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RESEARCH ARTICLE

Prodrugs for Nitroreductase Based Cancer Therapy- 1: Metabolite Profile, Cell Cytotoxicity and Molecular Modeling Interactions of Nitro Benzamides with Ssap-NtrB

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Abstract: ***Background*:** Directed Enzyme Prodrugs Therapy (DEPT) as an alternative method against conventional cancer treatments, in which the non-toxic prodrugs is converted to highly cytotoxic derivative, has attracted an ample attention in recent years for cancer therapy studies.

***Objective*:** The metabolite profile, cell cytotoxicity and molecular modeling interactions of a series of nitro benzamides with Ssap-NtrB were investigated in this study.

Method: A series of nitro-substituted benzamide prodrugs (**1-4**) were synthesized and firstly investigated their enzymatic reduction by Ssap-NtrB *(S. saprophyticus* Nitroreductase B) using HPLC analysis. Resulting metabolites were analyzed by LC-MS/MS. Molecular docking studies were performed with the aim of investigating the relationship between nitro benzamide structures (prodrugs **1-4**) and Ssap-NtrB at the molecular level. Cell viability assay was conducted on two cancer cell lines, hepatoma (Hep3B) and colon (HT-29) cancer models and healthy cell model HUVEC. Upon reduction of benzamide prodrugs by Ssap-NtrB, the corresponding amine effectors were tested in a cell line panel comprising PC-3, Hep3B and HUVEC cells and were compared with the established NTR substrates, CB1954 (an aziridinyl dinitrobenzamide).

Results: Cell viability assay resulted in while prodrugs **1**, **2** and **3** had no remarkable cytotoxic effects, prodrug **4** showed the differential effect, showing moderate cytotoxicity with Hep3B and HUVEC. The metabolites that obtained from the reduction of nitro benzamide prodrugs (**1-4**) by Ssap-NtrB, showed differential cytotoxic effects, with none toxic for HUVEC cells, moderate toxic for Hep3B cells, but highly toxic for PC3 cells.

***Conclusion*:** Amongst all metabolites of prodrugs after Ssap-NtrB reduction, *N*-(2,4-dinitrophenyl)-4-nitrobenzamide **(3)** was efficient and toxic in PC3 cells as comparable as CB1954. Kinetic parameters, molecular docking and HPLC results also confirm that prodrug **3** is better for Ssap-NtrB than **1**, **2** and **4** or known cancer prodrugs of CB1954 and SN23862, demonstrating that prodrug **3** is an efficient candidate for NTR based cancer therapy.

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1. INTRODUCTION

In the concept of prodrugs therapy, Directed Enzyme Prodrugs Therapy (DEPT) in which the non-toxic prodrugs is converted to highly cytotoxic derivative, has attracted an ample attention in recent years for cancer therapy studies [1]. Depending on the approach in that activating enzymes delivered to the tumour, DEPT was named accordingly, such as Antibody-Directed Enzyme Prodrugs Therapy (ADEPT), Gene-Directed Enzyme Prodrugs Therapy (GDEPT), Virus-Directed Enzyme Prodrugs Therapy (VDEPT), Polymer-Directed Enzyme Prodrugs Therapy (PDEPT) and *Clostridia*-Directed Enzyme Prodrugs Therapy (CDEPT). Whichever the way, the success of DEPT, however, depend on (but not limited) mainly three factors; the prodrugs and its cytotoxic metabolites, the activating enzyme and the delivery systems. Many studies have been directed to improvement in

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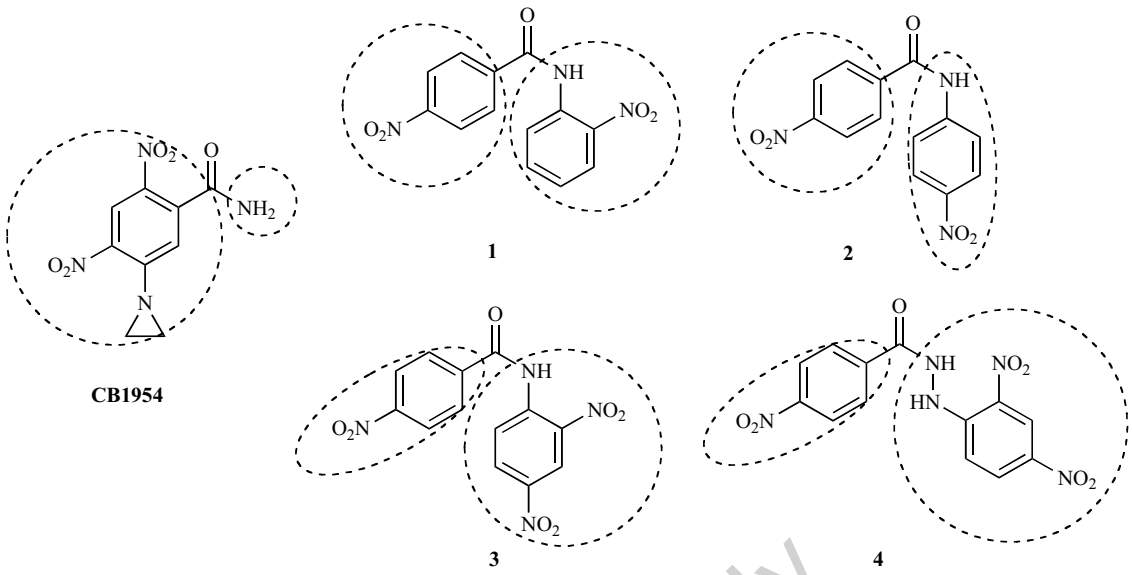

Fig. (1). Structures of synthesized nitro substituted benzamide prodrugs **(1-4)** and model prodrug CB1954.

features of the factors individually or collectively [1, 2]. Among the enzyme/prodrugs combination, type I nitroreductases (NTRs) from bacterial sources with the nitro group containing prodrugs have drawn attention for its capacity to destroy both actively dividing and dormant tumour cells.

Nitroreductases are enzymes from the oxidoreductase class and catalyzes reduction reaction of the nitro group containing organic compounds, using prosthetic groups as FMN or FAD, reducing power as NADH or NADPH [3, 4]. Pharmaceutical application of NTR based on the activation of prodrugs into active drug which forms cytotoxic DNA-crosslinking agents [5]. In particular, the combination of a nitroreductase (*NfsB*) from *E. coli* with CB1954 (the prodrugs, 5-aziridinyl-2,4-dinitrobenzamide) was appreciated as an 'unvoiced standard' procedure for works in which nitroreductases and prodrugs were used [6]. Activation of CB1954 by NTR forms hydroxylamine derivative which undergoes further reaction with a thioester, such as acetyl CoA, to form a final DNA reactive species. *E. coli* NTR/CB1954 combination of various types of cancer (such as colon, liver cancer *etc*.) in phase I/II clinical trials are performed [7]. The combination of *E. coli* nitroreductase/CB1954 was determined as highly cytotoxic and sensitive in V79 human lung cancer cells in a study conducted in the 1950s [8, 9]. Whereupon, *E. coli* nitroreductase was cloned for GDEPT treatments [9].

One of the latest additions to NTRs, we have reported a novel nitroreductase (Ssap-NtrB) from *S. saprophyticus* and modification of existing antibiotics in the form of precursor prodrugs previously [10a, 10b]. In combination with CB1954, it was demonstrated the enzyme's capability for prodrugs activation. Further in this direction, here, we aimed at getting profound understanding into the relationship between structure and Ssap-NtrB catalytic ability for prodrugs including CB1954 and nitro substituted aromatic amides as potential prodrugs.

The studies have shown nitro aromatic compounds are well characterized to be superior substrates for activation by *E. coli* nitroreductase and they can be simply metabolized to the reduced products [11]. It is well known that various benzamide derivatives are used as drug at a great number of therapeutic agents including some types of cancer [12a-e]. Herein, prodrugs were designed on the basis of model prodrugs CB1954 and benzamides. Our concept was to replace -$CONH_2$ group of the CB1954 with the nitro group containing -CONHAr groups (Fig. **1**). Thereupon we would compare the effectiveness of the changing groups on the enzymatic reactions and cytotoxic studies.

In this study, a series of nitro substituted aromatic amide prodrugs were selected among the known benzamides by in silico election and synthesized to explore their structure-activity relationships as substrates of Ssap-NtrB and as potential antiproliferative agents for cancer cells (Hep3B, HT-29 and PC3). In addition, the activities and the interactions of the compounds were visualized by using molecular modeling methods. The metabolites which are reduced form of benzamide prodrugs and CB1954 as standard were examined by *in vitro* cell assays in three cancer lines (Hep3B, HT-29 and PC3) for nitroreductase based cancer therapy. Consequently, the structure-activity relationships between model CB1954 and synthesized compounds are supported by molecular docking and biological evaluations.

2. MATERIALS AND METHOD

NADH and NADPH were obtained Roche Applied Science. CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) was kind gift from Professor Richard Knox (Morvus Technology Limited, UK). All the other chemicals in this study were of analytical grade and were purchased from Merck, Fluka and Sigma–Aldrich were used as supplied without prior purification. Cell cultures, Hep3B, HT-29, HUVEC and PC3, were obtained from the European Cell Culture Collection.

Melting points were determined with X-4 Melting-point Apparatus and are uncorrected. Synthesis reactions were

monitored by TLC on 0.25 mm silica gel plates ($60GF_{254}$) and visualized with ultraviolet light. Infrared spectra were measured using ATR techniques on a Perkin Elmer Spectrum 100 FTIR spectrophotometer. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded at Varian Mercury 500 MHz High Performance Digital FT-NMR spectrometer using TMS as an internal standard. Metabolites of enzymatic reaction were analyzed with HPLC (Shimadzu HPLC system; SPD-20A prominence UV/vis detector, DGU-20A5 prominence degasser, LC-20AT prominence liquid chromatograph, SIL-20AC HT prominence autosampler, CTO-10AS VP column oven) and LC-MS/MS (LC/MS 8040 Liquid Chromatograph Mass Spectrometer, Shimadzu).

Origin pro 8.5 program was used for drawing all cytotoxic activity graphs. The measurements of all cytotoxic activity experiments were performed triplicate and error bars are ± standard deviation.

3. EXPERIMENTAL

3.1. Synthesis

N-(2-Nitrophenyl)-4-nitrobenzamide (**1**), [13] *N*-(4-nitro phenyl)-4-nitrobenzamide (**2**), [14] *N*-(2,4-dinitrophenyl)-4-nitrobenzamide (**3**) [14, 15] were synthesized according to known methods with some modifications [16a] and were characterized by melting point, FT-IR, ^{1}HNMR and ^{13}C-NMR [16b]. The experimental data of *N'*-(2,4-dinitro phenyl)-4-nitrobenzohydrazide (**4**) are given in supporting information-2 (SI, slide 11).

3.2. Biological Evaluations

3.2.1. Enzyme Assays

Expression and purification of Ssap-NtrB were executed as previously mentioned [10a]. Ssap-NtrB activity of prodrugs **1** and **2** was determined constant concentration due to low solubility. Ssap-NtrB activity was measured with an assay mixture containing final concentration 20 µM for prodrug **1** (25 µM for prodrug **2**), 98 µM NADPH and 13 µg/mL Ssap-NtrB. Reaction was carried out in 25 mM Tris-CI buffer (pH= 7.5) containing 20% DMSO as cosolvent and continued for at least 3 min at 25 ºC. The kinetic parameters of Ssap-NtrB were determined spectrophotometrically at 21ºC in 25 mM Tris-CI buffer (pH= 7.5) containing 5% DMSO as cosolvent (21% DMSO for prodrug **3**) from the Michaelis–Menten. Various substrate concentrations ranging from 0 to 0.2 mM for prodrugs **3** and **4** were used to investigate kinetic parameters (K_M, k_{cat}) toward prodrugs with NADPH (215 µM NADPH for prodrug **3**, 228 µM for prodrug **4**) as a cofactor and 23 µg/mL Ssap-NtrB (1.8 µg/mL Ssap-NtrB for **3**). Reaction was started with the addition of NADPH and was measured by following the initial decrease in absorbance at 340 nm for all substrates as well as the substrate decrease in absorbance at 450 nm for prodrug **4**. NAD(P)H ε_{340}= 6220 $M^{-1}cm^{1}$, ε_{450}= 25632 $M^{-1}cm^{-1}$ [17]. Kinetics measurements were performed using Molecular devices Microplate Reader Spectro Max. Michaelis–Menten curve and Non-linear regression analysis were carried out using commercially available software (Origin v.7 software, Origin Lab, Northampton, MA). The amount of enzyme that catalyze the turnover of 1.0 µmol of substrate or NADPH per minute was defined as one unit. So the specific activity was expressed as µmol $min^{-1}mg^{-1}$ active enzyme.

3.2.2. Metabolite Analysis

Metabolites as reduction products of prodrugs **1-4** catalyzed by Ssap-NtrB was monitored by HLPC. In a typical reaction, final concentration of reaction components are 150 µM substrate (100 µM prodrug **3**), 289 µM NADH (429 µM NADH for prodrug **3**) and 33 µg/mL Ssap-NtrB (37 µg/mL Ssap-NtrB for prodrug **3**). Reaction was carried out in 25 mM Tris-CI buffer (pH= 7.5) containing 5% DMSO (10% DMSO for prodrug **3**) as co-solvent and continued for at least 10 min at 21ºC. To investigate time dependent product profile of prodrugs **2** and **3**, reaction was occurred with couple enzyme system (Ssap-NtrB/FDH) for cofactor regeneration. Reaction mixture was consisted of 70 µM prodrug **3** (25 µM for prodrug **2**), 101 µM NADPH (50 µM NADPH for prodrug **2**), 1000 µM NADP+ (500 µM NADP+ for prodrug **2**), 0.1 mg/mL BSA, 200 µM ammonium format, 20% DMSO, 0.4 µg/mL Ssap-NtrB (24 µg/mL Ssap-NtrB for prodrug **2**) and 6.4 µg/mL FDH (24 µg/mL FDH for prodrugs **2**). 50 µL samples were taken into a tube different time intervals of 1 min-48 h at 20ºC. The reaction was stopped with the addition of ice cold acetonitrile (1:1 v/v) and then incubated at least 1.5 hours at -80ºC. After that reaction mixture was centrifuged at 12,000 rpm for 10 min, 20 µL sample injected to RPC18 column ((250 mm x 4.6 mm, 5 µm), UK). HPLC Analyses were performed in acetonitrile (ACN)-HPLC grade water mixture as a mobile phase and flow rate was 1 mL/min also detecting at UV-Vis detector at 254 and 350 nm. HPLC gradient was 0-5 min 20% ACN, 15-22 min 80% ACN, 27-32 min 20% ACN. Metabolite mass detection was performed by LC-MS/MS. Reaction was occurred by incubation with 150 µM substrate (100 µM prodrug **3**), 76.5 µg/mL Ssap-NtrB (39 µg/mL Ssap-NtrB for prodrug **4** and 50 µg/mL Ssap-NtrB for prodrug **3**) and 580 µM NADPH (398 µM NADPH for prodrug **3**) in 25 mM Tris-CI buffer (pH= 7.5) containing 5% DMSO at 22 ºC. After centrifugation at 4000 rpm for 10 min, 5-10 µL reaction mixture was loaded to LC-MS/MS system and analyses were carried out using "quick scan" and "negative" mode in the range of 50-800 m/z. After molecular ion detection, product ion scan was produced in the same range.

3.2.3. Cell Cytotoxicity Assays

The cells (Hep3B, HT-29, HUVEC and PC3) were grown in monolayer culture in Dulbecco's modified Eagle medium (DMEM) with glutamine containing 10% fetal calf serum and maintained in a humidified atmosphere at 37°C with 5 % CO_2.

The viability of cancer cells was determined *via* the MTT assay as previously described [18]. Exponentially growing cells were obtained by plating 5×10^{5} cells/mL for the three human cell lines followed by 24 h of incubation. Various concentrations of prodrugs (150, 75, 39, 19 and 9 µM) were treated on Hep3B, HUVEC and HT-29 cells for 48 h. The effect of the vehicle solvent (DMSO) on the growth of these cell lines was evaluated in all the experiments by exposing untreated control cells to the maximum concentration (1%) of DMSO used in each assay by spectrophotometrical meas-

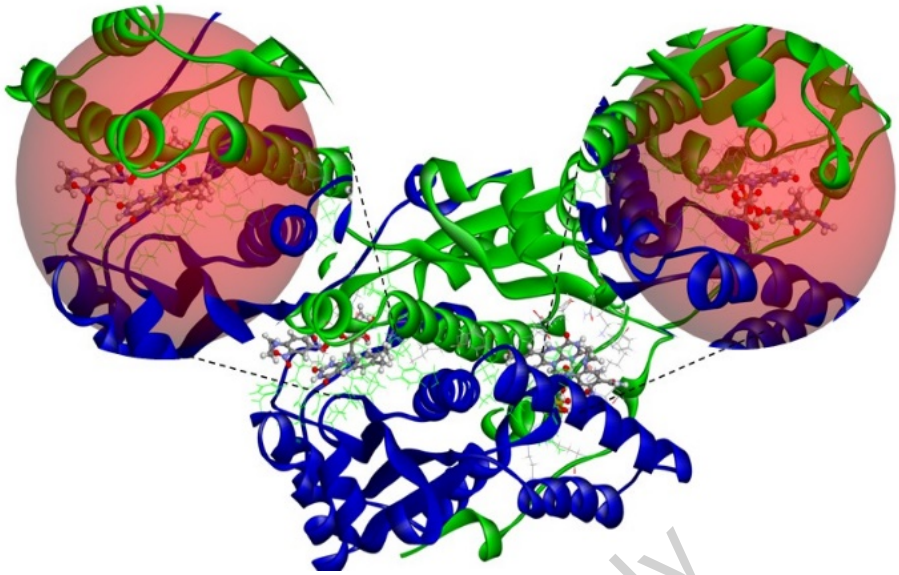

Fig. (2). The three dimensional (3D) form of two different active sites, A (right side) and B (left side) in Ssap-NtrB protein structure.

urement at 550 nm. The graphics and IC_{50} values were comprised using Origin pro 8.5 programme.

SRB assay was used to search the cytotoxic feature of prodrugs catalyzed by Ssap-NTR. The seeding density is dependent on the cell cycle time of each cell line. Therefore, the cells were seeded into 96 well plates appropriate density in which for PC3 and HUVEC: 10.000 cells, Hep3B: 30.000 cells per well in 100 µL Dulbecco's Modified Eagles Medium (DMEM) containing 10% FCS and were allowed to attach overnight in a CO_2 incubator. After 16 h incubation period, 100 µL of the reaction, control 1 or control 2 mixtures were added into the wells. The total volume of 100 µL reaction tube contained 150 µM substrate as prodrugs, 31 µg/mL Ssap-NtrB and 600 µM cofactor (NADH) in final concentration completed with 25 mM Tris-HCl buffer. CB1954 is an excellent substrate of *E. coli* nitroreductase and also is currently in clinical trials was used as a control in the experiments. Reaction condition for CB1954 was optimized with 150 µM CB1954, 46.5 µg/mL Ssap-NtrB and NADH (900 µM) in final concentration completed with 25 mM Tris-HCl buffer. After exposure time (24 h and 48 h), SRB assay was carried out as described [19].

3.2.4. Statistical Analysis

Minitab 14 software was used for all statistical analysis, and $p < 0.05$ were considered statistically significant. All data are expressed as mean ± SD.

3.3. Computational Studies

3.3.1. Molecular Structures and Optimization

The structures of selected prodrugs **1-4** were drawn and minimized using semi-empirical/PM3 and DFT/B3LYP/6-31G* basis set by using Gaussian 09 (G09) [20]. After that, Chemistry at HARvard Macromolecular Mechanics (CHARMm) [21] force field of Discovery Studio (DS) 2016 [33] was applied to define conformation of these compounds.

3.3.2. Homology Modeling of Ssap-NtrB

The crystal structure of Ssap-NtrB [10b] was not available. So it is a vital requirement to explain the 3D structure of the Ssap-NtrB to understand the mechanism and the functional role of the nitro substituted benzamide prodrugs on Ssap-NtrB. Therefore, the 3D structure of Ssap-NtrB was confirmed by using the computational homology modeling method in this study.

It was performed in a multiple sequence alignment to find out the most suitable templates throughout the known 3D structures of the proteins existing in National Centre for Biotechnology Information (NCBI) databases (http://www.ncbi.nlm.nih.gov/protein/). The protein sequence of Ssap-NtrB was Accession no: gi73661517. The results came out of the sequence alignment analysis of these proteins by means of the Basic Local Alignment Search (BLAST) [22]. Then DS Verify Protein verification protocol [23] and Ramachandran Plot were used to validate the obtained results. The Loop Refinement by the DS protocol was also performed to get rid of the steric clashes and to end up with an energetically better conformation of the 3D model of Ssap-NtrB.

3.3.3. Molecular Docking

Molecular docking was performed with Discovery Studio 2016 to supply an insight into the prodrugs **1-4** and the known cancer prodrugs, CB1954. Firstly, the nitro substituted benzamide prodrugs (Fig. **1**) and Ssap-NtrB were prepared using G09 and DS 2016 software for molecular docking study, as described above. Hydrogens were inserted, and water and ions as undesired agents were extracted from the enzyme. In the meantime, their positions in Ssap-NtrB were minimized using CHARMm forcefield and the adopted-basis Newton-Raphson (ABNR) method [22, 23] until the root mean square deviation (RMSD) gradient is <0.05 kcal/mol Å2. The active sites were described in literatures about NTR [10a]. So there are two different active sites, A and B in Ssap-NtrB, Fig. (**2**).

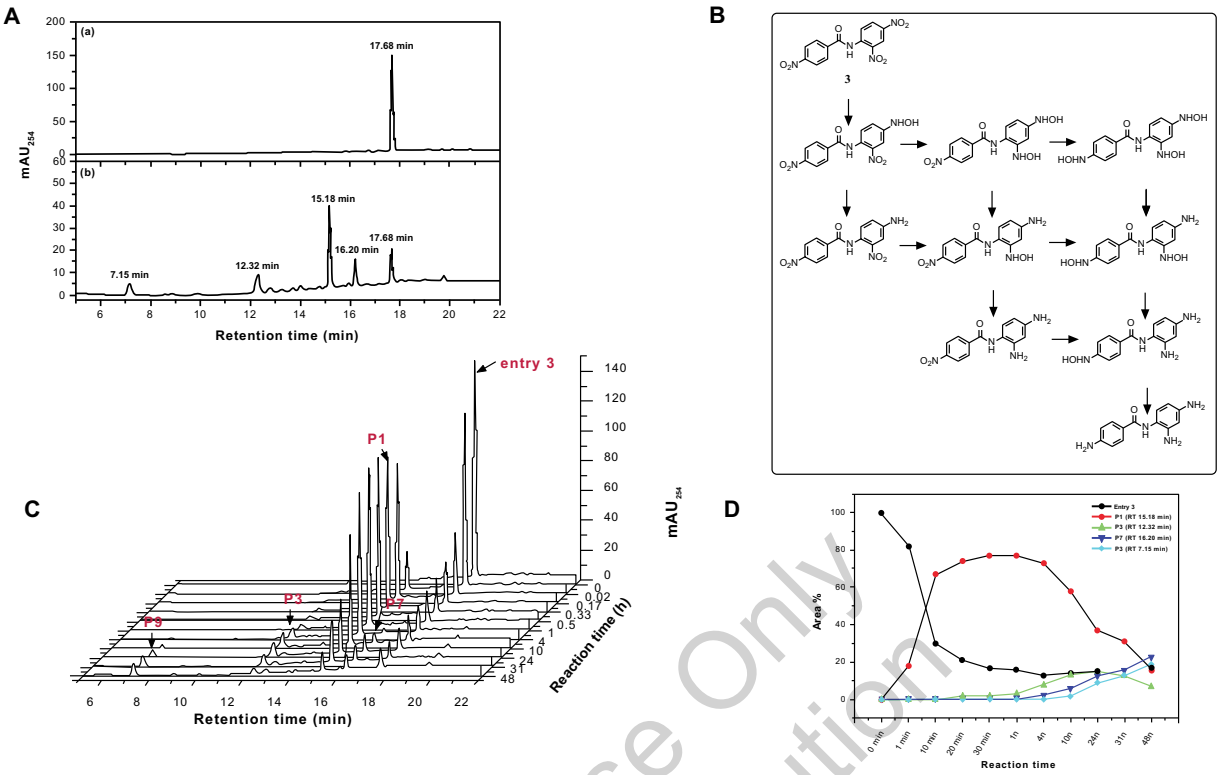

Fig. (3). (A) HPLC chromatogram of reduction reaction prodrug **3** catalyzed by Ssap-NtrB, (**a**) Control without Ssap-NtrB, (**b**) Enzymatic reduction reaction at 24 h, (**B**) Predicted reaction steps of NTR-catalyzed reactions for prodrug **3**, (**C**) HPLC chromatogram of different times of reactions, (**D**) Time dependent product profiles.

Ssap-NtrB was held rigid and the ligands were flexible in the docking study. CDOCKER, which is subprotocol of DS 2016, was applied using the default settings. The following other step, the Analyze Ligand Poses was used to calculate and define the non-bonding interactions between ligand atoms and the enzyme. After all, binding energies were also obtained in water solution by implemented the Calculate Binding Energy subprotocol of DS 2016. The largest minus CDOCKER energy, the lowest minus CDOCKER interaction energy and the lowest RMSD values are desired for the best result of each ligand-enzyme complex. In addition the non-bonding interactions in the each enzyme-ligand complex are important properties to determine the best-docked conformation of the investigated compounds in the docking study [24, 25]. Dissociation constant Kd is calculated for each prodrugs and the possible metabolites to evaluate their interactions and activities with Ssap-NtrB.

4. RESULTS AND DISCUSSIONS

4.1. Chemistry

CB1954 has an important position at the nitroreductase based GDEPT. We pay attention to design prodrugs with similar structural elements resemble to CB1954. According to this model compound, selected known nitro substituted benzamides (Fig. **1**) were prepared by substitution reaction of 4-nitrobenzoyl chloride and nitro groups containing aniline derivatives in various reaction conditions with some modifications [16a]. Structures of the prodrugs candidates were fully characterized by melting point, FT-IR, ^{1}H-NMR and ^{13}C-NMR analysis [16b].

Also, an important point for choosing these compounds, prodrugs **1-4** have nitro substituted aromatic groups that having electron-withdrawing properties connected to the nitrogen atom of the amide. The conjugation of prodrugs **1-4** is going to be increased as a result of the *N*-aryl substituted amide functional group by resonance effect. The presence of the nitro group in ortho or para position or in both at nitrogen atom of the amide is going to be tested how far effect on the enzyme activity. While prodrugs **1** and **2** have two electron-withdrawing nitro groups, **3** and **4** have three. Those nitro groups will cause to decrease electron density at the amide nitrogen atoms at the prodrugs **1-3**, the extra-NH group at hydrazide will cause increase electron density on the amide nitrogen of prodrug **4**. According to the results of the enzyme activity studies, proposed structure-activity relationships of these structural modifications are going to be discussed.

4.2. Metabolite Profiles of Prodrugs 1-4

Activation of prodrugs **1-4** by Ssap-NtrB was conducted under the standard assay condition described in the experimental section. The product profiles of prodrugs **1-4** were analyzed by HPLC. Initial screenings suggested that all the substrates (**1-4**) were converted to their corresponding metabolites. Single metabolite with retention times 14.64 and 14.16 minutes was observed for prodrugs **1** and **2** respectively while multiple products were detected for prodrugs **3** and **4** under the specified experimental conditions (Fig. **3** and **SI**).

The product profiles were dependent on the reaction conditions and time of sampling. That is, in fact, indicates the multistep reactions of NTR-catalyzed reactions and relative stability of the corresponding products (Fig. **3B**). For example, four major products were observed with retention times of 15.22-15.18 min, 12.34-12.25 min, 16.22-16.20 min and 7.17-7.15 min for prodrug **3**. The products' profiles versus time are shown in (Fig. **3C** and **3D**). A time-course profile for the biocatalytic conversion of prodrug **3** yielded metabolites labelled as P1-P9. The main reaction product (P1) rapidly increased with time, along with other metabolites; P3, P7 and P9. At the end of the 7-8 minutes, approximately 50% of prodrug **3** had been converted to P1, along with 1-2% of other bioconversion products. The formation of P1 peaked at the reaction time of 30-60 minutes. Then, a gradually decrease was observed for 48 h periods while the formation of P3, P7 and P9 was detected. At the end of 48 h reaction period, the percentage of prodrug **3** and its products (P1, P3, P7 and P9) were as follows: 18.0, 16.0, 7.0, 22.6, and 19.0%, respectively. If necessary, a coupled enzyme system using a formate dehydrogenase (CmFDH) and formate as an auxiliary enzyme and substrate, respectively were utilized to overcome the cofactor restriction by means of regeneration of NADH, *in situ*.

4.3. LC-MS/MS Analysis of Prodrugs 1-4

Analyses of prodrugs **1-4** and corresponding metabolites using LC-MS/MS with negative mode yielded very challenging spectrum to be analyzed, fully (SI). Analysis of nitro aromatics having more than one nitro-group itself is not straightforward. The LC-MS/MS analyses are summarized in Table S1, slide 10. It should be noted that Ssap-NtrB catalyzed reduction of nitro-groups is a reflection of product profile of a particular time. As stated previously, product profile is simultaneously changing and mostly depends on the reaction condition as well as time of sampling. Therefore, LC-MS/MS result is a snapshot for giving times of sampling and reaction conditions specified. ESI behavior of prodrugs **1-4** and their metabolites is investigated regarding emerging ion signals in the Q1 scan mode in the mass range of *m/z* 50-800. The ionization yield of the substrates was substantially higher using negative ionization mode in comparison to positive polarity due to the electronegative nitro groups. Ssap-NtrB catalyzed reduction of prodrug **1** yielded a single product (rt. 6.92 min) with the m/z of 256 under reaction condition specified. Since the prodrug **1** has got two nitro groups and each group can be potentially reduced to form nitroso, hydroxyl amine or amine derivatives. The m/z of the metabolite indicates that only one nitro group is reduced to amine containing metabolite. From MS/MS fragmentation analysis, we have tentatively determined that the nitro group of the second ring (ring B) was reduced to amine derivative via hydroxyl amine (SI, slide 9).

Similarly, prodrug **2** has been reduced by Ssap-NtrB catalyzed reaction into three metabolites with m/z of 272 (rt. 16.10 min) and both of m/z 256 (rt. 15.87 and 4.61 min), which are consisted with a hydroxyl derivative and amine derivative respectively. Once again, we believe that the nitro group in ring B has been reduced to form hydroxyl amine derivative (m/z of 272 $[M\text{-}H]^-$), which then further reduced to amine derivative (m/z of 256 $[M\text{-}H]^-$). There is no sign of nitro reduction observed for nitro-group on the ring A, but this does not necessarily mean that it is exempted for reduction but rather, not preferred initially.

In prodrug **3**, biocatalytic reduction has yielded at least three major metabolites and seven minor metabolites under the reaction condition specified and time of sampling. Major metabolites with m/z of 318, 257 and 241 are consisted with a single hydroxyl derivative, double amine and hydroxyl derivative, and triple amine derivative. It is not clear and complicated to determine the order of reductions, but what is clear that Ssap-NtrB has got potential to reduce all three nitro groups to their corresponding amines.

For prodrug **4**, there are three major metabolites with m/z of 332, 316 and 302 detected, corresponding to a single hydroxylamine (m/z of 332 $[M\text{-}H]^-$), a single amine (m/z of 316 $[MH]^-$), and hydroxylamine-amine (m/z of 302 $[M\text{-}H]^-$) metabolites.

4.4. Kinetic Studies

We have studied kinetic assay to determine kinetic parameters Ssap-NtrB catalyzed reactions of prodrugs (**1-4**). Due to the low solubility of prodrugs **1** and **2** in the buffer solution, we preferred to determine enzyme activity with fixed substrate concentration. On the other hand, it is possible to use Michaelis-Menten type kinetic for prodrugs **3** and **4**. The specific activity of Ssap-NtrB with prodrugs **1-4** was found 0.8, 0.9, 43.8 and 0.38 U/mg, respectively. Michaelis-Menten kinetic parameters were calculated for reduction reactions of prodrugs **3** and **4** catalyzed by Ssap-NtrB. Kinetic parameters of these prodrugs and common prodrugs (CB1954 and SN23862) with Ssap-NtrB and *E. coli* nitroreductase (NfsB) are compared in Table **1**. Kinetic parameters show us that prodrug **3** is a better substrate for Ssap-NtrB than known cancer prodrugs as CB1954 and SN23862. Ssap-NtrB showed increasing in specificity constant (for prodrug **3**), $k\text{cat}/K_M$, of approximately **40** fold compared to CB1954/*E. coli* NfsB. High difference between $k\text{cat}/K_M$ for Ssap-NtrB and NfsB may be due to reaction measuring directly or indirectly. Reaction of prodrug **3** was followed indirectly at 340 nm to decrease cofactor whereas reactions with CB1954 were measured directly at 420 nm to increase product.

4.5. Cytotoxic Activity of Prodrugs

To control for the possible biological effects of prodrugs, they were tested sole for their ability to inhibit cell proliferation. The prodrugs were evaluated by MTT assay in two different cancer cell lines, namely Hep3B, HT-29 in addition to non-cancer cell, HUVEC. Hep3B cell was chosen as Hepatocellular Carcinoma model that is one of the most common primary tumors of the liver [26]. HT-29 cell was included to our study due to undifferentiated state and high metastatic potential. On the other hand, we have also chosen healthy model, namely HUVEC, for screening the effect of compounds on healthy human cells. % Cell survival of prodrugs was determined in different concentrations for 48 hours. Non-treated cells were included to our study as a control. As shown in Fig. (**4**), prodrugs **1**, **2** and **3** had no remarkable cytotoxic effect. However, previous cytotoxicity analyses of the prodrug **2** showed that it has some cytotoxic effect in

Table 1. Comparison of kinetic parameters of nitroreductases toward CB1954, SN23862 and prodrugs 1-4.

Substrate	Enzyme	$K_{cat}(s^{-1})$	$K_M(\mu M)$	K_{cat}/K_M $(s^{-1}\mu M^{-1})$	U/mg	Relative to CB 1954/Ssap-NtrB	Reference
Prodrug 1	Ssap-NtrB	ND	ND	ND	0.8±0.00	0.18[a]	This study
Prodrug 2	Ssap-NtrB	ND	ND	ND	0.9±0.10	0.21[a]	This study
Prodrug 3	Ssap-NtrB	19.23±2.00	66.07±13.50	290975	43.8±2.0	137.25[b]	This study
Prodrug 4	Ssap-NtrB	0.19±0.00	26.64±2.70	7100	0.38±0.00	3.35[b]	This study
CB1954	Ssap-NtrB	2.26±0.07	1065.2±53.1	2120	4.37±0.01	1.00[b]	10a
SN23862	Ssap-NtrB	0.77±0.07	82.4±16.9	9350	1.14±0.04	4.41[b]	10a
CB1954	NfsB	26±2.6	3600±660	7300	-	3.44[b]	10c
SN23862	NfsB	26.4±7.2	2500±100	0.01	-	$<1.10^{-5}$[b]	10d

ND: Not determined. [a]: According to U/mg. [b]: According to *k*cat/K_M. (Specific activity values at 98 μM NADPH for prodrugs **1** and **2**; 215 μM NADPH for prodrug **3**; 228 μM NADPH for prodrug **4**, 151 μM NADPH for CB1954; 130 μM NADPH for SN23862. Reaction monitored at 340 nm (exception CB1954 at 420 nm)).

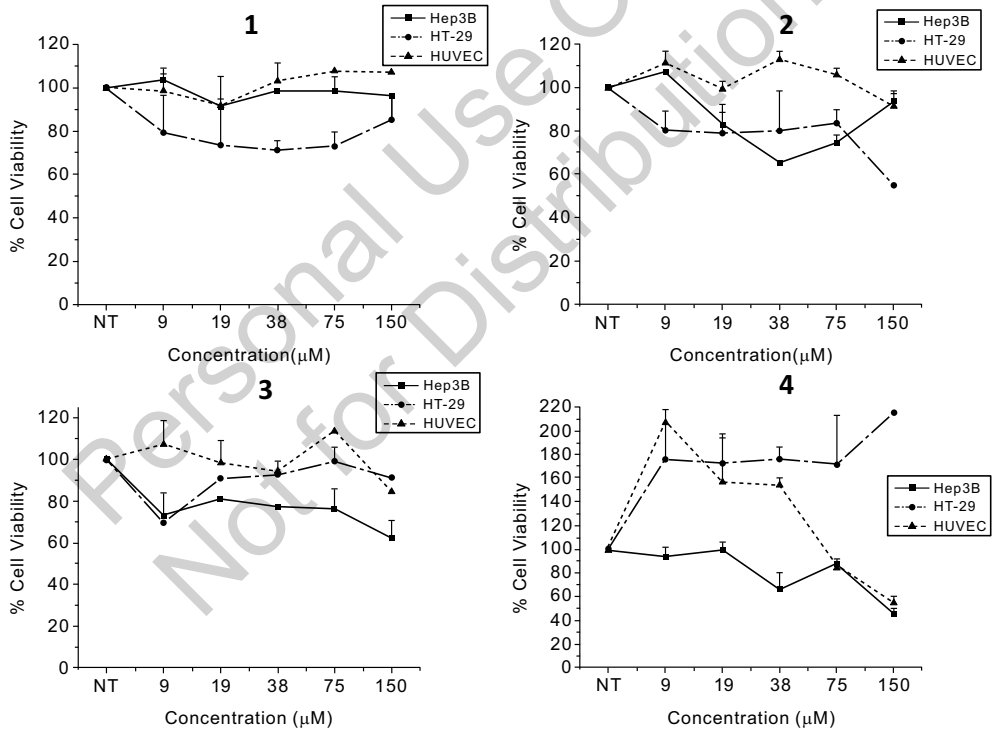

Fig. (4). Cytotoxic effects of prodrugs **1-4** that determined using MTT assay. (The cells were seed at a density of $5x10^4$ into 96 well plates. Following 24 h of incubation, five different concentrations of prodrugs were applied Hep3B, HT-29 and HUVEC cells. After 48 h incubation, cell viability was measured at 550 nm spectrophotometrically. Percentage cell survival at each prodrugs concentration was calculated (OD 550 prodrugs/OD 550 NT)*100 formula).

A375 (Melanoma Cancer Cells), K562 (Leukemia), 293T (Kidney), HepG2 (Liver Carcinoma), Hep-2 (Laringeal Carcinoma), EJ (Bladder Carcinoma) and Raji (Lymphoma) [27, 28]. It is common phenomenon that the compounds may show differential effects based on cell type specific. Although, HepG2 and Hep3B cell lines share many common characteristic, they can be differentially affected by the same compound [29]. The prodrug **4** showed the differential effect based on cell type. Specifically, prodrug **4** leads to the proliferative effect up to two fold in HT-29 cells. In addition, it has a concentration-dependent activation present in healthy cells, HUVEC. The prodrug **4** exhibited the moderate cytotoxicity effect up to 50% inhibition for 150 μM in Hep3B cells (Fig. **4**).

Cytotoxic feature of metabolites of the nitro substituted benzamide prodrugs activated by Ssap-NtrB were determined using SRB assay in two different cancer cells, Hep3B and PC3, as well as healthy model cells, HUVEC.

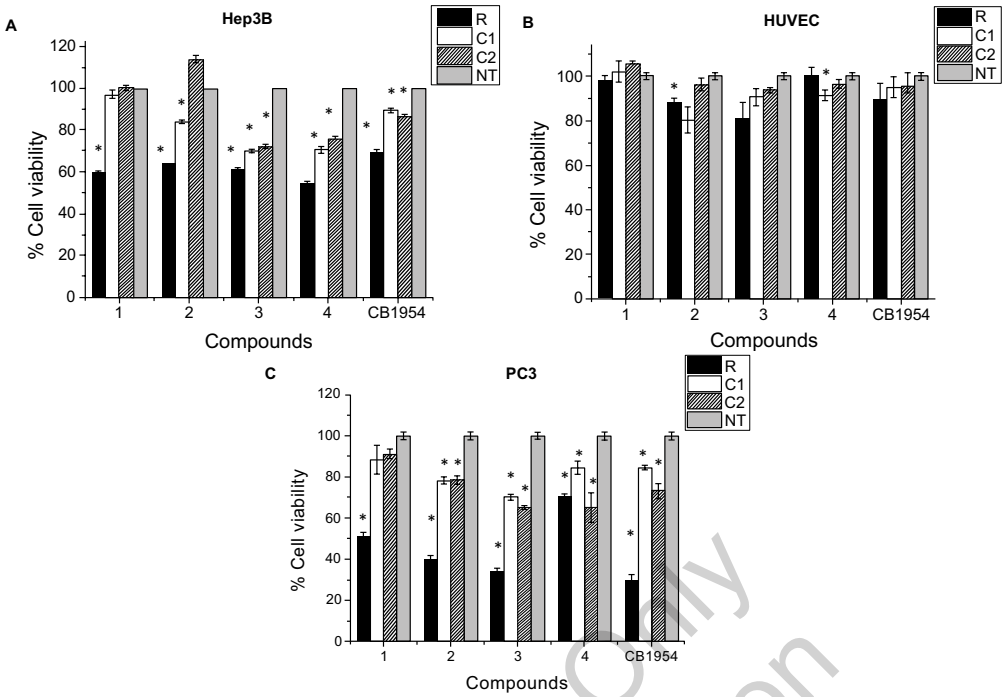

Fig. (5). Cytotoxic feature of metabolites of the nitro substituted benzamide prodrugs that catalyzed Ssap-NtrB reduction were determined using SRB assay. (The cells were seed in a 96 well plates. **A:** The cytotoxic effects of compounds were investigated in **A:** Hep3B, **B:** HUVEC and **C:** PC3 cells. **CB1954** was used as positive control. R represents reaction (Ssap-NtrB 31 µg/mL + Cofactor (NADH) 600 µM + prodrugs 150 µM, C1 stands for Control 1 (only prodrugs 150 µM), C2 represents Control 2 (Ssap-NtrB 31 µg/mL + prodrugs 150 µM), **NT** is None Treated cells as a negative control. * indicates $p < 0.05$).

and PC3, as well as healthy model cells, HUVEC. The results were evaluated not only for Ssap-NtrB ability as prodrugs acting enzymes but also nitro containing aromatic amides as prodrugs in tumor killing feature in the manner of 'by stander' effect.

Recent studies demonstrate that by stander effect of metabolites is the major toxic agent that used in gene therapy [30]. To achieve this strategy, recombinant Ssap-NtrB was used for the catalysis of prodrugs reaction *in vitro*. After *in vitro* reaction, the whole components of the reaction including the metabolites were applied on the three types of cells and further incubated for 48 h.

The cells that not to express *NtrB* gene, was infected with the active metabolites (R) and compound (C1) and Compound + Ssap-NtrB (C2) for 48 hour. The effect of metabolites was compared with those of CB1954 (150 µM). Our results showed that all compounds including CB1954 did not significantly affect the cell proliferation in healthy cells, HUVEC. In Hep3B cells, the metabolites of prodrug **1** are cytotoxic as much as CB1954. The metabolites of **2**, **3** and **4** also indicate the significant cytotoxic effects but these prodrugs are cytotoxic before going into the reaction. The metabolites of **1**, **2** and **3** show the comparable cell cytotoxic effect to PC3 cells like CB1954 (Fig. **5** C1s).

4.6. Computational Studies

4.6.1. Homology Modeling of Ssap-NtrB

Homology modeling is necessary to define and visualize the interactions of Ssap-NtrB with prodrugs **1-4** which include nitro group(s) in the different positions of the phenyl moieties in the framework. BLAST which is the first step of homology modeling, the protein sequence of Ssap-NtrB matches some of the PBD_nr95 protein database sequences. It performed multiple sequence alignment to determine the most optimal templates throughout the known 3D structures of proteins existing in the database. The best score of Ssap-NtrB protein structure similarity of 3GBH, 3GE6, 4LQX, 2HAY, 1DS7, 1IDT, 1YKI was given in Table **S1** in **SI** part. This protein was used as a template for the Ssap-NtrB protein model and the built 3D structure with the DS MODELLER protocol [25, 31-33]. Lastly, the homology model of Ssap-NtrB protein sequence region is 208 amino acids.

DS Verify Protein (Profiles-3D), Loop and Side chain refinement, and Ramachandran Plot processes were applied to endorse the generated model. Furthermore, the Probability Density Function (PDF) energy data [34], and DOPE (Discrete Optimized Protein Energy) scoring function [35] were used to reinforce the model. In the end, one model was chosen in twenty generated models; it is displayed in bold in Table **S2** in **SI** part.

As known that better alignment means the lowest energy value. Verify Score and Verify Expected Low Score values for the best model is 41.00 and 42.4671, respectively. After this, Loop Refinement [36] by DS protocol is performed and presented as given in Table **S3**. The Ramachandran Plot [37] provides for our model protein 85% of the residues in the favorable region and allowed region with 15% of the residues, were given in Fig. **S1**. The three dimensional (3D) homo dimer structure of Ssap-NtrB protein has also shown in Fig. **S2**.

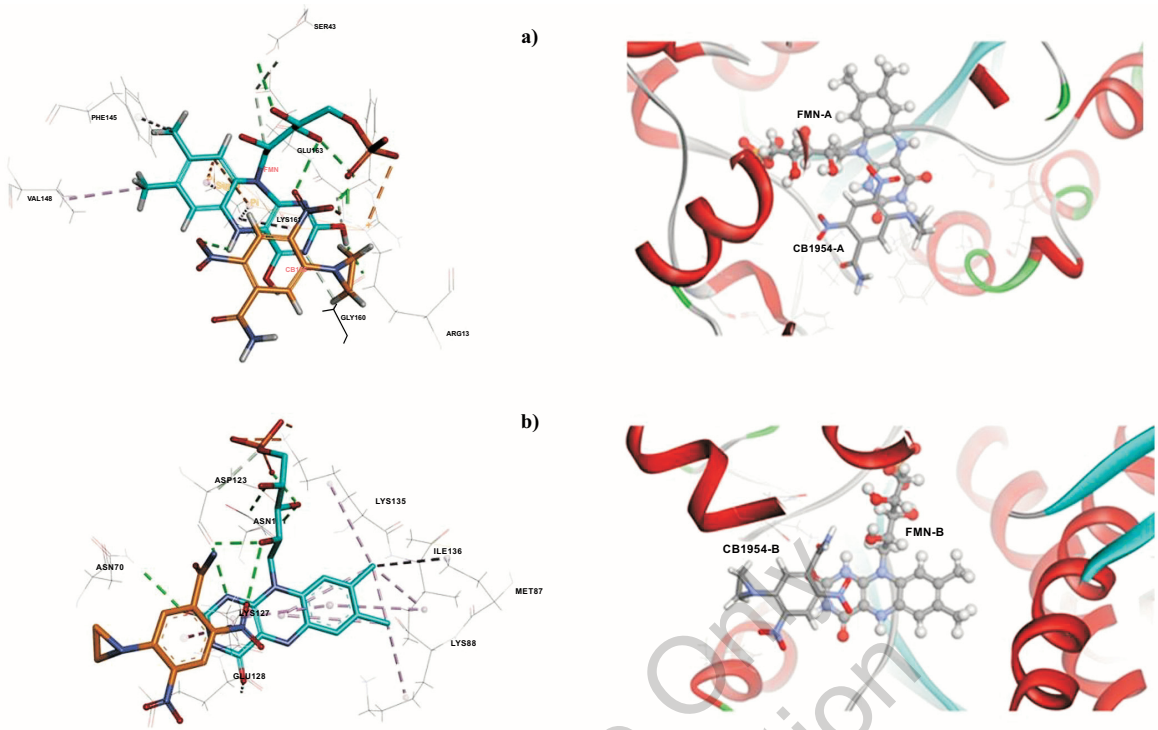

Fig. (6). Interactions between FMN and CB1954 on a 2D and 3D diagram in active site A (**a**) and active site B (**b**) of the Ssap-NtrB, respectively.

As a result, the first homology model of Ssap-NtrB protein included A and B chains which have total 416 amino acid and the molecular weight of the protein is 46,801.2 g/mol. The secondary structure defined by STRIDE [38] has four β-sheet, nine isolated β-bridge, sixteen coil, twenty turn and six 3-10 helix with thirteen α-helices (Table **S4**).

4.6.2. Molecular Docking

Based on kinetic and cytotoxic activity studies of prodrugs **1-4**, prodrug **3** is better substrate for Ssap-NtrB than known cancer prodrugs as CB1954 and SN23862. Therefore, molecular docking was performed to answer some questions in mind about this situation. For example, what are the binding pose and interactions of prodrug **3** and its metabolites in each different active site (A and B) of Ssap-NtrB. Another one is how affects the Ssap-NtrB activity, when the extra-NH group at hydrazide group is replaced on the amide nitrogen of prodrug **4**.

The obtained Ssap-NtrB model was validated by using docking technique of the known cancer prodrugs, (CB1954) with Ssap-NtrB model. It was shown that CB1954 has activity on the enzyme with flavin mononucleotide (FMN) as cofactor. Fig. (**6**) shows the docking of FMN and CB1954 into active site A and B of Ssap-NtrB. In active site A, the 4-nitro group of CB1954 was placed the active site, stacking above the FMN cofactor. The oxygen atom of 4-nitro group forms hydrogen bond and electrostatic interactions with FMN and Arg13 residue. The 2-nitro moiety forms hydrogen bonds with FMN. Moreover, FMN as cofactor of enzyme forms hydrogen bonds and lipophilic interactions with nitrogen atom of nitro moiety in CB1954 and A: Arg13, A: Gly160, A: Lys188, A: Glu163, B: Ser43, B: Phe145 and B: Val148 residues of the enzyme. In active site B, the 2-nitro group of CB1954 was oriented the binding site, stacks above the cofactor.

The 2-nitro moiety forms hydrogen bond and lipophilic interactions with FMN and protein backbone. On the contrary of FMN in the active site A, FMN interacts with different residues (A: Glu128, A: Lys88, A: Ile136, A: Met87, A: Asn131, A: Lys135, A: Asp123, A: Lys127 and B: Asn70) of the enzyme in active site B of Ssap-NtrB. These results have firstly supported the model structure of Ssap-NtrB and show the importance of the substrate conformations with FMN in each active site of the enzyme.

To get insights about interaction and mechanism of the prodrugs **1, 2** and **4** with both active sites (A and B) of the enzyme, binding energy (Ki) and dissociation constant (Kd) values for each prodrugs and possible metabolite were calculated. In addition, enzyme, prodrugs, complex structures and entropy energies which contributed to binding energy were calculated one by one. These calculated values are evaluation criteria for how well the enzyme and prodrugs or their metabolites do not interact (Fig. **S3-S4** and Tables **S5-S6**).

Based on these results, when we examine the binding and dissociation constants of the prodrugs **1, 2** and **4**, metabolite **4c** has the highest average Ki value (*7.400E+04*), denotes better and fitting binding with A and B active sites of the enzyme. Besides, the metabolite **4c** has the lowest Kd value (*3.388E-55 for A active site; 3.409E-55 for B active site*) amongst the prodrugs. It reveals that metabolite **4c** exhibited good interaction trend with Ssap-NtrB (Fig. **S3-S4**). It is desirable to obtain the highest Ki value and the lowest Kd value for any prodrugs with the enzyme, because the NTR interacts with the prodrugs efficiently and is easily degraded then catalytic processes take place.

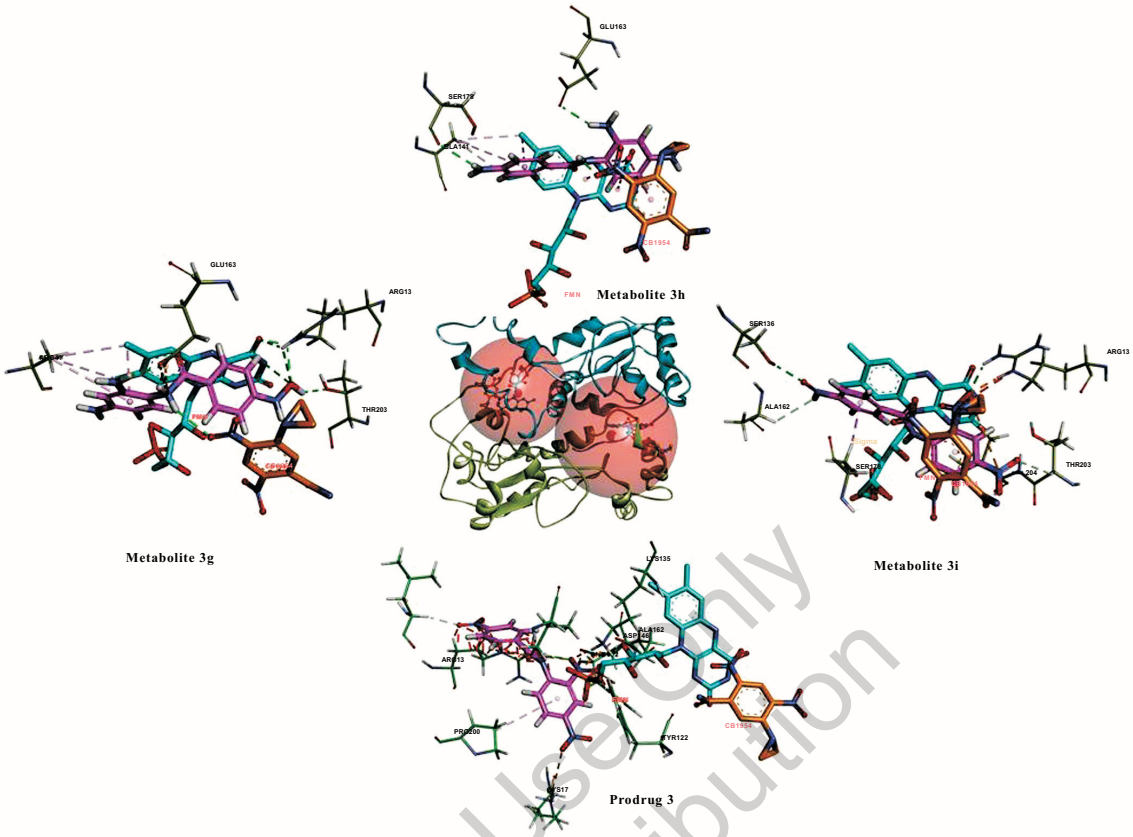

Fig. (7). Docking of prodrug **3** (poor position at the below part of the figure), metabolites **3g, 3h, 3i** with FMN into in active site A and B of Ssap-NtrB. (prodrug **3** and metabolites mentioned pink color, FMN and CB1954 were shown blue and orange color). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

Table 2. Binding energy (K_i) and dissociation constant (K_d) values of prodrug 3 and its possible metabolites 3a-3j with active site A and B of Ssap-NtrB.

Name (A site)	Average Ki (kcal/mol)	Average Kd (M)	Name (B site)	Average Ki (kcal/mol)	Average Kd (M)
3	73959,140	3,655E-55	**3**	73954,152	3,686E-55
3a	73984,949	3,499E-55	**3a**	73975,996	3,553E-55
3b	73992,872	3,452E-55	**3b**	73982,923	3,511E-55
3c	73971,917	3,577E-55	**3c**	73961,775	3,639E-55
3d	73971,817	3,578E-55	**3d**	73962,292	3,636E-55
3e	73974,592	3,561E-55	**3e**	73961,411	3,629E-55
3f	73976,175	3,551E-55	**3f**	73970,084	3,588E-55
3g	74013,954	3,331E-55	**3g**	74002,365	3,397E-55
3h	74015,460	3,323E-55	**3h**	74006,318	3,375E-55
3i	74016,304	3,318E-55	**3i**	74008,726	3,361E-55
3j	74008,699	3,361E-55	**3j**	73998,220	3,421E-55

On the other hand, same process was applied for prodrug **3** and its all possible metabolites (Scheme **S1**) in both active sites of Ssap-NtrB. The binding energy and dissociation constant values for prodrug **3** and its metabolites were obtained and shown in SI part, (Tables **S7-S8**). The results show that the high binding energy (Ki) and the low dissociation constant result from energetically favorable interaction between Ssap-NtrB and metabolites such as **3g, 3h** and **3i**, (Table **2**). These metabolites were also validated their activities by being visualize 3D interactions with active sites of the enzyme (Fig. **7**). The residue interaction histogram demonstrates how the mentioned metabolites interact with which residue(s) in

active site A and B of the enzyme (Tables **S9-S12**). Especially, metabolite **3i** has the best binding energy and dissociation constant numerical values in all prodrugs. The numerical values of Ki and Kd are 7.402E+04, 3.318E-55 for A active site; 7.401E+04 and 3.361E-55 for B active site, respectively.

Another point derived from these results is that the prodrugs and/or metabolites do not exhibit the same interaction with the active sites of the enzyme, but show a better interaction, especially with A active site than with B active site of the enzyme.

CONCLUSION

In summary, four nitro substituted benzamide prodrugs **1-4** were synthesized and evaluated for their enzymatic activities with Ssap-NtrB. According to the HPLC results, all prodrugs were activated by Ssap-NtrB. While one metabolite formed as a result of activation of prodrugs **1** and **2** with Ssap-NtrB, three metabolites occurred from the reaction of prodrugs **3** and **4** with Ssap-NtrB. When compared to the Michaelis-Menten kinetic parameters, prodrug **3** which is synthesized from 4-nitrobenzoyl chloride and 2,4-dinitroaniline was found more active than CB1954 and SN23862 (**137** and **31** fold for Ssap-NtrB, respectively). Moreover, in the time dependent experiment with HPLC, it was observed that product profile changes with the reaction time.

In addition, the compounds were subjected to cell cytotoxicity assays. Firstly, the cell cytotoxicities of the compounds were evaluated by MTT assay in two different cancer cells and healthy endothelial cells. The compounds displayed diverse antiproliferative activities against cancer cell lines. The most resistant was colon carcinoma, HT-29 cells. The majority of the tested prodrugs were not toxic for all cells. Moreover, the prodrug **4** increased cell viability. Following the reaction of prodrugs with Ssap-NTR, the metabolites of all prodrugs (**1-4**) showed differential cytotoxic effects, with non toxic for HUVEC cells, moderate toxic for Hep3B cells, but highly toxic for PC3 cells. Amongst all metabolites of prodrugs after Ssap-NtrB reduction, *N*-(2,4-dinitrophenyl)-4-nitrobenzamide (**3**) was efficient and toxic in PC3 and Hep3B cells as compared as CB1954.

According to theoretical perspectives, prodrugs **1-4** show different interactions with A and B active sites of the enzyme. Especially, prodrug **3** and its metabolites (**3g, 3h** and **3i**) have energetically favorable interaction with Ssap-NtrB based on their conformations, binding energy and dissociation constant values. However, the prodrug **4** has the second most favorable tendency among the prodrugs. As a result of docking, the optimum number of nitro groups and positions were identified. It was observed that a similar interaction was not exhibited for both active sites of the enzyme, especially A active site exhibited better interaction than B active site of the enzyme. Furthermore, these results consolidate the importance of the conformation between the binding sites of Ssap-NtrB and nitro groups of the prodrugs for high reactivity.

HPLC results, kinetic parameters, molecular docking and cytotoxic data also confirm that prodrug **3** is better for Ssap-NtrB than **1**, **2** and **4** or known cancer prodrugs of CB1954 and SN23862, demonstrating that it is an efficient candidate for NTR based cancer therapy.

LIST OF ABBREVIATIONS

CB1954	=	5-(aziridine-1-yl)-2,4-dinitrobenzamide
DMSO	=	Dimethyl Sulfoxide
ACN	=	Acetonitrile
DS	=	Discovery Studio
G09	=	Gaussian 09
CHARMm	=	Chemistry at HARVARD Macromolecular Mechanics
BLAST	=	Basic Local Alignment Search Tool
NCBI	=	National Centre for Biotechnology Information
ABNR	=	Adopted-Basis Newton-Raphson
RMSD	=	Root Mean Square Deviation
K_i	=	Binding Energy
K_d	=	Dissociation Constant
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
SRB	=	Sulforhodamine B
DMEM	=	Dulbecco's Modified Eagle Medium
FCS	=	Fetal Calf Serum

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base on this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article. This data includes Model structure of Ssap-NtrB, Molecular Docking data, HPLC and LC-MS/MS results of prodrugs **1-4**.

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