to room temperature and quenched into 1500 ml chilled water, the compound was extracted with diethyl ether (3x300 ml).The combined organic layer was distilled to get 5.6gm of 8-formyl-7- bromocoumarin (i.a) as a light brown powdered solid (yield 25 %)**[31].** mp:187-190^{o}C.

Compound(**i.a**) (10 .0 g, 0.0395 mol) and 50.0 ml6% w/w Methanolic ammonia solution in a Parr-Hydroginator, was charged , 2.0 gm pre washed Nickel was added in Nitrogen atmosphere. The reaction was maintained at 3.0-3.5 kg , temperature of 35-40°C. The consumption of hydrogen gas ceased after 150 min. cooled the temperature to below 25°C. Filtered the catalyst under Nitrogen atmosphere , the clear filtrate was evaporated to get a crude mass of7-bromo-8-methylamino coumarin(**ii.a**) , which was taken directly into next step.

Same procedure was followed to 7-bromo-4-methyl coumarin to get the corresponding compound(**ii.b**).

Scheme-1



R= H = 7-bromo coumarin
or
R= Me= 7-bromo-4-methyl coumarin

R= -H = i.a

R= -Me = i.b

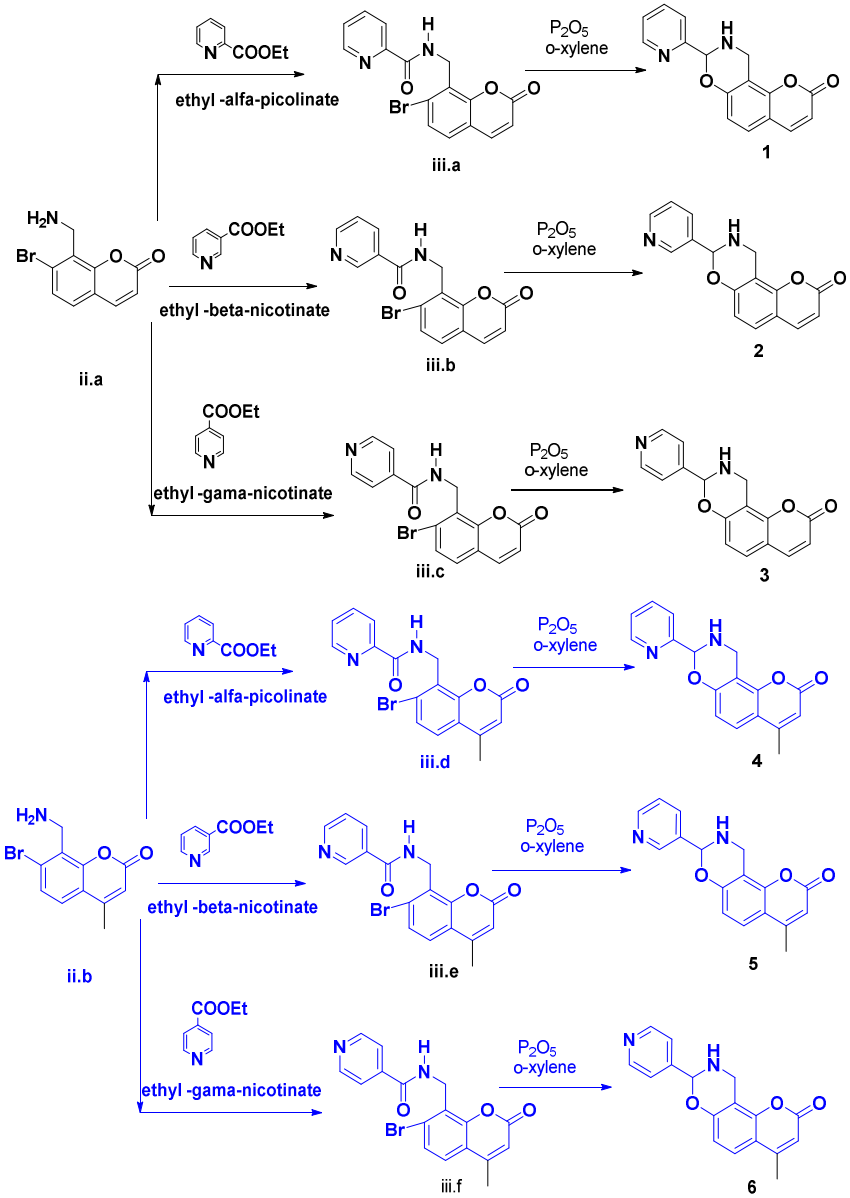
R= -H = ii.a

R= -Me = ii.b

2.1.2. Preparation of 8-(pyridin-2-yl)-9,10-dihydrochromeno [8,7-e][1,3]oxazin-2(8H)-one (4a-f)

The compound (**ii**) was treated with ethyl esters of picolinic acids in presence in toluene medium . The mass was maintained for 4.0 hr at reflux, the completion of the reaction was monitored by TLC. After the completion of the reaction, the reaction mass was cooled to room temperature , an off white solid of amide derivative**(iii)**was precipitated , which was separated by filtration. The amide solid was dried aerially. The amide was directly used in the final step of cyclisation where in the amide is treated with phosphorous pentoxide in xylene and pyridine medium. The crude compound was purified by column chromatography to get final desired compounds**(1-6).**

Scheme-2



Compound (**ii.a**) (2.0 g, 0.0078 mol.) is dissolved in ethanol (10 ml) , then added α-picolinic acid ethyl ester(1.78 g,0.0118 mol.), slowly increased the temperature to 55-60°C. The completion of the reaction was monitored by TLC. After TLC complies, cooled the mass to room temperature and filtered ,after drying at 50-55°C, a cream color solid (2.9 g) of amide compound**(iii.a)**obtained.

Compound**(iii.a)** (2.5 g,0.0069mol) was dissolved in 10 ml o-xylene, cooled the mass to 10°C. Slowly added P_2O_5(4.9 g, 0.0174 mol) below 10°C. After completion of the addition, slowly raised the temperature to 120°C.After 2.0 hr cooled the mass to room temperature. A dark brown color solid was isolated by filtration. The solid(**1**) was purified by column chromatography.

Same procedure was adopted to 7-bromo-4-methyl coumarin and with β and γ picolinic acid ethyl esters to isolate the final compounds **2** to **6**

Molecular docking studies

Molecular docking studies was performed to study the binding mode of synthesized coumarin derivatives with arylamide N-acetyltransferase 2 (NAT-2) and rho-associated kinase protein (ROCK-1) by using Schrödinger Maestro molecular modelling suite software (version 9.2; Schrödinger LLC, Newyork). Crystal structure of both the protein with PDB ID: 2PFR and 3TWJ were retrieved from protein data bank (PDB). Flurouracil and Raloxifene which are anti-cancer chemotherapy drugs for colon and breast cancer was used as reference ligand to undergo molecular docking studies against NAT-2 and ROCK-1 protein. Flurouracil is a pyrimidine analog which is a nucleoside metabolic inhibitor whereas Raloxifene is a known selective estrogen receptor modulator approved for the treatment of breast cancer.

Methodology

Protein preparation

For the use of molecular docking studies, protein preparation was performed before hand for the target proteins using protein preparation wizard of Schrödinger maestro. In this process, bond orders were assigned, hydrogens were added, selenomethionines was converted to methiones and missing side chains residues were filled. Water molecules which are beyond 5Å from the hetero groups are also deleted. Finally the target proteins were optimized and minimized by applying optimized potentials for liquid simulation (OPLS) 2005 force field.

Ligand preparation

The synthesized and reference ligands to be used as inputs for the study were sketch by using ChemBioOffice2014software and prepared using LigPrep module of Schrodinger maestro (version 2.5,Schrödinger Maestro).In this process, bonds orders were added, stereoisomers and tautomers of the ligands were generated. Ligands at possible state were also generated at possible state at a target pH 7.0+/-2.0 using Epik ionizer.

Receptor grid generation

Glide works with a grid generated on the protein for docking calculations and look for favorable hydrogen, hydrophobic, electrostatic interactions etc. between ligand molecules and target protein. A grid was generated for both the proteins by specifying the active site residues as retrieved from literature. van der Waals scaling factor for receptor was scale down with a factor of 1.0 Å and partial charge cut off was set to 0.25.

Molecular docking

The prepared synthesized and reference ligands were then allowed to undergo docking process with the target proteins by using GLIDE tool of Schrödinger Maestro. Docking studies were performed using Standard Precision (SP) mode.OPLS2005 force field was used for energy minimization the docking poses. The scaling of ligand van der Waals radii for non-polar atoms was kept at 0.80 with a partial charge cut off of 0.15. Based on the Glide score, the best docked ligands with the protein was determined and further analyzed to study the mode of binding interactions with the target protein.

RESULTS AND DISCUSSION

Chemical synthesis:

The synthesis of 8-amino methyl derivatives of 7-bromo-coumarin and 7-bromo-4-methyl coumarin was tried under different conditions, where in, the reaction was carried out at zero pressure and room temperature in a hydrogenator , the product conversion was very poor . The reaction was not successful in liquor ammonia evenat increased pressure and temperature conditions. Attempts were made at elevated temperatures and pressures, temperature was increased up to 45-50°C, and pressure was increased to 2-3 kgs , which in turn proved very poor conversion.

The reaction conditions were switched to non aqueous media. The reaction has been carried out in dry conditions by maintaining the ammonia assay between 15-20 % w/w in methanol medium. Here Raney Nickel was washed several times with methanol to knock out moisture under inert atmosphere.

At a hydrogen pressure of 3.0-3.5 kg , temperature of 35-40°C , in presence of dry Raney Nickel , completion of reaction taken place in 3.0 hrs. The formation of methyl amine group was confirmed by both mass and ^{1}H NMR.

Amide formation was established by IR by a sharp –CO stretching band at 1745cm^{-1} and Mass. The cyclization of 1,3oxazine ring is depicted in the below mechanism. During the course of the reaction an amide bond was initially

formed between pyridine moiety and coumarin molecule, which was the cyclized in presence of a P_2O_5. Disappearance of –CO bond formation of -C-O bond was confirmed by a sharp band(stretching) at 1188 cm^{-1} in IR spectra. A broad band at 3100-3250 cm^{-1} indicates the –NH bond in IR spectrum.

Spectral and characterization data of synthesized coumarin-oxazine derivatives:

8-(pyridin-2-yl)-9,10-dihydrochromeno [8,7-e][1,3]oxazin-2(8H)-one(1): IR (KBr, cm^{-1}): 3313(-NH), 1687 (-C=O), 1634 (-C=C), 1341(-N-O) (-C-O). **^{1}H NMR ($CDCl_3$):** δ 8.78(d,H,C-6'),8.00(t,H,C-4'), 7.68(d,H, C-3'),7.44(d,2H,C-5',C-4), 6.91(d,H,C-5), 6.5(d,H,C-6),6.22(s,H,C-3),6.10((d,H,-O-CH,oxazine), 4.0(s,2H,--CH_2). **^{13}C NMR ($CDCl_3$):** δ161.2,C-2), 157.1 (C-7), 155.2(C-2'), 150.1(C-6'), 151.3(C-9), 145.7(C-4), 135.6 (C-4'), 126.8(C,C-5),125(C,C-3'),122(C,C-5'),115.7(C-8),113.2(C-3),111.4(C-6),110.2(C-10),92.8(C,-OCH),44.9(C,N-CH_2). MS: M^+ at m/z :281.40 Anal.Calcd for $C_{16}H_{12}N_2O_3$: C, 68.56; H, 4.32; N, 9.99; O, 17.13, Found; C, 69.05; H, 4.44; N, 10.09; O, 17.25

8-(pyridin-3-yl)-9,10-dihydrochromeno[8,7-e][1,3]oxazin-2(8H)-one(2)

IR (KBr, cm^{-1}): 3351(-NH), 1700 (-C=O), 1621 (-C=C), 1350(-N-O),1150(-C-O). **^{1}H NMR ($CDCl_3$):** δ 8.65(d,H,C-6'),8.71(s,H, C-2'), 8.10(t,H,C-4'), 7.50(d,2H,C-5',C-4), 7.12(d,H,C-5), 6.9(d,H,C-6),6.52(s,H,C-3),6.15(s,H,-O-CH,oxazine), 4.22(s,2H,--CH_2). **^{13}C NMR ($CDCl_3$):** δ 162.1(C-2), 160.2 (C-7), 156.1(C-2'), 152 (C-6'), 149.5(C-9), 145.7(C-4), 140.2 (C-4'), 138(C,C-3'),130.1(C,C-5), 125(C,C-5'), 116(C-8),112(C-3),109.6(C-6),107.8(C-10),95.8(C,-OCH),41(C,N-CH_2). MS: M^+ at m/z :281.25 Anal.Calcd for $C_{16}H_{12}N_2O_3$: C, 68.56; H, 4.32; N, 9.99; O, 17.13, Found; C, 68.66; H, 4.54; N, 10.10; O, 17.20

8-(pyridin-4-yl)-9,10-dihydrochromeno [8,7-e][1,3]oxazin-2(8H)-one(3)

IR (KBr, cm^{-1}): 3385(-NH), 1710 (-C=O), 1652 (-C=C), 1344(-N-O),1109(-C-O). **^{1}H NMR ($CDCl_3$):** δ 8.74(d,2H,C-6',C-2'),8.71(d,2H, C-3',C-5'), 7.41(d,H,C-4), 7.08(d,H,C-5), 6.59(d,H,C-6),6.52(s,H,C-3), 6.25(d,H, C-3), 6.12(s,H,-O-CH,oxazine), 4.08(s,2H,-CH_2). **^{13}C NMR ($CDCl_3$):** δ160(C-2), 158.1 (C-7), 152(C-9), 152 (C-6'), 148(2C,C-2',C-6'), 145 (C-4'), 143.1(C-4), 130.1(C,C-5), 125(2C,C-3',C-5'), 115(C-8),112(C-10),110(C,C-3),108(C-6), 100(C,-OCH),35(C,N-CH_2). MS: M^+ at m/z :281.25 Anal.Calcd for $C_{16}H_{12}N_2O_3$: C, 68.56; H, 4.32; N, 9.99; O, 17.13, Found; C, 68.66; H, 4.54; N, 10.10; O, 17.20

4-methyl-8-(pyridin-2-yl)-9,10-dihydrochromeno [8,7-e][1,3]oxazin-2(8H)-one(4)

IR (KBr, cm^{-1}): 3375(-NH), 1700 (-C=O), 1621 (-C=C), 1370(-N-O),1180(-C-O). **^{1}H NMR ($CDCl_3$) :** δ 8.55 (d,H,C-6'),8.12(t,H,C-4'), 7.86(d,H, C-3'),7.55(d,H,C-5'), 6.98(d,H,C-5), 6.55(d,H,C-6), 6.25((d,H,-O-CH,oxazine), 6.12(s,H,C-3),3.88(s,2H,--CH_2),2.5(s,H,-NH),1.75(s,3H,-CH_3) **^{13}C NMR ($CDCl_3$):** δ 162.5(C-2), 159(C-2'), 156 (C-7), 155 (C-4), 152(C-9), 147(C-6'), 138(C-4'), 130(C,C-5),124(C,C-3'),121(C,C-5'), 116(C-8), 111(C-10),109(C-3),107(C-6), 90(C,-OCH),35(C,N-CH_2),22(C,-CH_3). MS: M^+ at m/z :295.30 Anal.Calcd for $C_{17}H_{14}N_2O_3$: C, 69.38; H, 4.79; N, 9.52; O, 16.31, Found; C, 70.02; H, 4.91; N, 10.02; O, 15.85

4-methyl-8-(pyridin-3-yl)-9,10- dihydrochromeno[8,7-e][1,3]oxazin-2(8H)-one(5)

IR (KBr, cm^{-1}): 3382(-NH), 1704 (-C=O), 1654 (-C=C), 1350(-N-O),1158(-C-O). **^{1}H NMR ($CDCl_3$) :** δ 8.80 (s,H,C-2'),8.60(d,H,C-6'), 7.80(d,H, C-4'),7.52(t,H,C-5'), 7.10(s,H,C-5), 6.85(s,H,C-6), 6.25(d,H,-O-CH,oxazine), 6.00 (s,H,C-3),4.18(m,2H,--CH_2),2.5(s,H,-NH),1.75(s,3H,-CH_3) **^{13}C NMR ($CDCl_3$):** δ 161(C-2), 158(C-2'), 155 (C-4), 152 (2C,C-2',C-9), 148(C-6'), 135(C-4'), 133(C,C-5),128(C,C-5),124(C,C-5'), 115(C-8), 112(C-10),110(C-3),109(C-6), 95(C,-OCH),3385(C,N-CH_2),20(C,-CH_3). MS: M^+ at m/z :295.25 Anal.Calcd for $C_{17}H_{14}N_2O_3$: C, 69.38; H, 4.79; N, 9.52; O, 16.31, Found; C, 69.25; H, 4.85; N, 9.65; O, 16.50

4-methyl-8-(pyridin-4-yl)-9,10-dihydrochromeno [8,7-e][1,3]oxazin-2(8H)-one(6)

IR (KBr, cm^{-1}): 3400(-NH), 1695 (-C=O), 1610 (-C=C), 1348(-N-O),1166(-C-O). **^{1}H NMR ($CDCl_3$) :** δ 8.77 (d,2H,C-6',C-2'),7.66(d,2H, C-3',C-5'), 7.15(d,H,C-5),6.85(d,H,C-6), 6.10(s,H,-O-CH,oxazine), 5.90(s,H,C-3),3.76(m,2H,-CH_2),2.2(s,H,-NH),1.65(s,3H,-CH_3). **^{13}C NMR ($CDCl_3$):** δ 161(C-2), 155 (C-7), 153 (C-4), 150(C-9), 148(2C-2',C-6'), 144(C-4'), 129(C,C-5),124(2C,C-3' ,C-5'), 117(C-8), 113(C-10),110(C-3),108(C-6), 95(C,-OCH),33(C,N-CH_2),22(C,-CH_3). MS: M^+ at m/z :295.33 Anal.Calcd for $C_{17}H_{14}N_2O_3$: C, 69.38; H, 4.79; N, 9.52; O, 16.31, Found; C, 69.85; H, 4.42; N, 9.65; O, 16.55

MOLECULAR DOCKING RESULTS

A molecular docking study was employed to investigate binding affinities and interactions of the reference ligands and coumarin based 1, 3-oxazine derivatives against NAT-2 and ROCK-1 protein to identify potent antagonists.

Glide tool of Schrodinger Maestro was used for docking studies and the best docked compounds were ranked accordingly based on GLIDE score which calculates the free energy of ligands bound to a receptor. This score is based on ChemScore empirical function which encompasses interactions such as hydrophobic, electrostatic, van der Waals, hydrogen bonding as well as penalizing steric clashes between atoms[**30**].

Docking results for reference ligands with NAT-2 and ROCK-1 protein

Reference ligand fluorouracil was observed to interact with NAT-2 protein with a glide score of -6.717 kcal/mol whereas raloxifene was docked to active site pocket of ROCK-1 protein with a score of -6.415kcal/mol. Both the molecules fit in the pocket formed by the active site residues as shown in **Figure(3).**

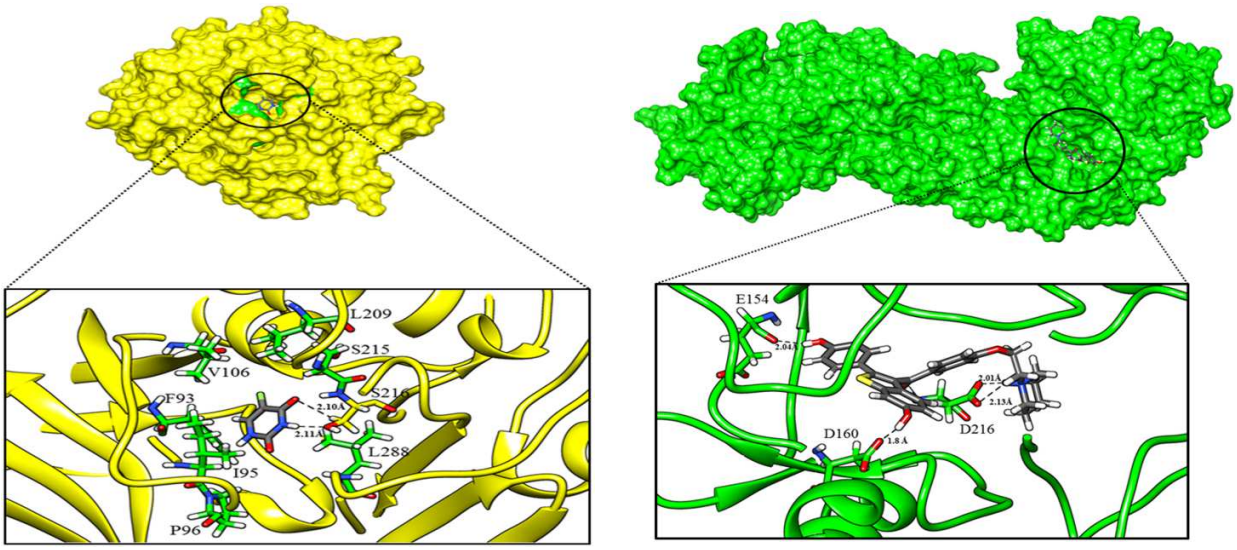


Figure 3: Molecular docking results for NAT-2 and ROCK1 protein with fluorouracil and raloxifene. Both the ligands fit into the active site pocket and hydrogen bond interacting residues are shown as yellow color stick representation where the black dash line represents the hydrogen bond. The ligand is displayed as grey colored stick representation

Fluorouracil interacts with substrate binding residue Ser216 of NAT-2 by forming two hydrogen bonds. The hydrogen atom of amino group of Ser216 acts as donor to oxygen atom of carbonyl group present at 4th position of the ligand whereas hydrogen attached to nitrogen atom at 3rd position of the ligand acts as donor atom to electronegative oxygen atom of Ser216 resulting in formation of two stable hydrogen bonds (**Figure 3**). Hydrophobic residues such as Phe93, Ile95, Pro96, Val106, Leu209, Ser215, Leu288 surrounding the ligand in the active site pocket aid in stabilizing the protein-ligand complex by forming van der Waal's interactions. Thus, fluorouracil occupies the substrate binding site near the active site residues and has the potential to interact with substrate binding site residues.

Whereas in case of ROCK-1 protein docking with raloxifene (**Figure 3**), the ligand fit into the active site pocket enclosed by hinge region residues and activation loop of ROCK1 protein with the formation of four hydrogen bonds. The hydrogen atom of 4-hydroxyphenyl attached to benzothiophene group of raloxifene acts as donor atom to oxygen atom of hinge region residue Glu154 forming a stable hydrogen bond. The ligand extend from the hinge region and binds with ATP binding site residue Asp160 through a hydrogen bond. Further, pyridine ring nitrogen of raloxifene interacts with Asp216 of DFG motif of ROCK-1 by forming two hydrogen bonds. The ATP binding site residues also assist in stabilizing protein-ligand complex by forming van der Waals interactions and charged interactions.

Docking results for coumarin based 1, 3-oxazine derivatives with NAT-2 and ROCK-1 protein

In case of docking studies of the compounds with NAT-2 protein, all coumarin based 1,3-oxazine derivativesinteracts with NAT-2 protein with better glide score above -8 kcal/mol as compared with reference ligand docking score of -6.717 kcal/mol. But out of all the six compounds, **compound 5** was the best docked ligand with a glide score of -8.611 kcal/mol resulting in formation of three hydrogen bonds with NAT-2 protein.

In compound 5, H14 of pyridine-3-yl group attached to [1,3]oxazin-2-one acts as donor atom resulting in formation of hydrogen bond with side chain oxygen atom of C terminal Ser216 of NAT-2 as shown in **Figure 4.** This particular hydrogen bond with Ser 216 was observed as in the case of reference molecule flurouracil. Whereas N atom of 1,3-oxazine molecule attached to coumarin backbone also participates in two hydrogen bond formation with hydroxyl group of Ser287 and Tyr94 contributing to stronger interaction with NAT-2 protein. C terminal residue Ser216 and Ser287 in NAT2 plays an important role in facilitating substrate to its binding site for participation in catalytic reactions. Thus compound 5 was able to interact with the residues present in the substrate binding region however, no hydrogen bonding of the compound with catalytic triad residues C68, H107 and D122 was observed. This compound was also able to interact with the substrate binding residues in the same manner as observed with the reference ligand (fluorouracil) and this interactions were further strengthen by forming van der Waals's and hydrophobic interactions with the residues surrounding the active site and substrate binding site as given in **Table 1**.

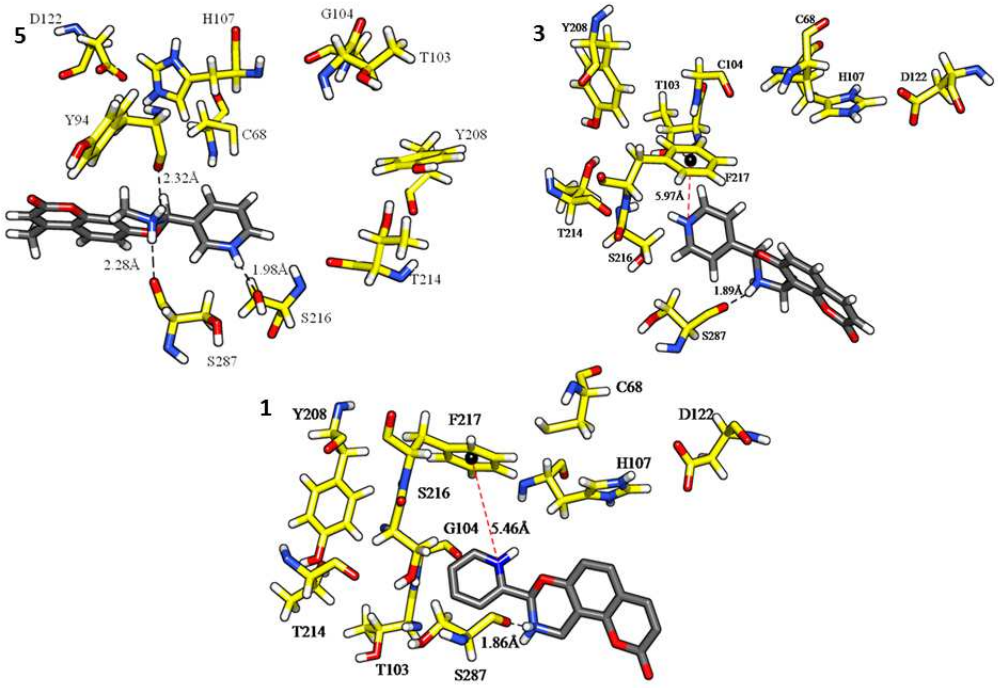


Figure 4: NAT-2 protein molecular-interactions with compound 5, 3 and 1. The ligand is displayed in grey color stick format whereas the residues are shown in yellow stick representations with black dash color representing the hydrogen bond. The pi-cation interaction is displayed as red dash line between the ligand and the residues respectively

Compound 3 and 1 binds to NAT-2 protein in a same binding mode whereas compound 4 and 6 were oriented in the active pocket in the same manner. In case of compound 3 and 1 as seen in **Figure 4**, the NH group of 1, 3-oxazine acts as donor atom and form hydrogen bond with the side chain oxygen atom of Ser287 of NAT2. This interaction is strengthened by a pi-cation interaction between N atom of pyridine-4-yl group of the compound with phenyl ring of Phe217 of NAT2 with a distance of 5.48Å.

However, compound 4 and 6 have a different binding mode as compared to previous binding interactions as shown in **Figure 5**. NH atom of 1, 3-oxazine acts as a donor atom leading to formation of a hydrogen bond with oxygen atom of Tyr 94. Compound 2 was observed to form a hydrogen bond with C terminal residue Ser287 with N atom of 1, 3-oxazine resulting in a stable bond with a distance of 1.82Å as shown in **Figure 5**.The interactions between ligands and NAT-2 protein were also strengthened by the formation of hydrophobic and van der Waals interactions with the active site residues.

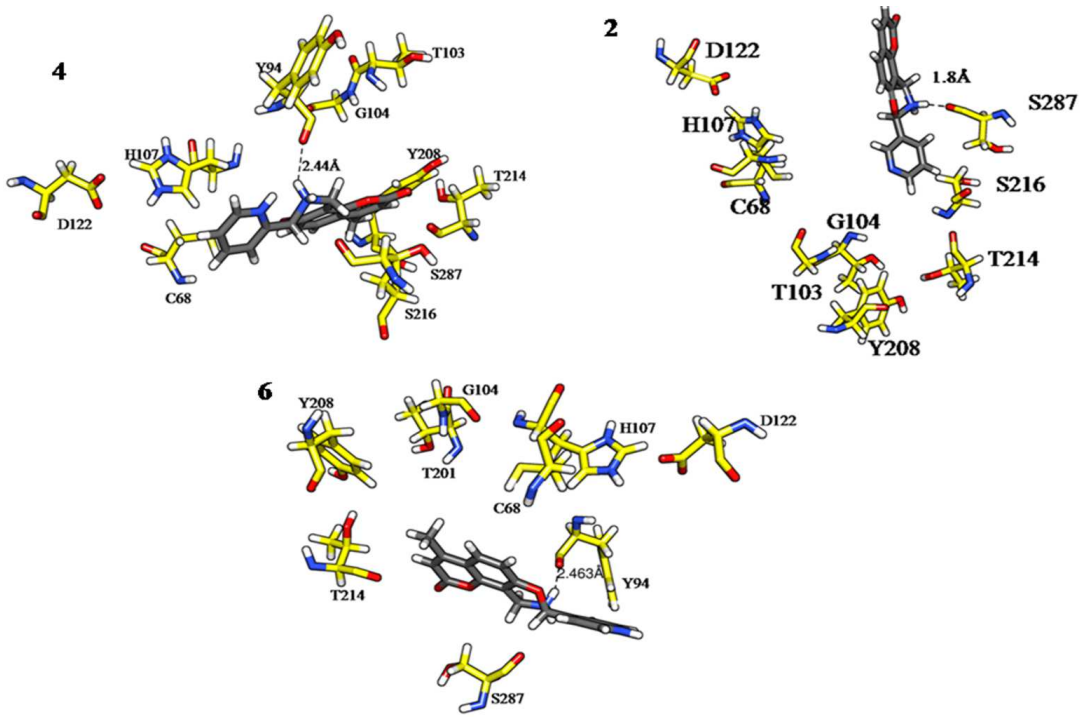


Figure 5: NAT-2 protein molecular-interactions with compound 4,2,and 6. The ligand is displayed in grey color stick format whereas the residues are shown in yellow stick representations with black dash color representing the hydrogen bond

Table 1: Detailed analysis of molecular docking studies of six coumarin based 1,3-oxazine derivatives with NAT-2 protein

Ligand	G score (kcal mol^{-1})	E model score	Hydrogen bonding interacting residues Donor Acceptor	Dist [Å]	Pi-cation interactions	Van der waals interactions	Hydrophobic interactions
Compound 5	-8.61	-65.1	UNK900:H14- S216: OG UNK900:H15 -Y94: O UNK900:H16-S287: O	1.9 2.3 2.2	None	T214,P96,Q163,S215,L209,F217,V106,P182,R165,S129,S128,I290,L288,F93,F222	S216,S287,L288,P93,Y94,P96,Q187,2129,I290,S127,S128,Q163,T289
Compound 3	-8.4	-64.5	UNK900:H14- S287: O	1.8	UNK900:N1-F217	S215,F217,I95,L288,F93,F222,P97,G126,R165,S127,S129,I290,S128,P182	S216,S287,L288,P93,Y94,P96,Q187,S129,S127,G126,T289
Compound 1	-8.3	-64.6	UNK900:H614- S287: O	1.8	UNK900:N1-F217	S128,I290,S129,S127,R165,G126,F222,F93,I95,S215,S216,P97,P182	S216,S287,L288,P93,T94,P96,Q187,S129,S127,G126,T289
Compound 4	-8.2	-64.9	UNK900:H16 - Y94: O	2.44	None	S125,G127,S127,S129,F222,P96,P97,I95,F93,L288,F217,L209,V106,S215,Y208	S129,T289,S127,G126,P93,L288,P96,Y94,S287,L209,S216,T214,I95
Compound 2	-8.1	-62	UNK900:H13 - S287	1.8	None	S128,I290,S129,S127,G126,F222,F93,I95,F217,S216,S215,P97,P182,R165	S216,F93,S287,P96,Q187,S129,Y94,T289,S127,G126,L288
Compound 6	-8.0	-63	UNK900:H16 - Y94: O	2.4	None	S125,F222,F93,L288,P96,I95,P97,F217,L209,V106,Y208,S215	P96,S216,L209,T214,F217,I95,Y94,S267,L288,T289,S127,G126
Flurouracil	-6.717	-32.238	S216: H -UNK900:O1 UNK900:H1-S216:OG	2.10 2.11	None	F93, I95,P96, V106,L209, S215,L288	I95,S215, S216,S287

Consequently, from docking studies of the coumarin based 1,3-oxazine derivatives with NAT-2**,** the importance of N atom of 1,3 oxazine group as donor atom in forming hydrogen bond with the substrate binding residue Ser287

can be inferred. Also, N atom of pyridinyl group plays an important role for the formation of pi- cation interaction with phenyl ring of Phe217. Even though the best docked compound 5 was able to form hydrogen bond in the same manner as the reference ligand, it was able to interact with important substrate binding site residue such as Ser287 of NAT-2 protein with strong binding affinities. Thus all the synthesized compounds have the potential to become better lead molecules to be used against NAT-2 proteinas well as the importance of residues 216 and Ser287 of NAT-2 protein can be inferred.

In respect to docking studies of coumarin based 1, 3-oxazine derivativeswith ROCK1 protein, all the six compounds were observed to be lying deep in active site pocket with animprovedglide scorethan reference ligand raloxifenein therange of -8.4 and -7.3 kcal/mol. Docking resultsindicates that compound **4 and 1**exhibit same binding modewith favorable molecular interactions whereas compounds **2, 3, and 6**interacts with active site residues in a similar binding orientation. Whereas compound **5**was shown to interact with active sites residues in a different binding mode as compared to rest of the compounds. The detailed analysis of the docking studies is given in **Table 2**.

Table 2: Detailed analysis of molecular docking studies of six coumarin based 1,3-oxazine derivatives with ROCK1 protein

Ligand	G score (kcal mol^{-1})	E model score	Hydrogen bonding interacting residues		Dist [Å]	Charged interactions	Van der waals interactions	Hydrophobic interactions
			Donor	Acceptor				
Compound 4	-8.4	-101.1	UNK900:H16-D160:OD1		1.8	Asp160, Asp 202	D160,I82,F368,D202, L205	S216,S287,L288,P93,Y94,P96,Q187,2129,I290,S127,S128, Q163,T289
Compound 1	-8.1	-96.2	UNK900:H14-D160:OD1		1.9	Asp160, Asp 202	V162,G83,V90,F368,L205,Y155,N203,A215, V137,A103	F368,A103,L205,D202,D160,I82
Compound 2	-7.9	-83.9	UNK900:H12-D202:OD2 UNK900:H13-D160:OD1 MET156:H-UNK900:O3		2.3 2.4 2.1	Asp160, Asp 202	G83,I82,D216,E154,M153,V137,A103,Y155, V90,F368,L205	D202,I82,L205,Y155,M156,A103,F368, V190,D160
Compound 3	-7.8	-81.9	UNK900:H12-D202:OD2 UNK900:H13-D160:OD1 MET156:H-UNK900:O3		2.3 2.4 2.1	Asp160, Asp 202	E154,A103,V137,M153,I82,D216,L205,V90, F368,G83,I82	L205,F368,Y155,D160,D202,I82,V190, A103
Compound 6	-7.4	82.1	UNK900:H14-D202:OD2 UNK900:H15-D60:OD1 MET156:H-UNK900:O3		2.4 2.0 2.3	Asp160, Asp 202	E154,A103,Y155,V137,M153,M128,L103,A215,V90,D216,G83,I83,L205,F368	F368,Y155,A103,M153,V90,L205,I82, D202,D160
Compound 5	-7.3	-85.8	UNK900:H14-D202:OD2 UNK900:H16-D160:OD1		1.6 1.7	Asp160, Asp 202	M153,A103,Y155,V137,L105,A215,M128,D216,V162,V90,G83,F368,I82,D369	D202,D160,F368,L205,I82
Raloxifene	-6.415	-72.723	UNK900:H26-D160:OD1 UNK900:H27-E154: O UNK900:H28-D216:OD1 UNK900:H28-D216:OD2		1.88 2.04 2.01 2.31	Lys105, Asp216	A103,V137, A215,I82, L205,F368,V90,N203, L200,G83,D202, R84,T219, G218,F120, F87	G218,T219,G85,V90, L105,D202,V137,M153,A103,L205, I82,P368, D160

For compound **4 and 1** as shown in **Figure 6**, nitrogen atom of 1, 3 oxazine group acts as donor atom and participates in forming hydrogen bond with ATP binding site residue Asp160. Charged interactionswere alsoformed between nitrogen atom of pyridinyl and 1, 3oxazinegroups with negatively charged Asp160 and Asp202 of ROCK1 protein stabilizing the protein-ligand complex further. Compound 2, 3 and 6 exhibit similar binding mode as depicted in **Figure 7 and 8**. These compounds were observed to interact with hinge region residue Met156 of ATP binding site through a hydrogen bond. ATP binding site residue Asp160 and Asp202 facilitate hydrogen bonds formation by acting as donor atom to pyridinyl and 1, 3-oxazine group of the ligands. But in case of compound 2, a pi- cation interaction between pyridine-3-yl ring of the ligand and positively charged Lys200 assisted in stabilizing the protein-ligand complex.

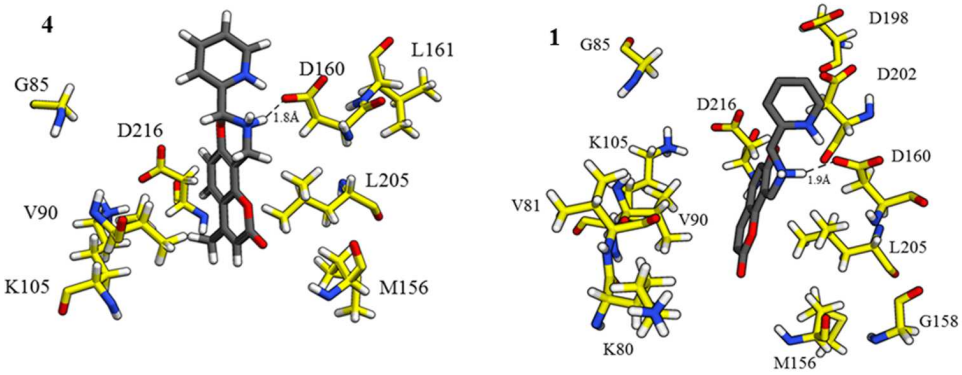


Figure 6: ROCK-1 protein molecular-interactions with compound 4 and 1. The ligand is displayed in grey color stick format whereas the residues are shown in yellow stick representations with black dash color representing hydrogen bond

However in case of compound 5 (**Figure 8)**,this ligand is anchored in active site pocket where pyridinyl and 1,3 oxazine group acts as donor atom leading to formation of two hydrogen bonds with Asp 202 and Asp 160 with a glide score of -7.3kcal/mol. The interaction is further stabilized by formation of charged interactions, hydrophobic and van der Waals interactions between them.

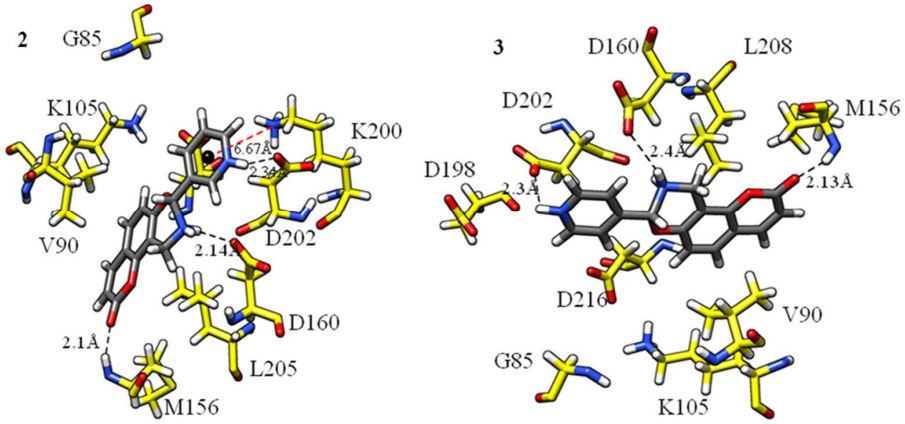


Figure 7: ROCK-1 protein molecular-interactions with compound 2 and 3. The ligand is displayed in grey color stick format whereas the residues are shown in yellow stick representations with black dash color representing hydrogen bond. The red dash line between the ligand and the protein represents the pi-cation interaction

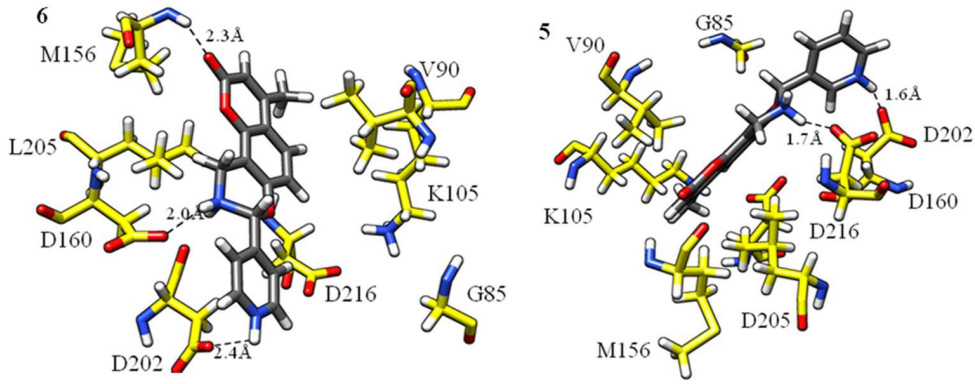


Figure 8: ROCK-1 protein molecular-interactions with compound 6 and 5. The ligand is displayed in grey color stick format whereas the residues are shown in yellow stick representations with black dash color representing hydrogen bond

Thus, all the 6 compounds were able to form strong interactions with ATP binding site residue Asp160 as observed in a similar mode as reference ligand raloxifene. 3 compounds were able to interact with the active site hinge region residue Met156. Even though hydrogen bond formed between Ser216 and Glu154 in the reference ligand was not observed in case of docked compounds, active site residue Ser216 plays proved to play a crucial rolein forming van der Waal's interactions with the ligands. Docking studies of the synthesized compounds with ROCK-1 protein also revealed the importance of nitrogen atom in 1,3oxazine and pyridinyl group in formation of strong hydrogen bond interactions.

CONCLUSION

The new series of compoundsbridging coumarin and pyridine molecules viaoxazine moiety were successfully synthesized as per the designed protocol. The characterizion of the synthesized compounds was proved the same.

This route of synthesis has been considered as simple and easily handled in laboratory with simple chemicals available. This new synthetic approach would help in designing several compounds for medicinal chemistry.Molecular docking studies were also carried out to check the binding affinities of the synthesized compounds against NAT-2 and ROCK-1 protein to be used as anti-cancer agents. Standard anti-cancer drug such as Fluorouracil (colon cancer) and Raloxifene (breast cancer) was used as reference ligands to compare the binding mode and interactions.In-silico studies concluded that all the synthesized compounds (**1-6**) have better binding interactions with a good glide score as compared to the standard drugs.Pyridinyl ring and 1,3oxazine group of the compounds played an important role in formation of hydrogen bond interactions acting both donor and acceptor atoms. In case of NAT-2 protein, the compounds were able to interact with the important substrate binding residues such as Ser216 and Ser287 whereas for ROCK-1 protein, the compounds interacts strongly with active site hinge region residues Met156 and Asp216 of the DFG activation loop region. Further favorable hydrophobic, van der Waals, charged and pi-cation interactions stabilized the protein-ligand complex and thus can be considered as a good antagonist against NAT-2 and ROCK-1. Henceforth this study has provided the scope for developing coumarin based 1, 3-oxazine derivatives as anti-cancer agents to be used for treatment against colon and breast cancer respectively.

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