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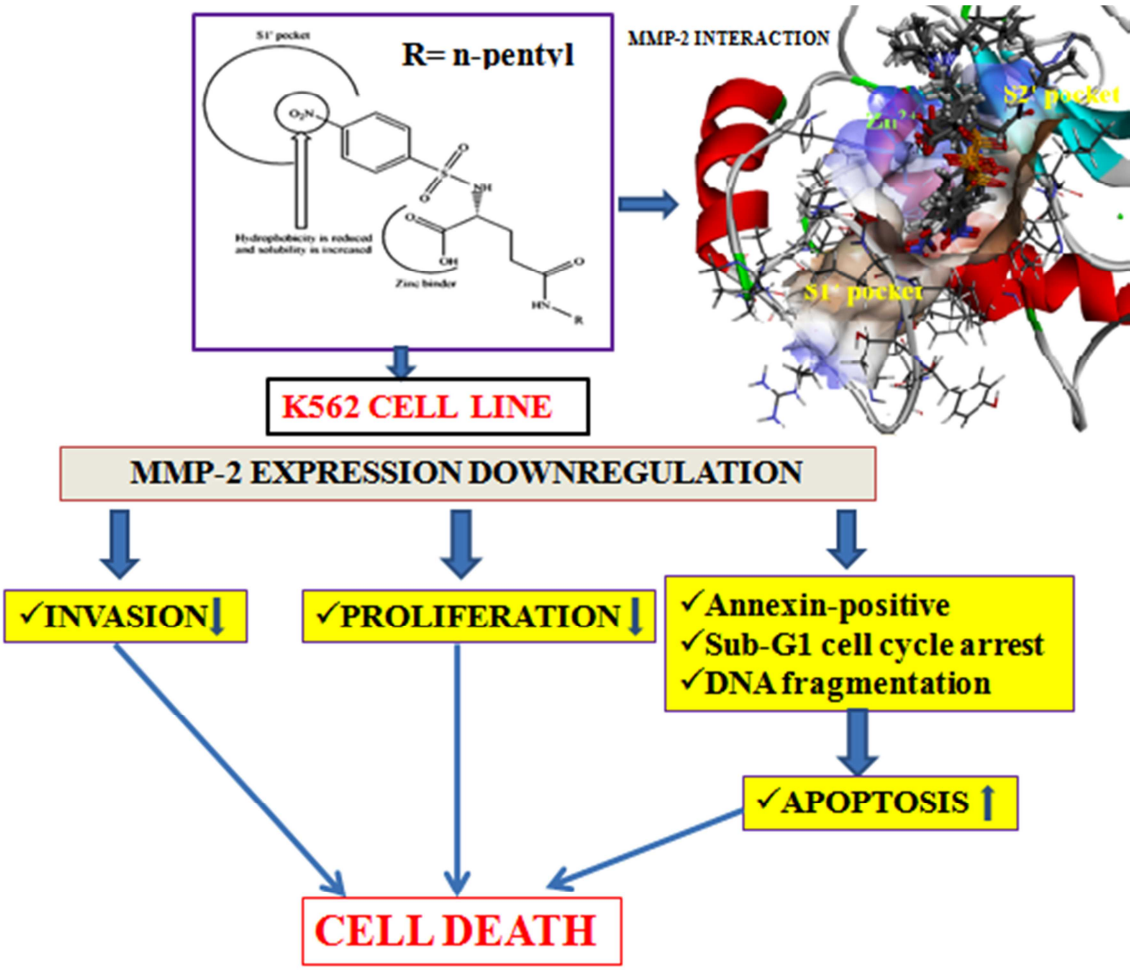
S1' pocket
R= n-pentyl
Hydrophobicity is reduced and solubility is increased
Zinc binder
MMP-2 INTERACTION
S2' pocket
S1' pocket
K562 CELL LINE
MMP-2 EXPRESSION DOWNREGULATION
✓INVASION
✓PROLIFERATION
✓Annexin-positive
✓Sub-G1 cell cycle arrest
✓DNA fragmentation
✓APOPTOSIS
CELL DEATH

A pentanoic acid derivative targeting matrix metalloproteinase-2 (MMP-2) induces apoptosis in a chronic myeloid leukemia cell line

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Abstract:

Depending on our previous observations, some compounds of pentanoic acid were designed and synthesized. Characterization of the synthesized compounds was done by mass, NMR and IR spectroscopy as well as elemental analysis. Among the synthesized molecules, (2*S*)-5-oxo-2-[(nitrobenzene-4-yl sulfonyl) amino]-5-(pentylamino) pentanoic acid (**Cpd 11**) was found as a lead and potent inhibitor of matrix metalloproteinase-2 (MMP-2). Molecular modeling and enzyme inhibition studies were done to confirm the interaction or inhibitory potential of this compound. Thereafter, the biological screening was done through cytotoxicity, anti-invasion and apoptosis-related assays. Docking analysis revealed that **Cpd 11** interact with the target molecule MMP-2 and with MMP-9. However, enzyme inhibition assay showed 3-fold MMP-2 inhibition compared to MMP-9. Cytotoxicity assay showed the inhibitory potential of **Cpd 11** against K562 cell line having IC_{50} value of 17.9±0.01 µM after 48 h of incubation. The cell death was apoptotic in nature as revealed from the annexin V and sub-G1cell cycle arrest assay. Besides this, **Cpd 11** also exhibited dose dependent anti-invasive activity into K562 cell line. On the other hand, flow cytometry and western blot data revealed **Cpd 11** induced downregulation of MMP-2 in K562 cell line after 48 h of incubation that might be linked with the anti-invasive and apoptotic activity furthermore. Therefore, the overall results validated each method and make this molecule as a potent MMP-2 inhibitor that blocked the invasion and could bring apoptosis at later stages in K562 cells sparing the normal ones.

Keywords: pentanoic acid, MMP-2 inhibition, enzyme assay, apoptosis, cytotoxicity, molecular docking study

1. Introduction

Metalloproteinases are associated with a variety of pathological and cellular functions including matrix degradation during cancer cell proliferation and migration [1]. Till now, 26 different matrix metalloproteinases (MMPs) are identified. These are categorized into six subgroups (namely collagenases, gelatinases, matrilysins, stromelysins, membrane type MMPs and other MMPs). Overexpression of MMPs is related to a number of diseases such as cardiovascular and neurological disorders as well as the formation of cancer through uncontrolled cellular proliferation, invasion and metastasis [2-4]. Among these MMPs, MMP-2 is found to be upregulated in most of the cancers particularly in leukemia where the leukemic cells proliferate in an uncontrollable manner [5]. In leukemia, leukemic cell blasts occur in an excess amount which later triggers invasion into various tissues [6]. Regarding this, MMP-2 expression plays a role in the increased vessel density found in the bone marrow of leukemia patients by accelerating *in vitro* endothelial cell migration [7]. Moreover, the cell surface association between MMP-2 and integrins is found to be involved in the growth and progression of acute and chronic myeloid leukemia cells [8]. Therefore, designing and synthesizing MMP-2 inhibitors may be a valuable strategy for blocking the leukemia cell growth. However, very few MMP-2 inhibitors have been reported to control leukemia particularly [9-12]. Apart from this, most of the MMP-2 inhibitors either failed to induce apoptosis against leukemia or lacks their target specificity.

In this context, some compounds of pentanoic acid have been designed and synthesized to judge their MMP-2 inhibitory properties along with cytotoxic and apoptotic features. As far as our earlier observations were concerned, a number of potent and selective MMP-2 inhibitors over other MMPs were reported [13, 14]. However, none of these derivatives were able to generate cytotoxicity in cancer cells (such as A549 and MDA-MB-231 cell lines, etc) at a lower dose (data not shown). Some of these compounds were found to be partially

cytotoxic in chronic myeloid leukemia K562 cell line over the other MMP-2 secretive cells like A549 (non-small cell lung cancer cell line), MDA-MB-231 (breast cancer cell line) (data not shown). It may be assumed that poor solubility of these compounds due to the hydrophobic aryl functions may hinder the cellular permeability and in turn, may result in a low degree of cytotoxicity though a higher MMP-2 inhibition was achieved. In search of the better cytotoxicity, some new compounds were designed and synthesized to achieve the better solubility (**Fig. 1**). It may also be assumed that due to free carboxylic acid function these analogs may bind to the zinc ion at the MMP-2 active site as evidenced in our earlier observations [13, 14]. In this article, only the hydrophobic aryl substituent directed towards the S1′ pocket was modified with the smaller substituent to enhance the solubility criteria without altering other structural features.

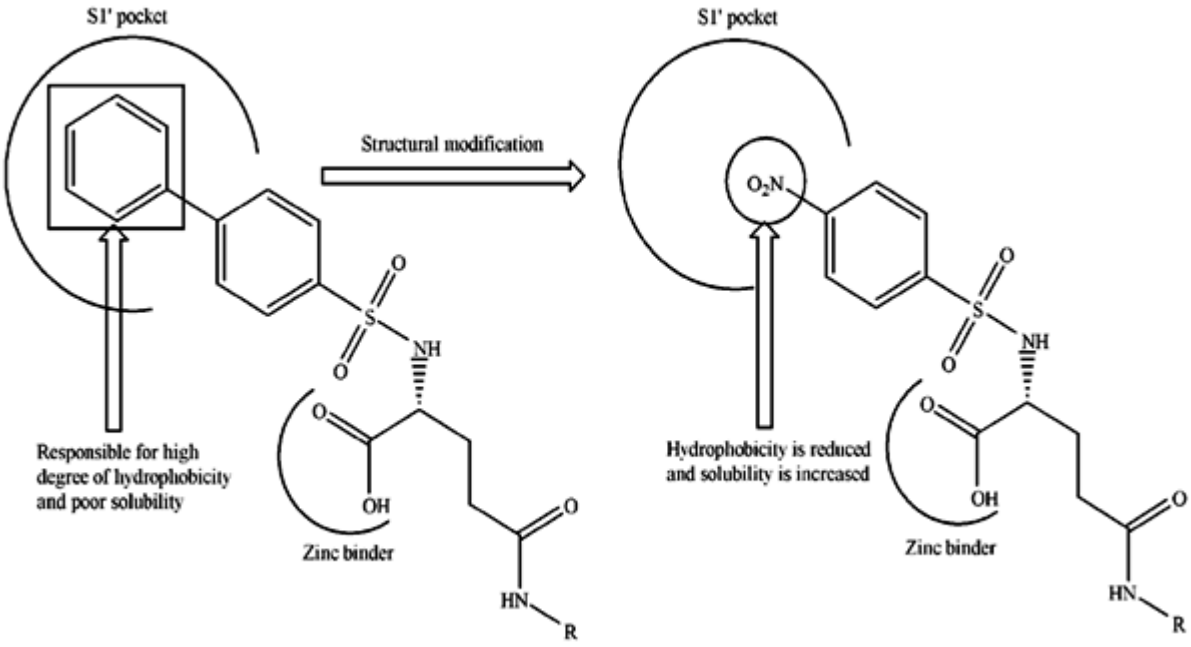


Fig. 1. Molecular modification of pentanoic acids for achieving higher solubility

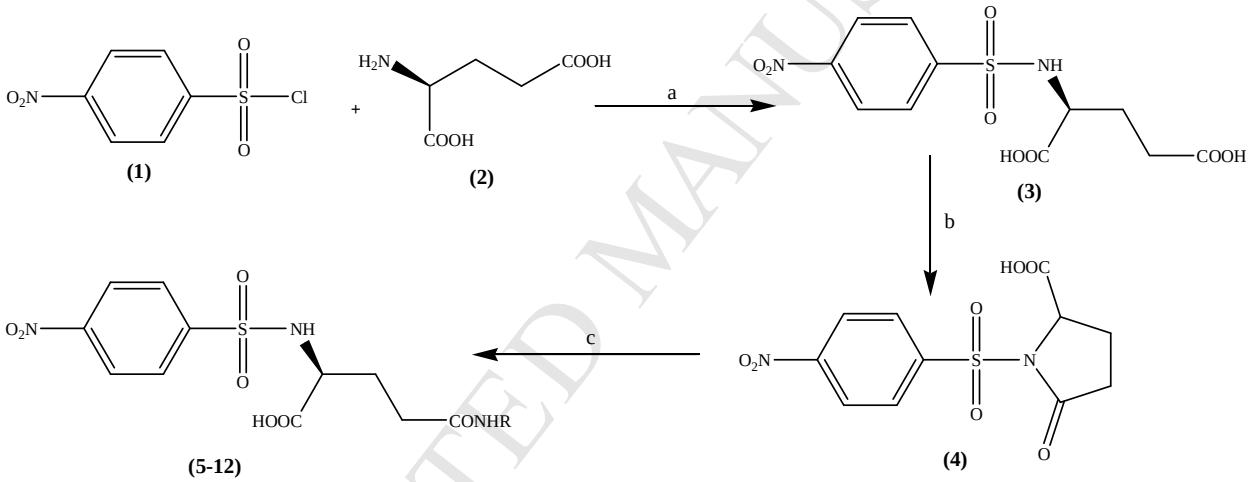
Among the designed and synthesized molecules, the lead compound was obtained by analyzing the results of enzyme assay and cytotoxicity profiling against a variety of cancer

cell lines (such as A-549, MDA-MB-231, U-937 and K562) keeping peripheral blood mononuclear cells (PBMC) as the positive control. Moreover, MMP-2 expression analysis and invasion assay were performed in K562 cell line by applying the best active molecule as these molecules exhibited comparatively better cytotoxicity in K-562 cell line. Besides this, the apoptotic efficacy of the best active MMP-2 inhibitor was also analyzed to establish the cytotoxic property in chronic myeloid leukemia.

2. Results and discussion

2.1. Synthesis

Derivatives of pentanoic acid (**Cpd 5-12**) were prepared according to Scheme 1.



a: 2(N) NaOH; b: CH_3COCl, Reflux, 2 hr.; c: RNH_2, 6(N) HCl

Scheme 1. Synthetic route of the designed molecules

The 4-Nitrobenzenesulfonyl chloride (**1**) was condensed with L(+)-glutamic acid (**2**) in presence of 2(N) sodium hydroxide solution to obtain 2-N-(4'-nitrobenzenesulphonyl)-L(+)-glutamic acid (**3**). The diacid (**3**) was cyclized with acetyl chloride to get 1-(4'-nitrobenzenesulphonyl)-5-oxopyrrolidine-2-carboxylic acid (**4**). The monoacid (**4**) was treated with different amines to get the desired pentanoic acids (**5-12**). All the final products were characterized by mass, ^{1}H NMR, ^{13}C NMR and FTIR spectroscopy as well as elemental analyses. The physicochemical properties of these compounds (**cpd 5-12**) are shown in **Table 1**.

Table 1. Physicochemical parameters of the final compounds (**Cpds 5-12**)

Cpd[a]	R	M.P.(°C)	% Yield	Molecular Formula	MW
5	H	181-183	63.24	$C_{11}H_{13}N_3O_7S$	331.30
6	Me	206-208	81.91	$C_{12}H_{15}N_3O_7S$	345.33
7	Et	219-221	78.72	$C_{13}H_{17}N_3O_7S$	359.36
8	*i-Pr*	235-237	92.06	$C_{14}H_{19}N_3O_7S$	373.38
9	*n-But*	209-211	89.25	$C_{15}H_{21}N_3O_7S$	387.41
10	*i-But*	204-206	76.86	$C_{15}H_{21}N_3O_7S$	387.41
11	*n-Pent*	215-217	86.82	$C_{16}H_{23}N_3O_7S$	401.44
12	Benz	191-193	88.24	$C_{18}H_{19}N_3O_7S$	421.43

[a]Compound number

2.2. Enzyme inhibition study

In enzyme inhibition study, MMP kits were taken and exposed to the synthesized compounds. Then the chromogenic substrate was applied after sufficient time of exposure. The concentration of these substrates was detected by its absorbance value at 410 nm in a microplate photometer. A gradual decrease in the absorbance indicated the compound induced inhibition of the MMPs action. As far as the MMP-2 and MMP-9 inhibitory activities of these final compounds (**Cpd 5-12)** were concerned, some compounds show MMP-2 selectivity over MMP-9 enzyme (**Cpds 5**, **6**, **8** and **12**) whereas other compounds are nonselective (**Cpd 7**, **9**, **10** and **11**) (**Table 2**). The **Cpd5** without having any substitution at the R position (**Table 2**) results in 4.24 folds MMP-2 selectivity over MMP-9 whereas substitution with the methyl group (**Cpd 6**) at R position (**Table 2**) yields about 2 folds loss in MMP-2 inhibition compared to **Cpd 5** though it shows 2.63 folds selectivity over MMP-9. Increasing the linear alkyl chain length (such as ethyl at R position, **Table 2**) results in nonselectivity rather it (**Cpd 7**) shows the better affinity towards MMP-9 compared to MMP-

2. Substitution with the branched alkyl function at R position (such as *i*-propyl, **Cpd 8**) shows more or less similar inhibitory profile compared to the ethyl analog (**Cpd 7**). It also shows 2.82 folds MMP-2 selectivity over MMP-9. Increasing more linearity (chain length) with the *n-butyl* function (**Cpd 9**) at the R position yields nonselectivity whereas branching (such as *i*-butyl, **Cpd 10**) rather exhibits comparatively better affinity towards MMP-9. For the higher linear alkyl substitution (such as *n-butyl* and *n-pentyl* at R position, **Table 2**), the MMP-2 inhibitory activity is found to be more or less similar. Moreover, the *n-pentyl* function (**Cpd 11**) at the R position (**Table 2**) also yields nonselectivity. Interestingly, aryl function (such as benzyl, **Cpd 12**) at the R position (**Table 2**) yields potent MMP-2 inhibition and more than 25 folds selectivity over MMP-9. Therefore, it may be assumed that smaller alkyl groups (methyl, ethyl) at R position favor MMP-2 inhibition and selectivity over MMP-9 whereas the higher bulky and linear substitution (such as *n-butyl*, *n-pentyl*) disfavors MMP-2 selectivity. Moreover, the bulky branched alkyl substitution (such as *i-butyl*) at R position disfavors activity. Interestingly, it is noticed that aryl substitution (such as benzyl) at R position may be necessary for maintaining the higher MMP-2 inhibitory potency as well as selectivity over MMP-9.

Besides this, MMP-1, MMP-8, MMP-12 and MMP-14 inhibitory assays were also done. The results of the other enzyme (apart from gelatinase sub group) inhibitory assays indicated that the **Cpd 5** and **Cpd 12** having the inhibitory potential against the other MMPs than gelatinase (Table 3) whereas **Cpd 11** shows the lower specificity upon the inhibition of the other MMPs than gelatinase subgroup enzymes.

Table 2. Enzyme inhibitory activity and cytotoxic activity profile of pentanoic acids (**Cpds 5-12**)

Cpd[a]	MMP-2 (IC_{50})[b]	MMP-9 (IC_{50})[b]	Cytotoxicity (IC_{50})[c]				
			A-549	MDA-MB-231	K-562	U-937	PBMC
5	1.65±0.52	7.00±0.7	>500	>500	>500	>500	>1000
6	2.85±0.2	7.50±0.73	>500	>500	>500	>500	>1000
7	5.34±0.23	4.80±0.81	221.10±0.09	>500	>500	89.37±0.12	>1000
8	2.12±0.33	5.97±0.43	>500	>500	19.80±0.2	95.12±0.23	>1000
9	4.07±0.11	6.36±0.56	276.10±0.12	>500	19.70±0.32	94.01±0.19	>1000
10	8.52±0.04	5.14±0.03	>500	>500	42.45±0.23	93.76±0.05	>1000
11	4.80±0.03	6.80±0.04	205.80±0.1	162.00±0.13	17.90±0.04	32.14±0.07	>1000
12	0.21±0.02	6.37±0.07	342.60±0.09	174.80±0.09	23.41±0.02	102.33±0.03	>1000

[a]Compound number; [b]Activity in µM; [c]Values taken after 48 h of incubation

Table 3. MMP-1, MMP-8, MMP-12, MMP-14 enzyme inhibitory activity of pentanoic acids (**Cpds 5-12**)

Cpd[a]	Enzyme Inhibition (IC_{50})[c]			
	MMP-1	MMP-8	MMP-12	MMP-14
5	>500	198.9±0.12	100.1±0.92	76.3±0.04
6	>500	401.1±0.23	>500	>500
7	>500	298.5±0.03	109.1±0.03	87.1±0.06
8	>500	>500	493.2±0.53	301.1±0.1
9	>500	345.8±0.04	198.3±0.01	111.35±0.09
10	>500	483.1±0.32	>500	309.3±0.12
11	>500	>500	493.25±0.98	435.8±0.1
12	>500	172.80±0.14	95.2±0.88	109.0±0.02

[a]Compound number

2.3. Cytotoxicity assay

In cytotoxicity assay, cells were incubated with the synthesized compounds. After the exposure, MTT was added which forms crystal violet coloration in live cells after addition of DMSO. A gradual decrease in the absorbance of the crystal violet coloration indicated compound induced cellular death. All these synthesized compounds are screened against some cancer cell lines (A-549, MDA-MB-231, K-562 and U-937) for their possible cytotoxicity along with normal peripheral blood mononuclear cell (PBMC) to judge whether these analogs are cytotoxic or not. All these compounds are found to be inactive in normal cell line PBMC (> 1000 µM).

Regarding the cytotoxic activity in A-549 cell line, most of these compounds are inactive (**Cpds 5**, **6**, **8** and **10**) whereas some of them exhibit cytotoxicity in high doses (**Cpds 7**, **9, 11** and **12**). It is interesting to notice that compounds with either linear alkyl (straight chain) functions (such as ethyl, *n-butyl*, *n-pentyl*) or aryl function (such as benzyl) may retain A-549 inhibitory activity at high doses. Branching at the R position completely abolishes the activity profile as far as the A-549 inhibition is concerned (> 500 µM). The higher linear (straight) function (such as *n-pentyl*, **Cpd 11**) is the highest cytotoxic in A-549 cell line (IC_{50} = 205.8±0.1 µM) whereas incorporation of aryl function such as benzyl (**Cpd 12**) may reduce the activity (IC_{50} = 342.6±0.09 µM). Therefore, regarding the cytotoxicity in A-549 cell line, the higher linear (straight chain) alkyl substitution at R position (**Table 2**) is favored compared to the aryl function. Regarding MDA-MB-231 cell cytotoxicity, only the highly linear *n-pentyl* (**Cpd 11**) and the benzyl (**Cpd 12**) analogs retain cytotoxicity in high doses (IC_{50} = 162±0.13 and 174.8±0.09 µM respectively). Therefore, it may be assumed that *n-pentyl* substitution (**Cpd 11**) should be optimum to retain MDA-MB-231 cellular cytotoxicity in high dose.

Compared to A-549 and MDA-MB 231 cell lines, these compounds are found to be better effective in K-562 and U-937 cell lines. Regarding the cytotoxic profile in K-562 cell line, the SAR data suggests that the smaller groups such as hydrogen (**Cpd 5**), methyl (**Cpd 6**) and ethyl (**Cpd 7**) at the R position (**Table 2**) result in inactivity. Interestingly, branching at the R position with the *i-propyl* moiety (**Cpd 8**) result in effective inhibition against K-562 (IC_{50} = 19.8±0.2 µM) whereas increasing the length with the *i-butyl* function (**cpd 10**) at R position yields more than 2 folds reduction in cytotoxicity (IC_{50} = 42.45±0.23 µM). The corresponding linear alkyl function, i.e., *n-butyl* (**Cpd 9**) results in more or less similar activity (IC_{50} = 19.7±0.32 µM) compared to the *i*-propyl analog (**Cpd 8**).Increasing the chain length with the n-pentyl function (**Cpd 11**) at the R position is found to be similar active (IC_{50} = 17.9±0.04 µM) compared to the i-propyl (**Cpd 8**) and *n*-butyl analog (**Cpd 9**). Apart from linear and branched alkyl substitution at R position, interestingly, it is observed that the aryl function such as the benzyl moiety (**Cpd 12**) produces comparatively effective inhibition (IC_{50} = 23.41±0.02 µM). Therefore, it may be assumed that aryl function may be important at this position to maintain the cytotoxic profile whereas long chain alkyl functions (namely *n-pentyl*) impart the highest cytotoxicity as far as these glutamine analogs are concerned.

These compounds (**Cpds 5-12**) are also screened against U-937 cell line. Except **Cpd 5** and **6**, other analogs (**Cpds 7-12**) exhibit moderate activity against U-937 cell line though the cytotoxicity of these molecules (**Cpds 7-12**) over U-937 is found better compared to the cytotoxicity in A-549 and MDA-MB-231 cell lines. It is interesting to notice that compounds with no substitution and smaller alkyl (methyl) substitution (**Cpds 5-6)** are inactive in U-937 cell line whereas increasing the chain length and bulkiness either linear or branched alkyl functions (**Cpds 7-10**) at R position yield cytotoxic feature though no such difference is observed regarding the cytotoxicity among them. Interestingly, the bulky linear group at the R position (such as *n-pentyl:* **Cpd 11**) is found to exhibit 3 folds higher potency (IC_{50} =

32.14±0.07 µM) compared to the lesser alkyl functions (**Cpds 7-10**). Moreover, the aryl moiety such as the benzyl substitution (**Cpd 12**) at the R position (**Table 2**) yields more than 3 folds loss in U-937 cytotoxicity (IC_{50} = 102.33±0.03 µM) compared to the *n-pentyl* analog (**Cpd 11**).

Therefore, combining results of all these cytotoxic activities found in these different cell lines, it may be assumed that the *n-pentyl* analog (**Cpd 11**) is the best one regarding producing cytotoxic effects in all these four cell lines though it produces nonselectivity in MMP assay (**Table 2**). As a result, **Cpd 11** was taken into consideration for further biological screening.

2.4. DNA nick generation assay

The 4',6-diamidino-2-phenylindole (DAPI) was used to stain nicked DNA, if any. Nicked DNA showed increased fluorescence of DAPI, which bind with the nicked region of DNA. As **Cpd 11** shows the best cytotoxic efficacy in K-562 cell line, it was further studied in K-562 cells for DNA nick generation. After staining with DAPI the fixed cells either untreated or treated with **Cpd 11** were visualized under fluorescence microscope. No fluorescence was observed in K562 cell line (**Fig. 2A**). Detectable increased fluorescence of DAPI was found after 48 h in K562 cells when treated with increasing doses (2.5-10 µM) of **Cpd 11** (**Fig. 2B-2D**). This dose dependent increase in fluorescence of DAPI indicates that this **Cpd 11** bears the ability to bring nick in cellular DNA which is a hallmark feature of apoptosis.

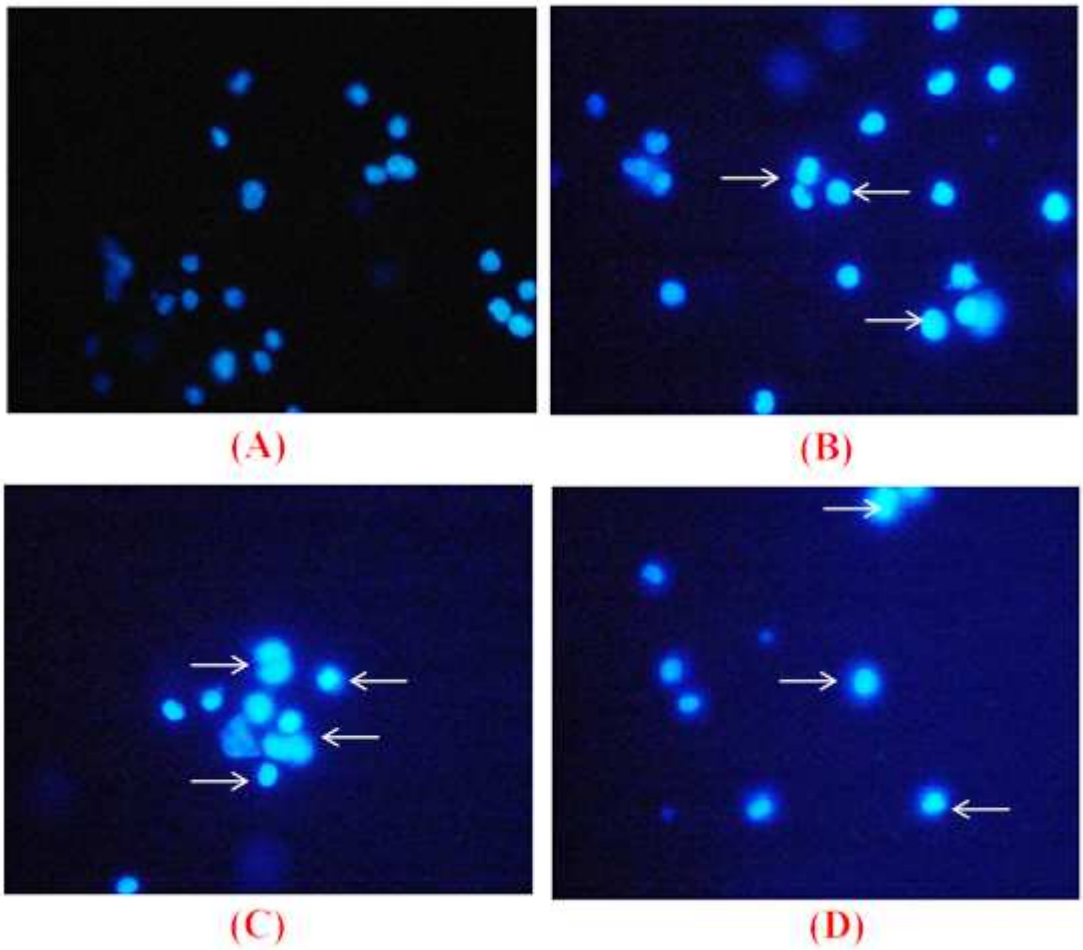


Fig. 2. Fluorescence imaging of K562 cells after DAPI staining. K562 cells was being treated with **Cpd 11** at the doses of (A) 0µM (B) 2.5µM (C) 5µM (D) 10µMand stained with DAPI to observe DNA nick generation. Arrow indicates nicked DNA with intense fluorescence.

2.5. Apoptotic assay after annexinV/propidium iodide staining

Flow cytometry by dual staining of annexin V and propidium iodide (PI) is a technique by which apoptotic or necrotic cell death percentage is being calculated. The increase in both annexin-V positive and PI negative cells indicate early apoptosis and both annexin-V and PI positive cells indicate late apoptosis when flow cytometric data are being analyzed after dual staining with annexin V-FITC and PI. The flow cytometric analysis reveals a progressive increase in the annexinV-FITC positive population of cells as compared to the untreated control (**Fig. 3**). **Cpd 11** induces almost upto 300 fold both early and late cellular apoptosis

when compared with the untreated control. This data suggests the state of drug induced apoptosis.

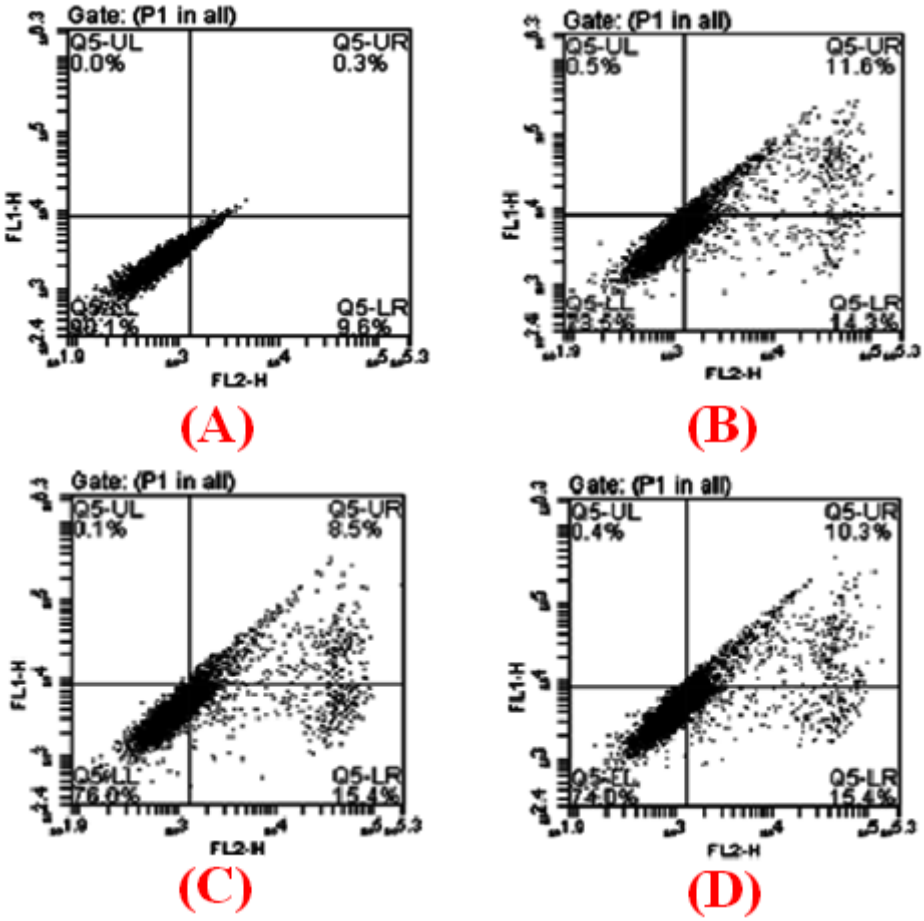


Fig. 3. AnnexinV-FITC/PI assay. Flow cytometric analysis after K562 either (A) untreated or treated with (B) 2.5 µM (C) 5 µM and (D) 10 µM doseof **Cpd 11**.

2.6. Cell cycle assay

Cell cycle stages were being identified through flow cytometry after PI staining followed by RNAse treatment. The increase in sub-G1 cell population in cell cycle indicated cellular apoptosis and arrest. In this experiment, flow cytometric analysis indicates a significant increase in sub diploid cell population (sub-G1) of K562 cell line after the treatment of the **Cpd 11**. An increase of sub-diploid cell population (sub-G1) from 1% (at 0 µM dose) to 4.6% (in 10 µM dose) suggests that the **Cpd 11** has the potentiality to block the cell progression and survivability by arresting the sub-G1cell cycle arrest (**Fig. 4)**.

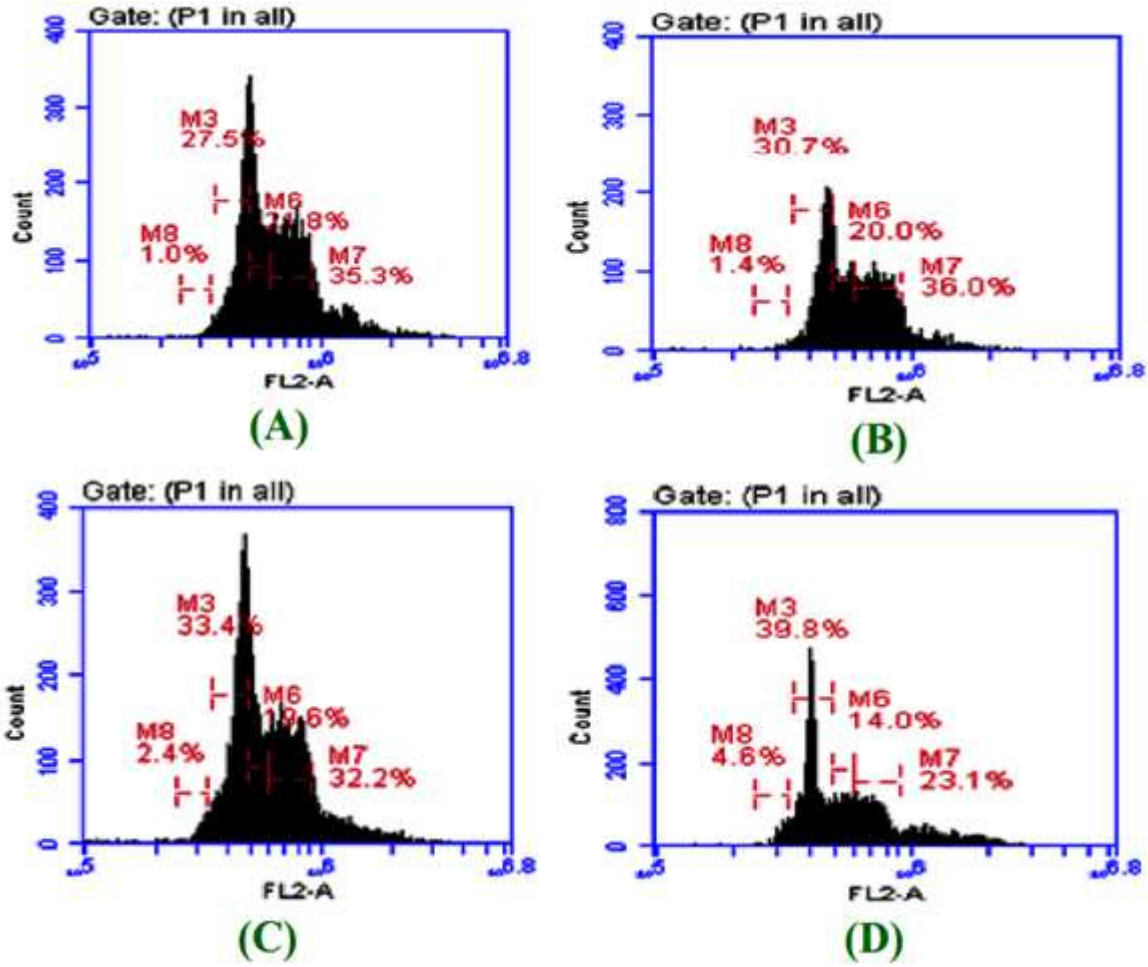


Fig. 4. Cell cycle analysis after PI staining. **Cpd 11** at the doses of (A) 0 µM (B) 2.5 µM (C) 5 µM and (D) 10 µM was given to K562 cells and sub-G1 cell cycle arrest (M8) was in flow cytometry after being stained with PI. G1, G1/S and G2/M stages are designated as M3, M6, M7 respectively.

2.7. Invasion assay of K562 cell line

The anti-invasiveness of the synthesized compounds was evaluated by a fluorimetric QCM EC Matrix Cell Invasion Assay kit (Millipore, USA) based on Boyden chamber principle. Modulation in the invasion process of K562 cell line was performed and the percentages of invaded cells are graphically presented in **Fig. 5A.** The inhibition was found to be 12.40±1.15%, 14.20±0.9%, 24.10±0.182% after induction of 2.5 µM, 5 µM and 10 µM of **Cpd 11** treatment respectively **(Fig. 5A)** which shows highly significant ($^{\$}P<0.001$) when

compared to untreated control (0±1.35%). These results indicate that the **Cpd 11** has the potentiality to be anti-invasive against K562 cell line.

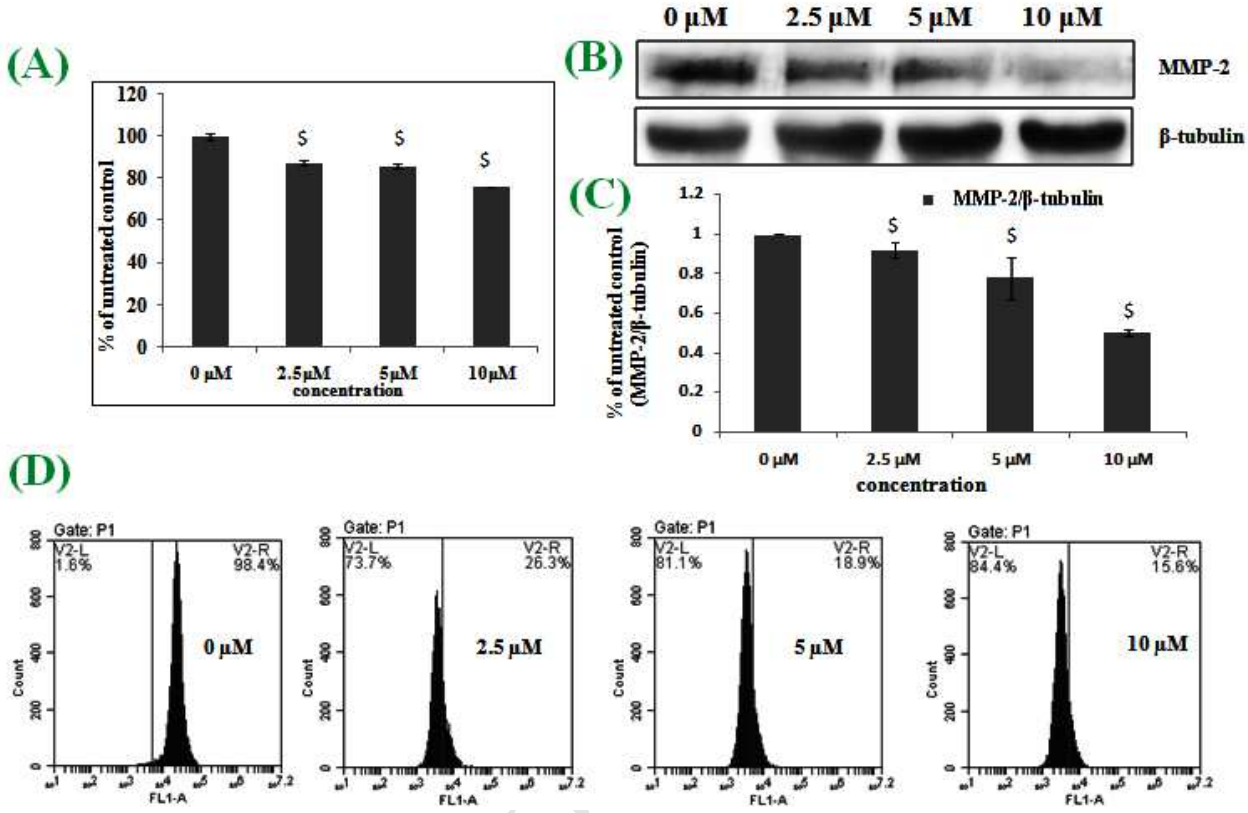


Fig. 5. Invasion assay and MMP-2 expression analysis. **(A)** Invasion assay of K562 cells after being treated with 0, 2.5, 5 and 10 µM of **Cpd 11** for 24 hours. Data are expressed as percentage of control and presented as mean ± SD. $^{\$}P<0.001$ were considered as statistically significant. **(B)** Western blots of MMP-2 and β-tubulin were being done. **(C)** Relative band intensities of MMP-2 and β-tubulin are calculated. β-tubulin was being used for loading correction. Data were expressed as percentage of control and presented as mean ± SD. $^{\$}P<0.001$ are considered as statistically significant. **(D)** Flow cytometric analysis of MMP-2 expression analysis. V2-L and V2-R were being considered as MMP-2 negative and MMP-2 positive cells respectively.

2.8. MMP2 expression assay and western blot analysis

MMP-2 expression was analyzed through western blot and flow cytometry. Western blot was done to confirm the **Cpd 11** induced downregulation of MMP-2. Results of western blot analysis indicated **Cpd 11** downregulated the expression of MMP-2 after 48 h of induction (**Fig. 5B**). Band intensity was found to be lowered after treatment of **Cpd 11** which is highly significant ($^{\$}P<0.001$) (**Fig. 5C**).

Furthermore, flow cytometry was done to check **Cpd 11** induced modulation of MMP-2 expression. Untreated or **Cpd 11** treated cells were fixed and stained with MMP-2 antibody. Thereafter, cells were stained with the secondary antibody labeled with fluorescein isothiocyanate (FITC). The MMP-2 positive cell population was found to be inhibited after treatment with **Cpd 11**. Flow cytometric data revealed that at 2.5 µM, 5 µM and 10 µM of **Cpd 11** treatment 26.30%, 18.90% and 15.60% cells were found to MMP-2 positive respectively whereas the untreated one showed 98.4% of MMP-2 positivity **(Fig. 5D)**. Therefore, this data indicates that the synthesized lead compound (**Cpd 11)** has the potentiality to inhibit the MMP-2 expression almost upto 80.10% at the highest dose (10 µM) which is significant.

2.9. Molecular docking study

The ligand-enzyme interaction study of these compounds (**Cpd 5-12**) with both MMP-2 and MMP-9 was done by molecular docking where the particular binding site and affinity of the ligand for the specific enzyme was calculated and interpreted. All these compounds were docked into the active site of MMP-2 and MMP-9 (**Fig. 6**).

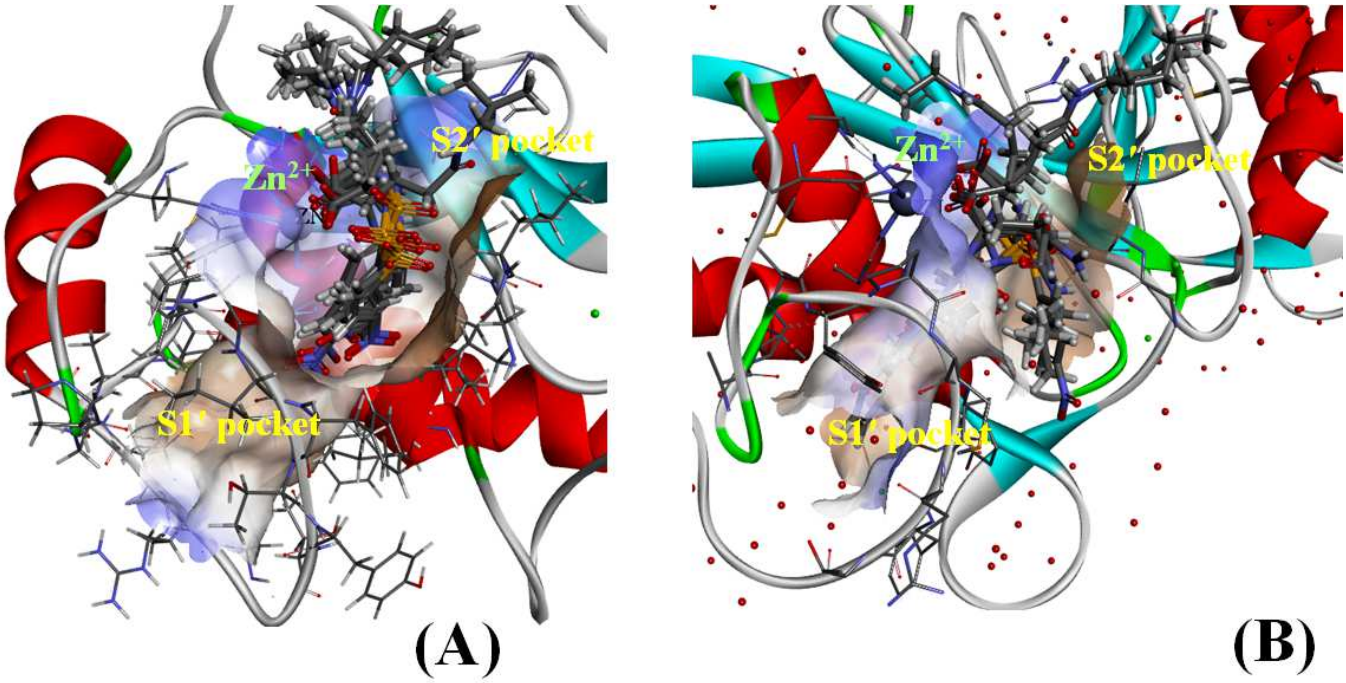


Fig. 6. Docked conformation of compounds (**Cpd 5-12**) along with the structure-based hydrophobic contour map in A) MMP-2 enzyme (pdb: 1HOV), B) MMP-9 enzyme (pdb: 2OW1)

Regarding the docking interactions of these compounds (**Cpd 5-12**) with both these enzymes, it is noticed that all these compounds coordinate with the catalytic Zn^{2+} ion at the active site of these enzymes through the carboxylic acid function (**Fig. 6A** and **Fig. 6B**). The 2D view of docking interactions for all these compounds (**Cpd 5-12**) with both MMP-2 and MMP-9 individually are shown in **Fig. 7** and **Fig. 8** respectively.

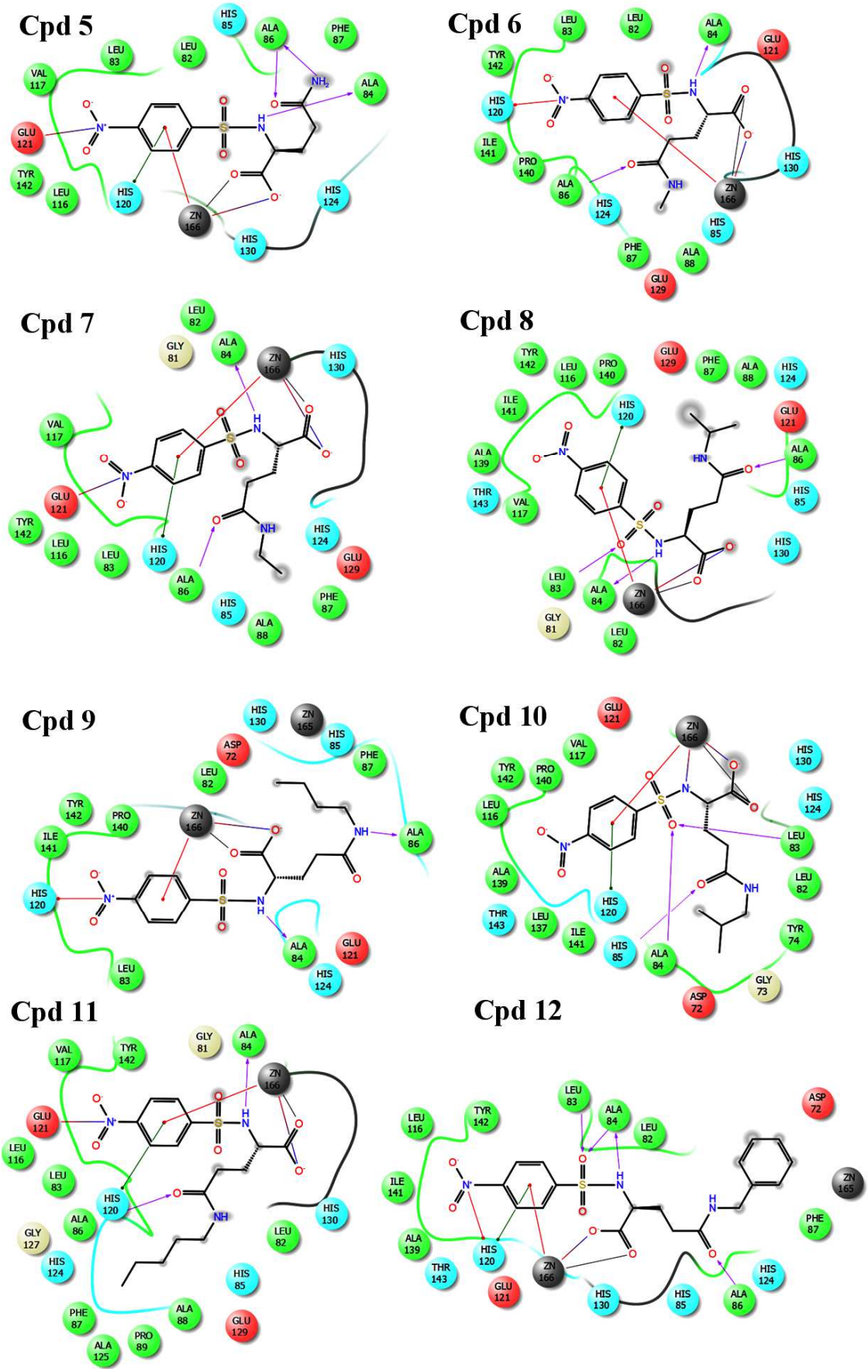


Fig. 7. Molecular docking interaction of compounds (**Cpd 5-12**) with MMP-2 enzyme (pdb: 1HOV)

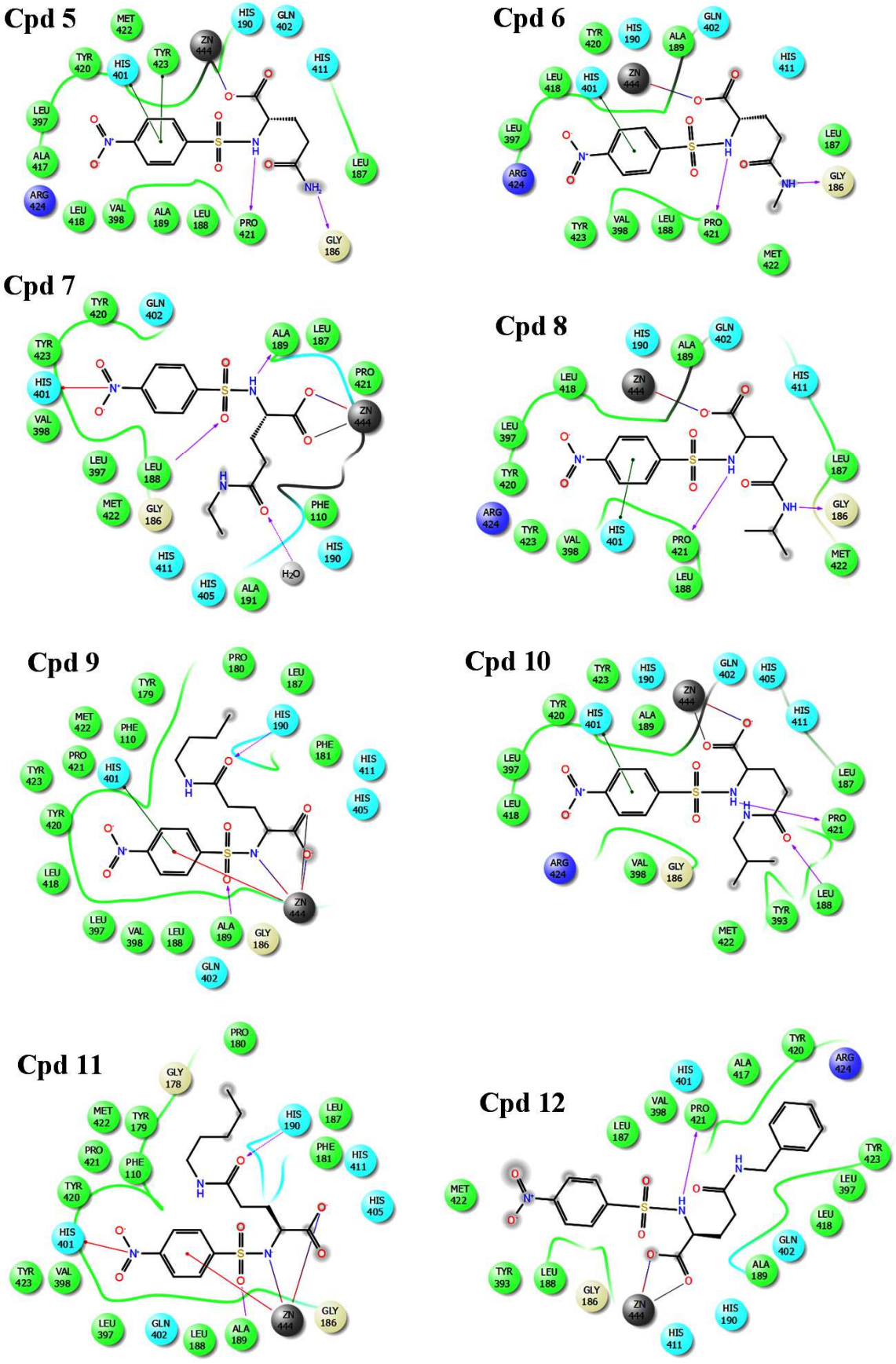


Fig. 8. Molecular docking interaction of compounds (**Cpd 5-12**) with MMP-9 enzyme (pdb: 2OW1)

Here, the ligand-enzyme interaction suggests that the carboxyl group of these compounds (**Cpd 5-12**) is making a salt bridge with the catalytic zinc for both MMP-2 (PDB: 1HOV) and MMP-9 (PDB: 2OW1) enzymes. In case of MMP-2, the carboxyl groups of these compounds (**Cpd 5-12**) interacts with Zn166 (**Fig. 7**) whereas the same carboxyl group interacts with Zn444 of MMP-9 (**Fig. 8**) at the enzyme active site. The *p*-Nitrophenylsulfonyl function enters into the hydrophobic S1′ pocket of MMP-2 enzyme formed by Leu82, Leu83, Val17, Tyr142, Ile141, Pro140, Ala139 and Leu116 (**Fig. 6** and **Fig. 7**) whereas the same function enters into the hydrophobic pocket formed by the amino acid residues namely Tyr423, Met422, Tyr420, Leu397, Ala417, Leu418, Val398 and Leu397 (**Fig. 8**). Regarding the docking interaction with the MMP-2 enzyme (**Fig. 7**), except **Cpd 6** and **9**, phenyl group associated with the sulphonyl function of other compounds (**Cpds 5**, **7**, **8**, **10**, **11** and **12**) forms a π-π interaction with His120 amino acid residue at the enzyme active site. Apart from π-π interaction, the phenyl group is found to form π-cation interaction with catalytic Zn^{2+} ion (**Fig. 7**). In some of these compounds (**Cpd 5**, **7**, **11**), the nitro group forms salt bridge interaction with the negatively charged Glu121 amino acid residue. Some compounds (**Cpd 6**, **9**, **12**) forms π-cation interaction with His120 by the nitro group substituted at the *para* position of the phenyl ring. Regarding the hydrogen bonding interaction, it is observed that one of the sulphonyl oxygen atoms acts as a hydrogen bond acceptor function through interacting with the backbone amino acid residues Leu83 and Ala84 for compounds 8, 10 and 12 (**Fig. 7**). Moreover, backbone amino acid Ala84 is also found to form a hydrogen bond with the amide function adjacent to the sulfonyl group (**Cpd 5-9**, **11-12**). The long chain alkyl functions associated with the carboxamide group tend towards the S2′ pocket surrounded by hydrophobic amino acid residues namely His85, Ala88, Pro89, Ala125 and Phe87 (**Fig. 6** and **Fig. 7**). Ala86 is another important backbone amino acid residue that is found to form hydrogen bonding interaction with the carbonyl group of the carboxamide

function (**Cpd 5-8**, **12**). Due to the higher flexibility of the higher alkyl function adjacent to the carboxamide group, the carbonyl group may form hydrogen bonding interaction with His85 (**Cpd 10**) or His120 (**Cpd 11**).

Regarding the molecular docking study of these compounds with MMP-9, The *p*-nitrophenyl group associated with the sulphonyl group enters into the hydrophobic S1′ pocket surrounded by amino acid residues Tyr423, His401, Tyr420, Leu397, Leu418, Val398 (**Fig. 6**). Moreover, the phenyl group is found to interact with His401 amino acid residue through π-π stacking interaction (**Cpd 5-6**, **8-10**). For **Cpd**s **7** and **11**, a π-cation interaction is noticed between the nitro group and His401 amino acid residue (**Fig. 8**). One of the sulphonyl oxygen atom forms hydrogen bonding interaction with backbone amino acids Leu188 (**Cpd 7**) or Ala89 (**Cpd 9**, **11**) (**Fig. 8**). Again, the amide group of the carboxamide moiety is found to form hydrogen bonding interaction with Gly186 (**Cpd 5-6**, **8**). The long chain alkyl function due to higher flexibility tends towards the hydrophobic S2′ pocket formed by amino acid residues namely Gly186, Leu187, Leu188, His190 and Gly178 (**Fig. 6** and **Fig. 8**).

3. Conclusion

In the present work, an MMP-2 targeted pentanoic acid having good cytotoxicity against leukemia cell line has been identified as the lead. Based on our earlier observations [13, 14], some new pentanoic acid analogs were designed and synthesized keeping in mind the solubility factor compared to the earlier analogs. All these analogs exhibited the gelatinase inhibition in lower micromolar concentration as it was presumed that these analogs were structurally related to the earlier reported compounds. The major issue is to judge whether the cytotoxicity has been achieved or not by incorporating the nitro function in the phenyl ring at the para position of the aryl moiety. Interestingly, these newer analogs exhibited higher cytotoxicity compared to the earlier reported analogs [14] though these newer analogs showed lesser gelatinase inhibition over the earlier reported compounds. Among all these

synthesized compounds, the *n*-pentyl analog (**Cpd 11**) may be considered as the lead as this compound produces comparatively better cytotoxic profile over other analogs in this series. Fluorescence imaging indicated that **Cpd 11** induced nicked DNA of K562 cell line which is a hallmark of apoptosis. Flow cytometric analysis studies also reveal that the compound (**Cpd 11**) induced cytotoxicity was apoptotic in nature as a significant number of cells are annexinV positive. This apoptotic efficacy is further being confirmed by cell cycle assay as the n-pentyl analog (**Cpd 11**) may arrest the sub-G1 stage of cell cycle in a significant proportion. Besides the apoptotic property, the **Cpd 11** is being found to block the invasion of K562 cell line as evidenced by the matrigel invasion assay.

Molecular docking analysis reveals a significant interaction of this compound with both MMP-2 and MMP-9. However, the enzymatic study indicates all these compounds including **Cpd 11** to have more or less inhibitory activity only on MMP-2 over MMP-9. Besides this, flow cytometry and western blot analysis show significant inhibition of MMP-2 expression after **Cpd 11** exposure against K562 cell line. Early studies of several workers have suggested that MMP-2 downregulation might be linked with the anti-invasive and apoptotic cell death property of cancer cells [15-19]. Therefore, our compound (**Cpd 11**) which is found to be a nonspecific inhibitor of MMP-2 and MMP-9 might lead to generate the anti-invasive and apoptotic nature of the compound against K562 cell line. As all these molecules have better solubility compared to the earlier reported molecules [14], these may exhibit better cytotoxicity against cancer cell lines such as K-562 and U-937. However, their gelatinase inhibitory activity may be lower as gelatinase inhibition may be mainly dependent on the bulky hydrophobic aryl group directed towards the S1′ pocket. Due to the presence of carboxylic acid function as zinc binding group, these molecules exhibit gelatinase inhibition but due to the presence of the *p*-nitrophenyl group instead of bulky higher aryl function, these molecules show lower gelatinase inhibition. Therefore, the lower hydrophobic character is

the main reason controlling the cytotoxicity of these molecules. Even though all these compounds (**Cpd 5-12**) exhibit lower gelatinase inhibition, compounds with higher linear or branched chain alkyl and aryl functions (**Cpd 8-12**) exhibit cytotoxicity against K-562 and U-937 cell lines. Interestingly, it may be the long chain alkyl (*n*-pentyl) function of **Cpd 11** that may be able to bind properly with both these MMP-2 and MMP-9 enzymes and produce nonselective gelatinase inhibition. The unique elongated linear *n*-pentyl function may, therefore, be able to exhibit cytotoxic feature along with the downregulation and reduced expression of the MMP-2 enzyme. Therefore, this study may help to validate the mechanism of MMP-2 inhibition mediated cytotoxicity against chronic myeloid leukemia cell line (K562). Regarding this, there is no significant compound has been reported so far which is found to be a potent MMP-2 inhibitor and also an apoptosis inducing agents against leukemia cells [9-12]. Therefore, this study particularly emerges the significance. Furthermore, this analysis may be taken into consideration for advance exploration of the lead candidate to design the better gelatinase inhibitor with the higher cytotoxic profile in future.

4. Experimental section

4.1. Chemicals and reagents

All major chemicals/culture/antibodies media were of the analytical/extra-pure grade. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin-neomycin (PSN) antibiotics, trypsin and ethylenediamine tetraacetic acid (EDTA) were obtained from Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were purchased from BD Bioscience (USA).

All organic solvents used were of HPLC grade. [3-(4, 5-dimethyl-thiazol-2-yl)-2, S-diphenyltetrazolium bromide (MTT), propidium iodide (PI), 4', 6-diamidino-2- phenylindole (DAPI) etc were purchased from Sigma Aldrich (USA). Annexin V-FITC apoptosis detection kit was purchased from Calbiochem, Germany. The MMP-2 and MMP-9 enzyme

assay kit were purchased from Enzo Life Science International, Inc. (Plymouth Meeting, PA, USA). Anti-MMP-2 monoclonal antibodies and anti-rabbit secondary antibody were purchased from Santa Cruz Biotechnology Inc, USA. The primary antibodies of β-tubulin and FITC-conjugated secondary antibody were obtained from Cell Signalling Tech (USA). The DAB substrate system was purchased from Amresco, USA.

4.2. Synthesis

The designed compounds were synthesized and characterized. Chemical reactions were checked by analytical thin layer chromatography (TLC) using silica gel G plates (TLC silica gel $GF_{254,}$ Merck, Germany). Spots were developed using iodine chamber. Melting points were uncorrected and measured on a capillary melting point apparatus and mel-temp electro-thermal apparatus. These were checked in CTRONICS digital melting point apparatus. Optical rotations of compounds were seen in Parkin-Elmer type 141 polarimeter. Mass spectroscopic analysis was done in an LC MS/MS instrument [The LC, Agilent coupled to Electrospray ion (ESI) mass spectrometer having Mass Hunter Quantitative Analysis software], Nuclear magnetic resonance (NMR) spectra were obtained from an AC Bruker 400 MHz FT-NMR spectrometer with tetramethylsilane (TMS) as the internal standard. Compounds were dissolved in dimethyl sulfoxide-d6 (DMSO-d6). Splitting patterns are mentioned as s (singlet), d (doublet), t (triplet) and m (multiplet). ^{13}C NMR of these compounds was performed in Bruker 400 MHz FT-NMR spectrometer. Infrared (IR) spectra were recorded on a Bruker alpha 11960095 FT-IR instrument. Elemental analyses of these compounds were conducted in a PerkinElmer 2400 Series II CHNS/O Elemental Analyzer. As the diacid (**3**) and the monoacid (**4**) were reported earlier [20], general procedures for preparations of these compounds are not discussed here.

*4.2.1. General procedure for preparation of pentanoic acid analogs (**Cpd 5-12**):*

The monoacid (**4**) was subjected to amination with some amines to get the desired products (**Cpd 5-12**). In a conical flask, a slurry of the monoacid compound (**4**) was prepared in 20 ml distilled water. Excess amines are added individually to these suspensions. The reaction mixture was kept overnight and heated on a water bath to remove the unreacted excess amines. Finally, the mixture was cooled down to room temperature. 6(N) hydrochloric acid was added dropwise to the reaction mixture in an ice bath for liberation of pentanoic acids. The precipitate was filtered off and washed with cold water thoroughly to obtain the desired compounds (**Cpd 5-12**). These compounds were recrystallized with hot ethanol with charcoal treatment.

All the final products were characterized through mass spectroscopy, ^{1}H NMR, ^{13}C NMR and FTIR analysis as well as elemental analysis. These analytical data are given below:

*4.2.2. (2S)-5-amino-5-oxo-2-[(nitrobenzene-4-yl sulfonyl) amino] pentanoic acid (**5**)*

Yield 63.24%, mp 181-183^{o}C, MS (ESI Positive) m/z [M+H^{+}]: 332.06. ^{1}H NMR (DMSO-d6, 400 MHz, ppm): δ 8.40-8.37 (d, *J* = 8.8 Hz, 2 Ar-H), δ 8.02-8.00 (d, *J* = 8.8 Hz, 2 Ar-H), δ 7.26 (m, 1H, SO_2NH), δ 6.75 (s, 2H, $CONH_2$), δ 3.78 (m, 1H, CH), δ 2.09-2.05 (t, *J* = 7.2 Hz, 2H, CH_2), δ 1.91-1.63 (m, 2H, CH_2). ^{13}C NMR (DMSO-d6, 101 MHz, ppm) δ 173.47, 172.80, 149.84, 147.18, 128.54, 124.80, 55.86, 31.08, 28.14. Anal. Calc. for $C_{11}H_{13}N_3O_7S$: C, 39.88; H, 3.96; N, 12.68; S, 9.68. Found. C, 40.14; H, 3.79; N, 12.89; S, 9.86. FTIR (KBr, cm^{-1}): 3324 (NH str of SO_2NH), 3125 (NH str Of CONH), 3041 (Ar-C-H str), 2857, 2825 (ali C-H str), 1685 (CO str of COOH), 1565, 1518 (N=O str of Ar-NO_2Asym.), 1428 (ali C-H def), 1334, 1228 (S=O str of SO_2NH), 855, 834, 798, 724 (Ar C-H def)

*4.2.3. (2S)-5-oxo-2-[(nitrobenzene-4-yl sulfonyl) amino]-5-(methylamino) pentanoic acid (**6**)*

Yield 81.91%, mp 206-208^{o}C, MS (ESI Positive) m/z [M+H^{+}]: 346.10, [M+H^{+}+Na^{+}] 368.10. ^{1}H NMR (DMSO-d6, 400 MHz, ppm): δ 8.61-8.59 (m, 1H, CONH), δ 8.40-8.38 (d, *J* = 8.8 Hz, 2 Ar-H), δ 8.02-8.00 (d, *J* = 8.8 Hz, 2 Ar-H), δ 7.70-7.69 (m, 1H, SO_2NH), δ 3.80-3.79

(m, 3H, CH_3), δ 3.45-3.40 (m, 1H, CH), δ 2.09-2.05 (t, *J* = 7.6 Hz, 2H, CH_2), δ 1.94-1.62 (m, 2H, CH_2). ^{13}C NMR (DMSO-d6, 101 MHz, ppm) δ 172.79, 171.70, 149.84, 147.13, 128.51, 124.79, 55.76, 31.24, 28.20, 25.88. Anal. Calc. for $C_{12}H_{15}N_3O_7S$: C, 41.74; H, 4.38; N, 12.17; S, 9.29. Found. C, 41.42; H, 4.69; N, 12.45; S, 9.51. FTIR (KBr, cm^{-1}): 3372 (NH str of SO_2NH), 3254 (NH str of CONH), 3068 (Ar CH str), 2934 (ali CH strAsym), 2867 (ali CH str), 1712 (CO str of COOH), 1582 (N=O str of Ar-NO_2Asym.), 1428 (ali C-H def), 1298 (asym. S=O str of SO_2NH), 1137 (sym. S=O str of SO_2NH), 958, 865, 826, 756 (Ar C-H def)

*4.2.4. (2S)-5-oxo-2-[(nitrobenzene-4-yl sulfonyl) amino]-5-(ethylamino) pentanoic acid (**7**)*

Yield 78.72%, mp 219-221^{o}C, MS (ESI Positive) m/z [M+H^+]: 360.0, [M+H^++Na^+] 382.0. ^{1}H NMR (DMSO-d6, 400 MHz, ppm): δ 8.61-8.59 (m, 1H, CONH), δ 8.40-8.38 (d, *J* = 8.8 Hz, 2 Ar-H), δ 8.02-8.00 (d, *J* = 8.4 Hz, 2 Ar-H), δ 7.78-7.75 (m, 1H, SO_2NH), δ 3.83-3.78 (m, 1H, CH), δ 3.05-2.95 (m, 2H, N-CH_2-1), δ 2.11-2.01 (m, 2H, CH_2), δ 1.94-1.62 (m, 2H, CH_2), δ 0.98-0.94 (t, *J* = 7.2 Hz, 3H, CH_3). ^{13}C NMR (DMSO-d6, 101 MHz, ppm) δ 172.78, 170.90, 149.83, 147.19, 128.53, 124.79, 55.80, 33.75, 31.38, 28.26, 15.09. Anal. Calc. for $C_{13}H_{17}N_3O_7S$: C, 43.45; H, 4.77; N, 11.69; S, 8.92. Found. C, 43.83; H, 5.07; N, 12.03; S, 8.76. FTIR (KBr, cm^{-1}): 3334 (NH str of SO_2NH), 3220 (NH str of CONH), 3015 (Ar CH str), 2845, 2812 (ali CH str), 1674 (CO str of COOH), 1563 (N=O str of Ar-NO_2Asym), 1462 (ali C-H def), 1266 (asym. S=O str of SO_2NH), 1134 (sym. S=O str of SO_2NH), 943, 862, 824, 786 (Ar C-H def)

*4.2.5. (2S)-5-oxo-2-[(nitrobenzene-4-yl sulfonyl) amino]-5-(isopropylamino) pentanoic acid (**8**)*

Yield 92.60%, mp 235-237^{o}C, MS (ESI Positive) m/z [M+H^+]: 374.0. ^{1}H NMR (DMSO-d6, 400 MHz, ppm): δ 8.59 (m, 1H, CONH), δ 8.40-8.38 (d, *J* = 8.8 Hz, 2 Ar-H), 8.02-8.00 (d, *J* = 8.8 Hz, 2 Ar-H), 7.68-7.66 (m, 1H, SO_2NH), 3.81-3.78 (m, 1H, CH of *i*-Propyl), 3.74-3.71

(m, 1H, CH), 2.08-2.04 (t, *J* = 7.8 Hz, 2H, CH_2), 1.93-1.61 (m, 2H, CH_2), 1.01-0.98 (m, 6H, $2CH_3$). ^{13}C NMR (DMSO-d6, 101 MHz, ppm) δ 172.74, 170.26, 149.82, 147.19, 128.54, 124.75, 55.83, 40.66, 31.51, 28.34, 22.76. Anal. Calc. for $C_{14}H_{19}N_3O_7S$: C, 45.03; H, 5.13; N, 11.29; S, 8.59. Found. C, 45.23; H, 4.85; N, 10.88; S, 8.78. FTIR (KBr, cm^{-1}): 3360 (NH str of SO_2NH), 3246 (NH str of CONH), 3025 (Ar CH str), 2886 (ali CH strAsym), 1695 (CO str of COOH), 1549, 1519 (C=C str of Ar), 1314 (S=O str of SO_2NH), 1261 (CO str and OH def of COOH), 1125 (sym. S=O str of SO_2NH), 824, 733 (Ar C-H def)

*4.2.6. (2S)-5-oxo-2-[(nitrobenzene-4-yl sulfonyl) amino]-5-(butylamino) pentanoic acid (**9**)*

Yield 89.25%, mp 209-211^{o}C, MS (ESI Positive) m/z [M+H^+]: 388.0. ^{1}H NMR (DMSO-d6, 400 MHz, ppm): δ 8.60-8.59 (m, 1H, CONH), 8.40-8.38 (d, *J* = 8.4 Hz, 2 Ar-H), 8.02-8.00 (d, *J* = 8.8 Hz, 2 Ar-H), 7.76-7.73 (m, 1H, SO_2NH), 3.81 (m, 1H, CH), 3.00-2.94 (m, 2H, N-CH_2-1), 1.93-1.61 (m, 2H, CH_2), 1.34-1.21 (m, 4H, N-CH_2-2 and N-CH_2-3), 0.87-0.83 (t, *J* = 7.0 Hz, 3H, CH_3). ^{13}C NMR (DMSO-d6, 101 MHz, ppm) δ 172.78, 171.05, 149.80, 147.21, 128.54, 124.75, 55.82, 38.58, 31.61, 31.41, 28.34, 19.99, 14.05. Anal. Calc. for $C_{15}H_{21}N_3O_7S$: C, 46.50; H, 5.46; N, 10.85; S, 8.28. Found. C, 46.27; H, 5.32; N, 11.18; S, 8.16. FTIR (KBr, cm^{-1}): 3312 (NH str of SO_2NH), 3120 (NH str of CONH), 2965, 2935 (Ar CH str), 2833 (ali CH str), 1682 (CO str of COOH), 1512 (C=C str of Ar), 1298, 1185 (S=O str of SO_2NH), 842, 798, 735 (Ar C-H def)

*4.2.7. (2S)-5-oxo-2-[(nitrobenzene-4-yl sulfonyl) amino]-5-(isobutylamino) pentanoic acid (**10**)*

Yield 76.86%, mp 204-206^{o}C, MS (ESI Positive) m/z [M+H^+]: 388.10. ^{1}H NMR (DMSO-d6, 400 MHz, ppm): δ 8.61-8.59 (m, 1H, CONH), 8.40-8.38 (d, *J* = 8.8 Hz, 2 Ar-H), 8.02-8.00 (d, *J* = 8.8 Hz, 2 Ar-H), 7.79-7.77 (m, 1H, SO_2NH), 3.81-3.80 (m, 1H, CH), 2.84-2.77 (m, 2H, N-CH_2-1), 2.13-2.09 (t, *J* = 7.4 Hz, 2H, CH_2), 1.94-1.85 (m, 1H, CH of *i*-Butyl), 1.71-

1.58 (m, 2H, CH_2), 0.81-0.79 (m, 6H, $2CH_3$). ^{13}C NMR (DMSO-d6, 101 MHz, ppm) δ 172.77, 171.18, 149.82, 147.21, 128.55, 124.78, 55.84, 46.45, 31.42, 28.46, 28.42, 20.53. Anal. Calc. for $C_{15}H_{21}N_3O_7S$: C, 46.50; H, 5.46; N, 10.85; S, 8.28. Found. C, 46.79; H, 5.82; N, 10.48; S, 8.54. FTIR (KBr, cm^{-1}): 3325 (NH str of SO_2NH), 3155 (NH str of CONH), 3036 (Ar CH str), 2871, 2826 (ali CH strAsym), 1702 (CO str of COOH), 1562 (N=O str of Ar-NO_2), 1438 (C=C str of Ar), 1316, 1137 (S=O str of SO_2NH), 916, 872, 794, 712 (Ar C-H def)

*4.2.8. (2S)-5-oxo-2-[(nitrobenzene-4-yl sulfonyl) amino]-5-(pentylamino) pentanoic acid (**11**)*

Yield 86.82%, mp 215-217^oC, MS (ESI Positive) m/z [$M+H^+$]: 402.00, [$M+H^++Na^+$] 424.00. ^1H NMR (DMSO-d6, 400 MHz, ppm): δ 8.60-8.58 (m, 1H, CONH), 8.40-8.38 (d, *J* = 8.8 Hz, 2 Ar-H), 8.02-8.00 (d, *J* = 8.8 Hz, 2 Ar-H), 7.76-7.74 (m, 1H, SO_2NH), 3.81-3.80 (m, 1H, CH), 3.02-2.90 (m, 2H, N-CH_2-1), 2.10-2.04 (m, 2H, CH_2), 1.93-1.61 (m, 2H, CH_2), 1.37-1.16 (m, 6H, N-CH_2-2, N-CH_2-3 and N-CH_2-4), 0.87-0.83 (t, *J* = 7.0 Hz, 3H, CH_3). ^{13}C NMR (DMSO-d6, 101 MHz, ppm) δ 172.78, 171.01, 149.82, 147.21, 128.54, 124.78, 55.82, 38.87, 31.40, 29.19, 29.05, 28.34, 22.28, 14.36. Anal. Calc. for $C_{16}H_{23}N_3O_7S$: C, 47.87; H, 5.77; N, 10.47; S, 7.99. Found. C, 46.60; H, 5.59; N, 10.08; S, 8.26. FTIR (KBr, cm^{-1}): 3362 (NH str of SO_2NH), 3192 (NH str of CONH), 3025 (Ar CH str), 2865 (ali CH str), 1683 (CO str of COOH), 1582, 1525 (N=O str of Ar-NO_2), 1437 (Ali C-H def), 1358, 1142 (S=O str of SO_2NH), 968, 878, 826, 741 (Ar C-H def)

*4.2.9. (2S)-5-oxo-2-[(nitrobenzene-4-yl sulfonyl) amino]-5-(benzylamino) pentanoic acid (**12**)*

Yield 88.24%, mp 191-193^oC, MS (ESI Positive) m/z [$M+H^+$]: 422.00. ^1H NMR (DMSO-d6, 400 MHz, ppm): δ 8.61 (m, 1H, CONH), 8.39-8.37 (d, *J* = 8.8 Hz, 2 Ar-H), 8.34-8.32 (m, 1H, SO_2NH), 8.02-7.99 (d, *J* = 8.4 Hz, 2 Ar-H), 7.32-7.28 (t, *J* = 7.0 Hz, 2 Ar-H of benzyl), 7.24-7.20 (t, *J* = 7.4 Hz, 3 Ar-H of benzyl), 4.22-4.20 (m, 2H, CH_2 of benzyl), 3.83 (m, 1H,

CH), 2.19-2.16 (t, *J* = 7.4 Hz, 2H, CH_2), 1.96-1.68 (m, 2H, CH_2). ^{13}C NMR (DMSO-d6, 101 MHz, ppm) δ 172.76, 171.32, 149.84, 147.21, 139.95, 128.70, 128.54, 127.60, 127.17, 124.79, 55.84, 42.47, 31.40, 28.33. Anal. Calc. for $C_{18}H_{19}N_3O_7S$: C, 51.30; H, 4.54; N, 9.97; S, 7.61. Found. C, 51.45; H, 4.89; N, 10.13; S, 7.93. FTIR (KBr, cm^{-1}): 3307 (NH str of SO_2NH), 3112 (NH str of CONH), 3026 (Ar CH str), 2834, 2807 (ali CH str), 1695 (CO str of COOH), 1532 (N=O str of Ar-NO_2), 1345, 1178 (S=O str of SO_2NH), 845, 798, 724 (Ar C-H def)

4.3. Matrix metalloproteinase (MMP) inhibition assay

MMPs (MMP-2, MMP-8, MMP-9, MMP-12 and MMP-14) inhibition assays were carried out using MMP inhibitor profiling kits (Enzo Life Science International, Inc., Plymouth Meeting, PA, USA) following the manufacturer's protocol. The concentration of chromogenic substrates was detected by its absorbance value at 410 nm in a microplate photometer (Thermo Scientific Multiscan FC, USA). Enzyme reactions were performed at 37^0 C for 1 h in a 100 ml final volume of solutions with at least six concentrations of inhibitors (except the control) in triplicate. After addition of the substrate, the increase in absorbance was recorded at 1 min time intervals for 30 minutes at 410nm with the help of the microplate photometer (Thermo Scientific Multiscan FC, USA). An inhibitor, N-isobutyl-N-(4-methoxyphenylsulfonyl)glycylhydroxamic acid (NNGH) was included as a prototype control inhibitor. The concentrations of compounds that provide 50% inhibition of enzymatic activity (IC_{50}) were determined by semi-logarithmic dose-response plots.

4.4. Cell lines

Human cancer cell line, A549 (non-small cell lung cancer cell line), MDA-MB-231 (breast cancer cell line), K562 (chronic myeloid leukemia cell line), U937 (acute leukemia cell line) were collected from National Centre for Cell Science, Pune, India. Human normal peripheral blood mononuclear cell (PBMC) was isolated from normal healthy human. A549, and MDA-

MB-231 cells were cultured in DMEM and K562, U937 and PBMC were cultured in RPMI-1640 with 10% heat inactivated foetal bovine serum (FBS) and 1% antibiotic (PSN) and maintained at 37ºC with 5% CO_2 in a humidified incubator. Adherent cells (A549 and MDA-MB-231) were harvested with 0.025% trypsin and 0.52 mM EDTA in phosphate buffer saline, plated at required cell numbers and allowed to adhere for minimum ~ 24h before treatment.

4.5. Treatment with the synthesized compounds

For MTT assay, the cell lines were exposed to the synthesized compounds (**Cpd 5-12**) with different concentrations at 37^0C in DMEM or RPMI-1640 supplemented with the serum. As positive control, equal volume of medium was added to the untreated cells.

4.6. Cell viability assay

Cell viability was determined using MTT assay. Briefly, A549, MDA-MB-231, K562, U937 and PBMC cells were seeded in 96 well plates at a density of 1×10^3 cells per well. These cells were allowed to settle for 24 h before treatment. The cells were treated with synthesized compounds (**Cpd 5-Cpd 12**) for 48 h with 5% CO_2 at 37^0C. Cell viability was measured by MTT assay. The control values corresponding to the untreated cells were taken as 100% and the viability data of the treated ones were expressed as the percentage of control. The IC_{50} values were determined as the concentration that reduced the cell viability by 50%. Among all the synthesized compounds, the lead compound (**Cpd 11**) was found after analyzing the IC_{50} values.

4.7. DNA nick generation assay

K562 cells (~2×10^4) were plated in 40 mm culture dish and allowed to grow for 24 h. Both untreated and treated (by **Cpd 11** at the dose of 2.5, 5 and 10 µM) cells were collected and fixed with 70% chilled ethanol for 1 h. Thereafter, the fixed cells were rinsed twice with PBS

and stained with 10 µM DAPI for 30 min to determine drug induced DNA nick generation, if any, under a fluorescence microscope (Zeiss Axiovert 40 CFL).

4.8. Apoptotic assay by flow cytometry after annexin V/PI staining

The apoptosis assay was done by using an annexin V-FITC apoptosis detection kit which was purchased from Calbiochem, Germany. Briefly, the K562 cells (~2×10^4) were treated with the **Cpd 11** for 48 h. Cells were harvested and washed with phosphate buffer saline (PBS). The harvested cells were incubated with annexin V-FITC (100 ng/ml) and PI (50 µg/ml) at room temp for 15 min in the dark and analyzed using a FACS Calibur (BD Bioscience) taking minimum 10,000 cells in each sample.

4.9. Cell cycle analysis

Briefly, ~2×10^4 K562 cells were seeded and treated with the **Cpd 11** for 24 h. Cells were recovered, washed twice with cold PBS and fixed in 70% chilled ethanol. Cells were washed twice in PBS, incubated for 1 h at room temp with 100µg/ml RNAse A. After that, 50 µg/ml PI was added and cells were incubated for 15 min in the dark and were analyzed using a FACS calibur flow cytometer (BD Bioscience) [21]. Ten thousand events were analysed for each sample using the appropriate gating to select single cell population and the same gate was used for all these samples.

4.10. Invasion assay

Inhibitory effect of the **Cpd 11**, if any, on K562 cells was estimated through fluorimetric QCM EC Matrix cell invasion assay (ECM 555, Millipore). Briefly, K562 cells ($1X10^6$ cells/ml) were starved overnight in FBS-free media. The cells were harvested and resuspended in each well of the insert within FBS free media with 5% bovine serum albumin (BSA). The lower tray which is called the feeder tray contained media with 5% FBS as a chemoattractant. The **Cpd 11** with the concentrations of 2.5, 5 and 10 µM was added in

triplicates to these cells and incubated for 24 h taking an untreated control. Invasive cells are able to invade through a basement layer of matrix membrane solution and cross the pores of polycarbonate membranes to adhere at the bottom of the inserts. The cell detachment solution was used to dissociate the adhered cells. CyQuantGR dye was used to detect the detached cells. The fluorescence was measured with a multi-mode microplate reader at 480 nm excitation and 520 nm emission (SpectraMax, Molecular Devices, USA) spectra.

4.11. MMP-2 expression analysis

The K562 cells ($1x10^4$cells/ml) were plated and after 24 h, incubated with 2.5, 5, 10 µM of **Cpd 11** (except the untreated control). After 48 h of the compound treatment cells were collected and washed with PBS and fixed in 70% chilled ethanol. After 1 hour of fixation, cells were washed with PBS and exposed to blocking solution (PBS containing 5% bovine serum albumin and 0.2% Tween 20) for 1 h at room temp. The cells were washed with PBS and incubated overnight with anti- MMP-2 primary antibody (1: 250 dilutions), washed with PBS and subsequently treated with the secondary antibody tagged with FITC (1:250 dilutions) for 1 h [13, 14, 22]. Thereafter, the cells were washed once with PBS and analyzed in flow cytometry for MMP2 expression modulation, if any.

4.12. Cell extract and protein isolation

K562 cells (~2×10^6) were plated in 90 mm culture dish and were allowed to grow for 48 h. Following the **Cpd 11** treatment, cells were collected and washed twice with ice cold PBS. These cells were lysed in 20 µl of ice-cold lysis buffer, made up of 10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 0.5% CHAPS and 10% glycerol. Cells were incubated for 30 min on ice and centrifuged for 30 min at 5000 g at 4^0C. After centrifugation, the supernatant was collected and stored at -20^0 C for further use.

4.13. Western blot analysis

For western blot analyses, an equal amount (50 µg) of the protein was loaded and samples were denatured in 12% SDS-PAGE for MMP2 and 15% for β tubulin. The separated proteins were transferred separately onto PVDF membranes and were probed with anti-MMP-2 (1:1000), and anti- β tubulin (1:1000) primary antibodies overnight at 4^0C followed by 1 h incubation with ALKP conjugated secondary antibody (1:500) and were developed by DAB substrate system [23]. β tubulin was used as housekeeping gene control. Immunoblot of proteins of interest was analyzed in three separate set of experiments. Quantification of proteins by calculating the band intensities was done by densitometry using image J software [24].

4.14. Molecular docking study

Molecular docking study of all these molecules (**Cpd 5-12**) was performed by Glide docking tool [25-26] of Schrödinger [27] software. The NMR solution structure of MMP-2 (PDB ID: 1HOV) and the crystal structure of MMP-9 (PDB ID: 2OW1), collected from Protein Data Bank [28], were considered for predicting the binding pattern of these molecules (**Cpd 5-12**) at the active site of these enzymes. These compounds were energy minimized and prepared by using the *LigPrep* module of Schrodinger software. By using Epik-based ionization process, all probable ionization states of each molecule were generated. These enzymes (MMP-2 and MMP-9) were minimized using the OPLS2005 force field and the *protein preparation wizard* of Schrodinger software for docking study. Zinc coordinating histidine amino acid residue was assigned an epsilon protonation state whereas the two other histidines were provided a delta protonation states [14]. Depending on the inbound ligand molecule, generation of a grid box of 15Åx15Åx15Å size was done. Finally, these prepared molecules (**Cpd 5-12**) were docked into the enzyme active sites by using the Glide tool through XP mode. Depending on the highest docking score, the best-docked conformation of every ligand was chosen.

4.15. Statistical analysis

All these data reported were the arithmetic mean of data of independent experiments performed in triplicate where each group was six in number. Results were expressed as mean ± SD (standard deviation) unless otherwise stated. Statistical analysis was made by the one-way analysis of variance (ANOVA) with LSD post-hoc test using SPSS 16 software. Statistical significance was considered as $^{\$}P<0.001$.

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Conflict of interest

None declared.

Figure Legend

Fig. 1. Molecular modification of pentanoic acids for achieving higher solubility.

Fig. 2. Fluorescence imaging of K562 cells after DAPI staining. K562 cells was being treated with **Cpd 11** at the doses of (A) 0µM (B) 2.5µM (C) 5µM (D) 10µMand stained with DAPI to observe DNA nick generation. Arrow indicates nicked DNA with intense fluorescence.

Fig. 3. AnnexinV-FITC/PI assay. Flow cytometric analysis after K562 cells either (A) untreated or treated with (B) 2.5 µM (C) 5 µM and (D) 10 µM dose of **Cpd 11**.

Fig. 4. Cell cycle analysis after PI staining. **Cpd 11** at the doses of (A) 0µM (B) 2.5µM (C) 5µM and (D) 10µM was given to K562 cells and sub-G1cell cycle arrest (M8) was in flow cytometry after being stained with PI. G1, G1/S and G2/M stages are designated as M3, M6, M7 respectively.

Fig. 5. Invasion assay and MMP-2 expression analysis. (A) Invasion assay of K562 cells after being treated with 0, 2.5, 5 and 10 µM of **Cpd 11** for 24 hours. Data are expressed as percentage of control and presented as mean ± SD. $^{\$}P<0.001$ were considered as statistically significant. (B) Western blots of MMP-2 and β-tubulin were done. (C) Relative band intensities of MMP-2 and β-tubulin were calculated. β-tubulin was used for loading correction. Data were expressed as percentage of control and presented as mean ± SD. $^{\$}P<0.001$ are considered as statistically significant. (D) Flow cytometric analysis of MMP-2 expression analysis. V2-L and V2-R were being considered as MMP-2 negative and MMP-2 positive cells respectively.

Fig. 6. Docked conformation of compounds (**Cpd 5-12**) along with the structure-based hydrophobic contour map in A) MMP-2 enzyme (pdb: 1HOV), B) MMP-9 enzyme (pdb: 2OW1)

Fig. 7. Molecular docking interaction of compounds (**Cpd 5-12**) with MMP-2 enzyme (pdb: 1HOV)

Fig. 8. Molecular docking interaction of compounds (**Cpd 5-12**) with MMP-9 enzyme (pdb: 2OW1)

Table Legend

Table 1. Physicochemical parameters of the final compounds (**Cpds 5-12**)

Table 2. Enzyme inhibitory activity and cytotoxic activity profile of pentanoic acids (**Cpds 5-12**)

Table 3. MMP-1, MMP-8, MMP-12, MMP-14 enzyme inhibitory activity pentanoic acids (**Cpds 5-12**)

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Highlights

- Some pentanoic acid derivatives reported as MMP-2 inhibitors
- The *n*-pentyl analog showed highest cytotoxicity in various cancer cell lines
- The *n*-pentyl compound effectively arrests sub-G1 cell cycle
- *n*-Pentyl analog induced MMP-2 downregulation in K562 cell line