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Research Article

Pharmacophore modelling and neuraminidase inhibitors identification from 4-hydroxy-3-methoxybenzaldehyde and 4-formylbenzoic acid derivatives

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Abstract

A 3D pharmacophore model was developed using Discovery Studio (DS) 2.5, Accelrys, with a training set comprising ten established influenza neuraminidase (NA) inhibitors. The best pharmacophore hypotheses consist of a hydrogen bond acceptor (HBA), a hydrogen bond donor (HBD) and a negative ionisable (NI). The pharmacophore generated hypotheses validated the test set comprises 4-hydroxy-3-methoxybenzaldehyde and 4-formylbenzoic acid derivatives. The chemical structures of the test compounds were established by FTIR and ^{1}HNMR spectral analyses. The 4-hydroxy-3-methoxybenzaldehyde derivatives (**1-2**) were found to map well to the pharmacophore model features compare to 4-formylbenzoic acid derivatives (**3-4**) and all compounds were further evaluated subjected to biological assay against MUNANA assay with *N*-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) as positive control. The two 4-hydroxy-3-methoxybenzaldehyde derivatives showed significant NA inhibition activity compared to both 4-formylbenzoic acid derivatives which was lack from activity.

Key Words: Pharmacophore, neuraminidase, training set, hypotheses, assay.

Introduction

Human flu epidemic causes great problems worldwide due to ineffective control by available antiviral drugs[1]. The virulent virus strains may lead to mass fatality and many countries have stockpiling the anti-influenza drugs[2-4]. The recent available anti-influenza drugs such as zanamivir and oseltamivir was facing some resistant to new influenza viral strains and therefore, we need to develop new novel and selective anti-influenza drugs to combat the resistance and fast mutations in influenza viral antigens[5].

To develop new anti-influenza drugs, neuraminidase (NA) has been the focused target for anti-influenza agents[6]. NA is a glycoprotein at the flu virus surface responsible for viral release from infected cells[7-8]. Compounds that inhibit NA will be considered to stop viral infections[9].

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The amino acid residues in the NA active site are conserved in all influenza viruses and this promotes NA as an excellent target for new lead NA inhibitors[10-11].

The aim of this study was to generate the best pharmacophore model for the search of NA inhibitors based on common features. The pharmacophore models are suitable for 3D database searching and validating to discover new potential lead compounds. The virtual screening will potentially selected some prospective compounds from 4-hydroxy-3-methoxybenzaldehyde (vanillin)and4-formylbenzoic acid (carboxybenzaldehyde) derivatives. We attempted to identify hypothetical pharmacophore model by using HipHop module[12].

In particular HipHop algorithm uses common feature pharmacophore models among a set of highly active compounds and carry out a qualitative model regardless biological activity data. The essential 3D arrangement of functional groups common to the set of molecules explains the specific activity of NA inhibitors. This approach is useful in building 3D pharmacophore models from the activity data and conformational structure as well as easy visualization with good prediction[13].

Material and Methods

Training set

Chemical feature-based 3D pharmacophore models was built within the Discovery Studio 2.5 software and used as queries for 3D database searching[14]. A training set of molecules having NA inhibitory activity was selected from established reported data. The selection of a suitable training set is essential for the quality of automatically generated pharmacophore model[15].

A set of training set as shown in Figure 1 represents the best known reported NA inhibitors. All structures were generated using 2D/3D and minimized by CHARMM force field and Poling Algorithm using ChemSketch and Discovery Studio 2.5 software package and building conformational models of up to 250 conformers for each molecule subjected the “best conformer generation” option and 20 kcal/mol energy above the lowest energy conformation found[16].

Hypothesis generation

The pharmacophore analysis was carried out to evaluate the common features required and the hypothetical geometries to search the most active compounds. HipHop module identifies 3D spatial arrangements of chemical features that are common to active molecules in a training set and provides feature-based alignment of a collection

of compounds regardless activity. Active training set compounds were evaluated on the basis of the types of their chemical features with the ability to adopt a conformation allows those features to be superimposed on a particular configuration.

Conformation-generating algorithms were adjusted to produce a diverse set of conformations without any repetitious groups of conformations. This matches the chemical features of molecules against drug candidate molecules. In this study, positive ionisable (PI), negative ionisable (NI), hydrogen-bond donor (HBD), hydrogen-bond acceptor (HBA), and hydrophobic (HY) were selected in order to broaden the search for deprotonated and protonated atoms or groups at physiological pH. HipHop generates ten hypotheses in a single run and hypotheses are ranked as they are built. The best generated pharmacophore model was used to predict and select the potential NA inhibitors from vanillin and carboxybenzaldehyde derivatives.

Synthesis

General methods

Chemicals were purchased from Aldrich, Fluka, Acros and J. T. Baker. The reactions of Schiff base condensation were carried out in Schlenk apparatus under nitrogen and the condensation was monitored by silica gel TLC. Detection was done with UV (254 nm). Infrared spectra were recorded on a Perkin Elmer Spectrum GX Fourier Transform Spectrphotometer using KBr disc from 4000-375 cm^{-1}. Electronic spectra were recorded on a Perkin Elmer Lambda25 from 260-550 nm in DMF. ^{1}H- and ^{13}C-NMR was recorded on a BRUKER 400 MHz, tetramethyl silane (TMS) was used as an internal standard spectrometer. The elemental analysis was performed using Flesh EA 1112.

General methods of preparation of compounds

All synthetic manipulations were conducted in the dry and nitrogen atmosphere. Solvent for synthesis were reagent grade. The starting materials used in reactions are 4-hydroxy-3-methoxybenzaldehyde (vanillin) and to 4-formylbenzoic acid (carboxybenzaldehyde).

Chemical Synthesis

All vanillin and carboxybenzaldehyde derivatives were prepared by Schiff-base condensation reactions. An ethanolic solution (20ml) of amine compounds (0.01mol) was added to an ethanolic solution (20ml) of vanillin/ carboxybenzaldehyde (0.01mol) and the solution was refluxed for about 5 hours with vigorous stirring and allowed to cool. Then, it was cool down in crushed ice for the compounds to be formed. The compounds were filtered and recrystallized from ethanol. The reaction was monitored by TLC.

Preparation of compounds

(4R,5R)-4-{[(E)-(4-hydroxy-3-methoxyphenyl)methylidene]amino}-1,5-dimethyl-2-phenylpyrazolidin-3-one (**1**)

Following the procedure of reaction of Schiff based gave 80% yield of **1**: IR (KBr) υ 3444 (O-H), 1630 (C=N), 1037 (C-O), 1732 (C=O) cm^{-1}; ^{1}HNMR (400 MHz, MeOD) δ 2.44 (s, 3H, -CCH_3), 3.14 (s, 3H, -NCH_3), 3.84 (s, 3H, -OCH_3), 6.84 (d, 1H, *J*=8 Hz), 7.18 (dd, 1H, *J*=2,2,8 Hz), 7.34 (s, 1H), 7.36-7.39 (m, 2H), 7.43 (d, 1H, *J*=2 Hz), 7.51-7.55 (m, 2H), 9.47 (s, 1H, -N=CH) ppm. Calc. For $C_{19}H_{21}N_3O_3$: C, 67.24; H, 6.24; N, 12.38%. Found: C,67.20; H,6.19; N, 12.32%.

4-(3,4-dimethyl-5-phenyl-1,3-oxazolidin-2-yl)-2-methoxyphenol (**2**)

Following the procedure of reaction Schiff based gave 70% yield of **2**. IR (KBr) υ 3403 (O-H), 1614 (C=N), 1037 (C-O), 1214 (C-N) cm^{-1}; ^{1}HNMR(400 MHz, MeOD) 1.21 (d, 3H, *J*=7 Hz, -NCH_3), 2.19 (s, 3H, -CCH_3), 2.50-2.57 (m, 1H), 3.91 (s, 3H, -OCH_3), 4.71 (d, 1H, *J*=8 Hz), 4.90 (s, 1H), 6.82 (d, 1H, *J*=8 Hz), 6.98 (dd, 1H, *J*=2, 2, 8 Hz), 7.18 (d, 1 H, *J*=2 Hz), 7.31-7.34 (m, 1H), 7.37-7.41 (t, 2H, *J*= 8,8 Hz), 7.44 (s, 1H), 7.45 (d, 1H, *J*=2 Hz) ppm. Calc. for $C_{15}H_{21}NO_3$: C, 86.01; H, 8.42; N, 5.60%. Found: C, 85.97; H, 8.37; N, 5.54%.

4-{(*E*)-[(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)imino]methyl}benzoic acid (**3**)

Following the procedure of reaction Schiff based gave 70% yield of **3.** IR (KBr) υ 3440 (O-H), 1695 (C=O), 1230 (C-N), 1536 (C=N), 3371, 3440 (N-H), 1050, 1091, 1119, 1156, 1230 (C-N), 1491, 1536 (Ar), 1695 (C=O), 2578-3056 (COOH) cm^{-1}. ^{1}HNMR (400 MHz, MeOD) δ 2.49 (s, 3H), 2.50 (m, 2H), 3.22 (s, 3H), 7.37-7.56 (m, 5H), 7.90 (d, J=8 Hz, 2H), 8.00 (d, J=8 Hz, 2H), 9.63 (s, 1H) ppm. Anal. Calc. for $C_{19}H_{17}N_3O_3$: C, 79.41; H, 5.96; N, 14.62%. Found: C, 79.36; H, 5.91; N, 14.61%.

4-(3,4-dimethyl-5-phenyl-1,3-oxazolidin-2-yl)benzoic acid (**4**)

Following the procedure of reaction Schiff based gave 70% yield of **4.** IR (KBr) υ 3064 (O-H), 1127 (C-O), 1221 (C-N) cm^{-1}; ^{1}HNMR (400 MHz, MeOD) δ 1.11 (d, *J*=6 Hz, 3H, -CH_3), 2.10 (s, 3H, -NCH_3), 2.46 (m, 1H), 4.70 (d, *J*=9 Hz, 1H), 4.99 (s, 1H), 8.00 (d, *J*=9 Hz, 2H), 7.60 (d, *J*=8 Hz, 2H), 7.42 (m, 2H), 7.37 (m, 2H), 7.30 (m, 1H) ppm. Anal. Calc. for $C_{18}H_{19}NO_3$: C, 86.70; H, 7.68; N, 5.62%. Found: C, 85.97; H, 7.62; N, 5.58%.

Neuraminidase (NA) inhibition assay

Reagents and apparatus

2-(4-methylumbelliferyl)-α-*D*-acetylneuraminic acid (MUNANA), *N*-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA), 2-*N*-Morpholino-ethanesulfonic acid (MES), Neuraminidase from c*lostridium perfringens* (*C. welchii*) were purchased from Sigma. $CaCl_2$, NaOH and ethanol were purchased from R&M Chemicals. Microplate 96 F was purchased from BMG Co., Germany.

Biological evaluation

The MUNANA inhibition assay was done based on the method of Maki Kiso *et al*.[17] (altered). MUNANA, a fluorogenic substrate 20-(4-methylumbelliferyl)-α-*D*-*N*-acetylneuraminic acid was used where DANA was introduce as positive control. Cleavage of this substrate by NA produces a fluorescent product which emits an

emission wavelength of 460 nm with an excitation wavelength of 355 nm. The intensity of fluorescence can reflect the activity of NA sensitively. Each assay was done in triplicate. Each NA was diluted in MES assay buffer according to the factor determined in the MUNANA assay. The fluorescence involving 4-MUNANA at excitation 365 nm and emission at 450 nm was measured using MODULUS Multi-well Plate Reader.

Results and discussion

The qualitative top ten hypotheses were generated based on the ten training set molecules. Ten hypotheses (1-10) were obtained using the default parameters of catalyst. Direct and partial heat mask value of '1' and '0' for hypothesis indicates that the molecules present in the dataset are well mapped to all the chemical features in the hypothesis specified in Table 1.

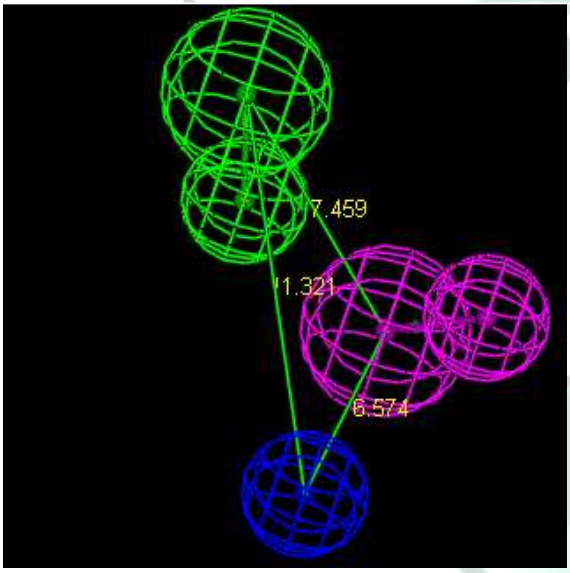

Figure 2. Pharmacophore model of the top rank score generated from training set

The hypothesis shows rank score ranges from 76.069 to 80.004. The best choice for the pharmacophore model was the hypothesis with the highest rank score (80.004). The best pharmacophore model consist three chemical features: one hydrogen bond acceptor (HBA-green), one hydrogen bond donor (HBD-magenta) and one negative ionisable (NI-dark blue). Thus, this model was employed as 3D search query against NA inhibitors using the 'fast flexible search' approach.

The pharmacophore model was selected as the best qualitative model based on the chemical features similarities, ranking score and shows good alignment with the training set molecules. The rank score range over the ten generated models is 3.9 and the small rank score range observed was due to molecules in the training set have a high degree of structural homology and good similarity in 3D spatial shape[18] and therefore, this hypotheses are considered to be equivalent. Figure 2 displayed the pharmacophore model generated with geometric parameters (distance point).

The hypotheses overlays the common features for which the fit of individual molecules to a hypothesis correlated with the molecule's activity. All compounds associated with their conformations were submitted to HipHop module for ligand pharmacophore mapping to the generated model to explain the specification of the NA inhibitors which are the best significant results among the biological evaluation against NA bioassay. The pharmacophore mapping and its fit values of the vanillin derivatives (**1**-**2**) and carboxybenzaldehyde derivatives (**3**-**4**), respectively were shown in Figure 3.

It was obviously shown that both carboxybenzaldehyde derivatives (**3**-**4**) mapped to HBA and NI features but lack of HBD feature mapping and exert lower fit values (1.59738 and 1.51319) compare to vanillin derivatives (1.98806 and 1.89851) which mapped to HBA and HBD features. The ligand mapping results revealed that the conformational properties of the compounds are important for the activity against viral NA and thus, the observation predicted the synthesized compounds.

Table 1. Detail of the top ten hypotheses generated using training set 1 of HYPO A

Hypothesis	Features	Rank	Direct hit	Partial hit	Max fit
1	HBA, HBD, NI	80.004	1111111111	0000000000	3
2	HBA, HBD, NI	79.900	1111111111	0000000000	3
3	HBA, HBD, NI	79.604	1111111111	0000000000	3
4	HBA, HBD, NI	79.005	1111111111	0000000000	3
5	HBA, HBD, NI	78.438	1111111111	0000000000	3
6	HBA, HBD, NI	78.060	1111111111	0000000000	3
7	HBA, HBD, NI	77.654	1111111111	0000000000	3
8	HBA, HBD, NI	77.289	1111111111	0000000000	3
9	HBA, HBD, NI	77.179	1111111111	0000000000	3
10	HBA, HBD, NI	76.069	1111111111	0000000000	3

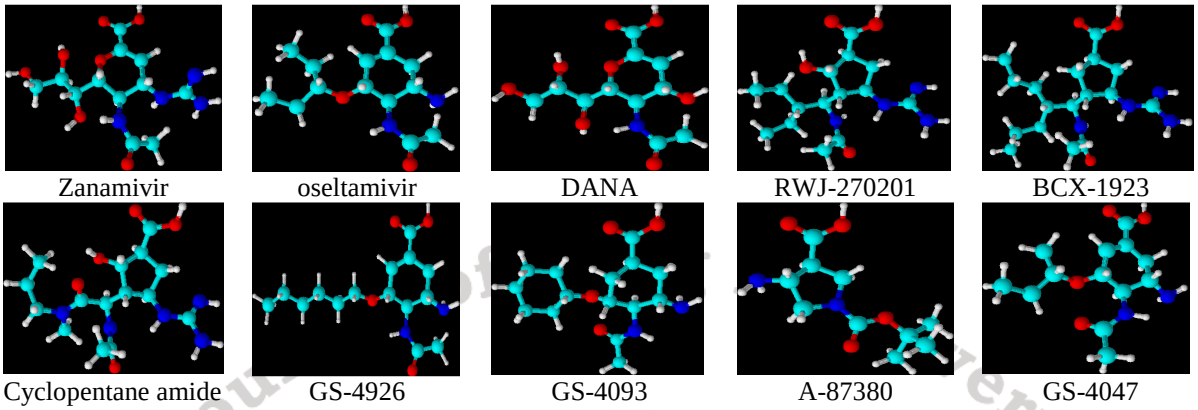

Figure 1. Structure of training set molecules

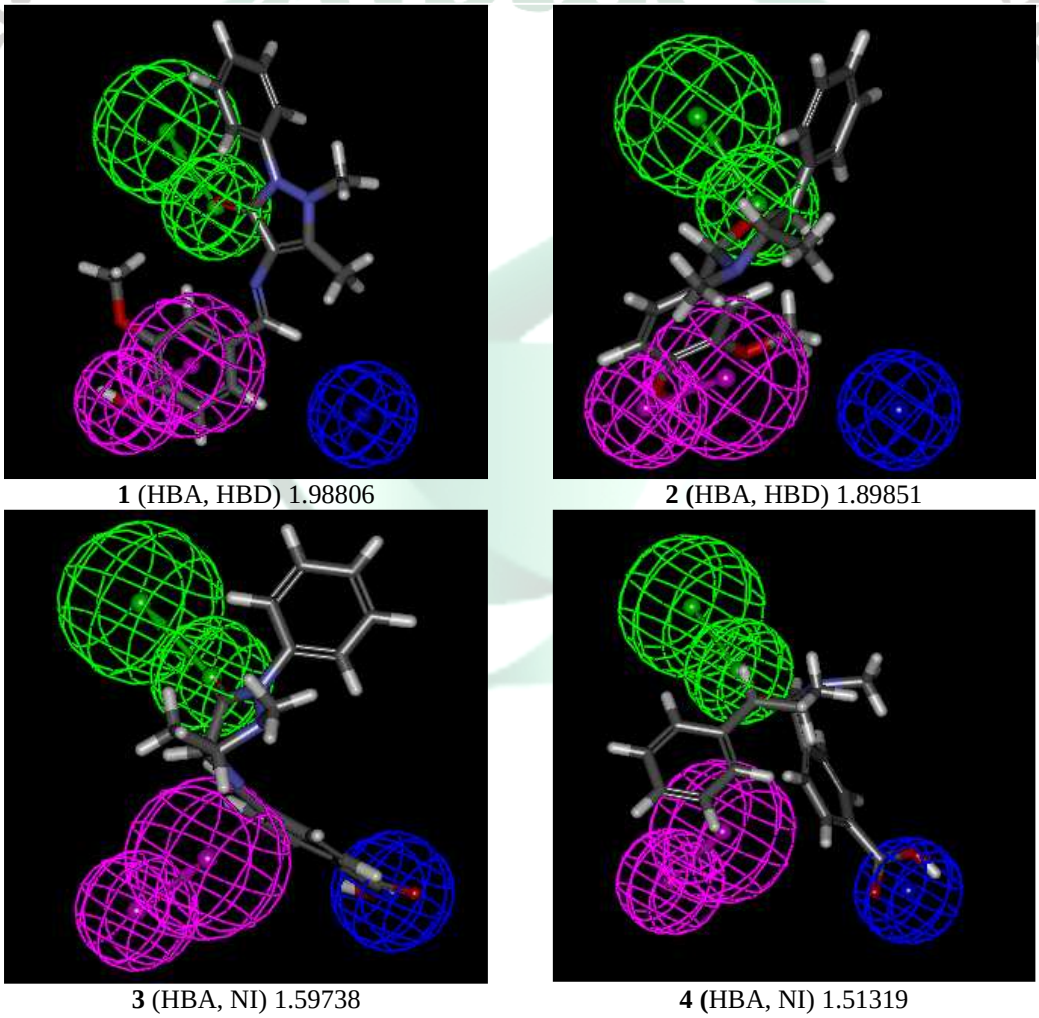

Figure 3. The mapping of compounds **1-4** to pharmacophore model with mapping features and fit

The synthesized vanillin derivatives (**1-2**) are lack of NI feature due to the absent of carboxyl (-COOH) group compare to carboxybenzaldehyde derivatives (**3-4**) which possess the NI feature. Both vanillin derivatives (**1-2**) bearing hydroxyl (-OH) group mapped HBD to all hypotheses. This information proposed that HBD feature is crucial for NA inhibition activity. The methoxy (-OCH_3) group as well plays an important role to exert NA inhibitory activity. The lower fit value of carboxybenzaldehyde derivatives indicates that although the chemical features of particular hit overlap with the corresponding pharmacophore features, the centres of the functional groups of the hits are slightly displace from the distance point of the pharmacophore features.

The virtual fact regarding the pharmacophore modelling results was performed *via* biological evaluation against MUNANA assay. In MUNANA assay, all newly synthesized compounds as shown in Figure 4 were tested against NA of *clostridium perfringens* in a dose dependent manner. The inhibitory effects of the synthetic compounds against NA were reported and Table 2 shows the inhibitory activity of vanillin derivatives (**1-2**) and carboxybenzaldehyde derivatives (**3-4**), respectively.

The NA inhibition MUNANA assay results showed that vanillin derivatives (**1-2**) were active against NA activity. Compound **2** showed the strongest activity, inhibited NA of IC_{50} 0.016 mg/mL compare to DANA (0.041 mg/mL) and compound **1** (0.147 mg/mL). Both, carboxybenzaldehyde derivatives (**3-4**) showed no NA inhibition activity. The use of pharmacophore modelling approach was capable to search NA inhibitors and in this paper we found that vanillin derivatives potentially to be explored and manipulated as NA inhibitors. Interestingly, vanillin derivatives (**1-2**) showed activity and carboxybenzaldehyde derivatives (**3-4**) are devoid from activity indicates that NI feature which map to carboxylic (-COOH) group is not essential for NA inhibition activity.

Acknowledgments

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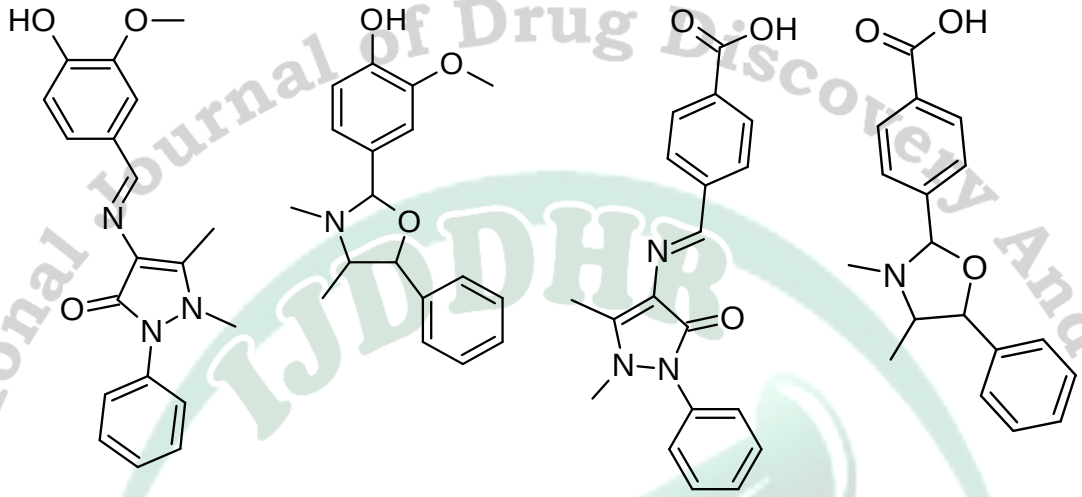

1 **2** **3** **4**

Figure 4. Synthesized vanillin (**1**-**2**) and carboxybenzaldehyde derivatives (**3**-**4**)

Table 2. Vanillin and carboxybenzaldehyde derivatives tested against MUNANA assay

compound	MUNANA inhibition assay (IC_{50} = mg/mL)
2	0.016
DANA	0.041
1	0.147
3	No activity
4	No activity