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A new small molecule that directly inhibits the DNA binding of NF-κB

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ABSTRACT

Nuclear factor-κB (NF-κB) has been considered as a good target for the treatment of many diseases. Although a lot of NF-κB inhibitors have already been reported, many of them have several common problems. Thus, we attempted to identify novel NF-κB inhibitors to be unique lead compounds for creating new pharmaceuticals. In the present study, we screened our chemical library for compounds that directly inhibit the DNA binding of NF-κB by using fluorescence correlation spectroscopy (FCS). Consequently, we identified a promising compound, 4,6-dichloro-*N*-phenyl-1,3,5-triazin-2-amine, referred to as NI241. It mediated a dose-dependent inhibition of the DNA binding of NF-κB p50. Its analogues also showed dose-dependent inhibition and their inhibitory effects were altered by the substituents on the *N*-phenyl group. Furthermore, we predicted the binding mode of NI241 with p50 in silico. In this model, NI241 forms three hydrogen bonds with Tyr60, His144, and Asp242 on p50, which are important amino acid residues for the interaction with DNA. These results suggest that NI241 with structural novelty may serve as a useful scaffold for the creation of new NF-κB inhibitors by rational optimization.

1. Introduction

Nuclear factor-κB (NF-κB) is a transcription factor that regulates gene expression by binding to discrete DNA sequences, known as κB elements. NF-κB regulates a wide variety of genes involved in diverse and key cellular and organismal processes, including cell proliferation, cell survival and death, innate immunity, and inflammation.[1–5] Because of these functions, the dysregulation, especially the activation, of NF-κB has been implicated in many diseases, such as cancers[6–9] and chronic inflammatory diseases.[10,11] Thus, selective NF-κB inhibitors would be useful in the investigation and the treatment of these diseases. Although a lot of NF-κB inhibitors have already been reported,[12–14] they share several problems.[12,13] First, many NF-κB pathway inhibitors are cell type- or stimulant-dependent. Second, in many cases, these compounds are likely to affect other targets and pathways. Third, the concentrations of compounds used to inhibit NF-κB in vitro are substantially different and often much higher than could ever be used or achieved in vivo. Therefore, the identification of specific and potent inhibitors of NF-κB has been pursued by researchers and pharmaceutical companies.

Two distinct signaling pathways, the canonical pathway and the non-canonical pathway, are implicated in the activation of NF-κB.[15–17] NF-κB specifically activates these pathways through the recognition of κB elements with a consensus sequence of 5′-GGGRNYYYCC-3′ and induces the expression of target genes. Because NF-κB signaling pathways converge at the level of DNA binding, the compounds that directly inhibit the DNA binding of NF-κB may be highly effective in regulating the expression of target genes. Here, we attempted to identify small molecules in our chemical library that directly inhibit the DNA binding of NF-κB. Interestingly, a scaffold compound, 4,6-dichloro-*N*-phenyl-1,3,5-triazin-2-amine, named NI241, was identified. Furthermore, we compared the inhibitory effects of NI241 analogues and predicted the binding mode of NI241 with p50 in silico. These results raise the possibility that NI241 is a novel seed small molecule for the development of potent and specific inhibitors of NF-κB.

2. Results

2.1. The inhibitory effect of NI241 on the DNA binding of NF-κB

We screened hundreds of chemical compounds from our chemical library by using fluorescence correlation spectroscopy (FCS).[18–21] We found a unique compound, 4,6-dichloro-*N*-phenyl-1,3,5-triazin-2-amine, named NI241, that inhibits the DNA binding of NF-κB (Fig. 1).

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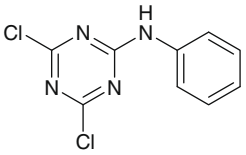

Figure 1. Chemical structure of NI241. NI241; 4,6-dichloro-*N*-phenyl-1,3,5-triazin-2-amine.

We used a single-molecule fluorescence detection system, MF20, for our detailed FCS evaluation of the inhibitory effect of NI241.[20,21] This system allows us to simultaneously measure and distinguish the individual signals of both fluorescence-free and fluorescence-bound molecules in the same solution. The data measured by this system are shown as diffusion times of fluorescence molecules. When the fluorescence-labeled molecules bind targeted molecules, the diffusion times are extended. The degrees of the extension of diffusion times are correlated with the ratio of interacting molecules.

We used MF20 to measure the changes of diffusion times of Alexa647-labeled NF-κB probe bound to human GST-NFκB p50 subunit (Δ434-969) (GST-hp50) with various concentrations of NI241. As shown in Figure 2A, the diffusion time of Alexa647-labeled NF-κB probe by itself was 908.2 ± 18.4 μs. When GST-hp50 was added to the Alexa-647-labeled NF-κB probe, the diffusion time was extended to be 1648.2 ± 64.9 μs, which indicates that GST-hp50 binds to NF-κB probe. To evaluate the inhibitory effect of NI241, it was added to this reaction mixture. As shown in Figure 2A, the diffusion time was shortened in a dose-dependent manner. The diffusion time with 300 μM NI241 was 922.2 ± 31.1 μs, which is almost the same as that of the control.

To verify the inhibitory effect of NI241 on the DNA binding of NF-κB, we performed an electrophoretic mobility shift assay (EMSA) by using a ^{32}P-labeled NF-κB probe and GST-hp50. EMSA is a conventional method for assaying sequence-specific DNA binding in vitro. EMSA also revealed that NI241 inhibited the DNA binding of GST-hp50 in a dose-dependent manner (Fig. 2B), although the inhibitory effect was weaker than that observed with FCS.

2.2. The comparison of the inhibitory effects among NI241 analogues

Next, we investigated the inhibitory effects of six NI241 analogues (Fig. 3) screened from our chemical library by FCS. To compare the inhibitory effects of these analogues, we used a two-component fit analysis. This method analyzes the difference in each molecular mass and discriminates the protein-bound DNA from the free DNA.[20] The fraction (%) in Figure 4 indicates the amount of Alexa-647-labeled NF-κB probe that is bound to GST-hp50 in the solution.

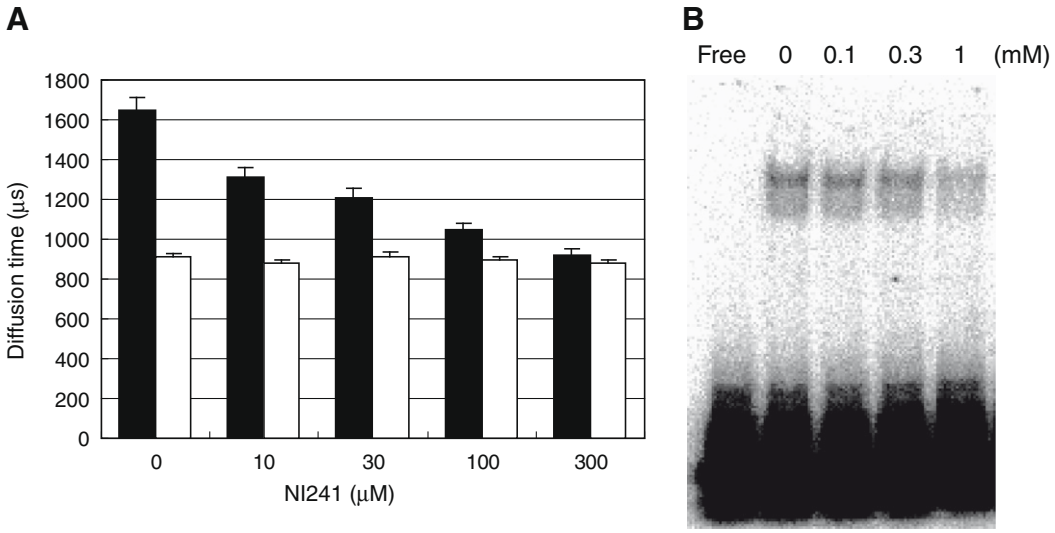

Figure 2. The inhibitory effect of NI241 on the DNA binding of GST-hp50. (A) The inhibitory effect of NI241 on the DNA binding of GST-hp50 was measured by FCS and shown as the change in diffusion times. The black and white bars indicate the diffusion times of Alexa647-labeled NF-κB probe bound to GST-hp50 and unbound Alexa647-labeled NF-κB probe as a control, respectively. Values represent the mean ± SD of five measurements. (B) The inhibitory effect of NI241 was analyzed by EMSA.

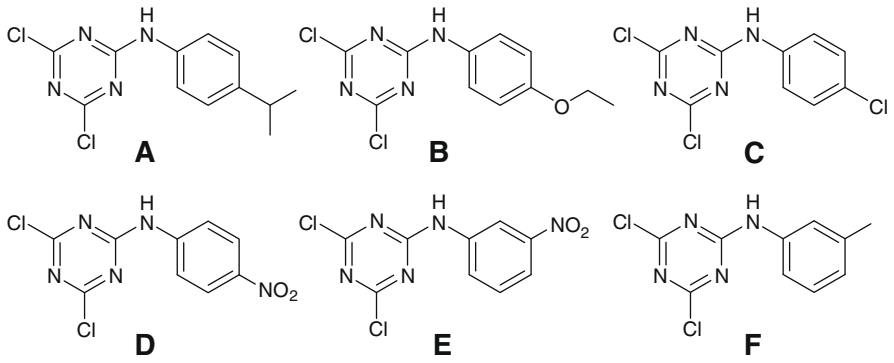

Figure 3. Chemical structures of the NI241 analogues (Compounds A–F).

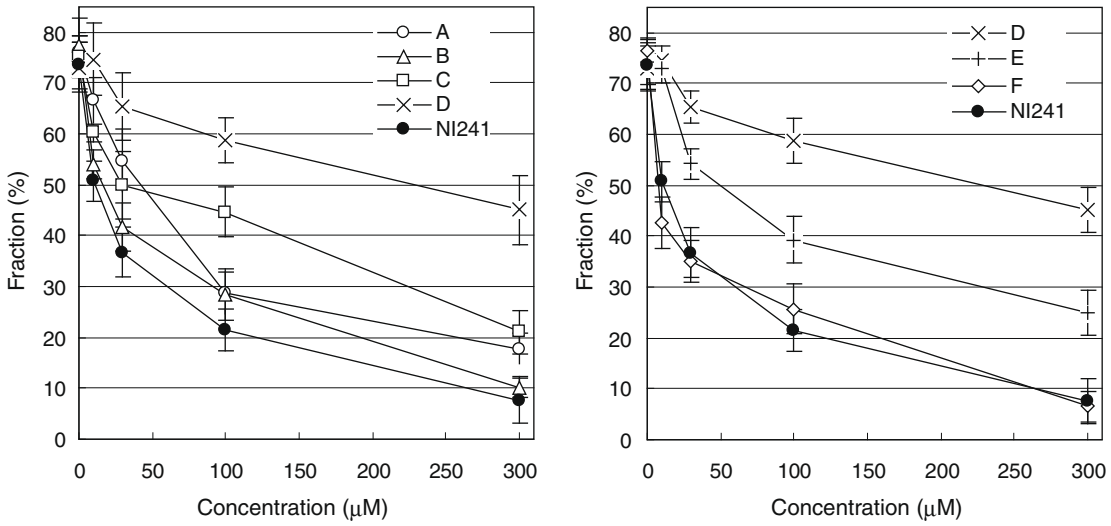

Figure 4. Comparison of the inhibitory effects among NI241 analogues. The inhibitory effects of the NI241 analogues were compared by a two-component fit analysis. Fraction (%) represents the amount of Alexa647-labeled NF-κB probe that is bound to GST-hp50. Values represent the mean ± SD of five measurements.

We examined the inhibitory effects of NI241 analogues that contain a functional group at the C4-position of the *N*-phenyl group (Fig. 4, left panel). NI241 and compounds A, B, and C had IC_{50} values of 28.6 μM, 74.4 μM, 35.2 μM, and 110 μM, respectively. Compound D, which has a nitro group, had a weaker inhibitory effect (IC_{50}, >300 μM) on the DNA binding of GST-hp50. We then investigated the inhibitory effects of compounds E and F (Fig. 4, right panel). Compound E is the isomer of compound D and has a nitro group at the C3-position of the *N*-phenyl group. Compound F has a methyl group at this position. The IC_{50} value of compound E was 119 μM, which indicates an inhibitory effect that is stronger than that of compound D but weaker than that of NI241. Compound F had an IC_{50} value of 19.0 μM, which indicates a slightly stronger inhibition than NI241.

2.3. The binding mode of NI241 with p50

To find more potent and specific NF-κB inhibitors, we predicted the binding mode of NI241 with p50 by applying molecular modeling and computational analyses. In the case where small molecules behave as good inhibitors, it is plausible that these molecules interact with the important amino acid residues for the DNA binding of NF-κB.[22–25] The region constructed by Arg59-Pro71 appears to be a DNA binding domain, referred to as DBR.[26,27] Based on this information, we performed docking simulations between NI241 and p50 and predicted the binding mode of this complex. In the complex of p50 with DNA, Tyr60 and Glu63 of p50 forms a hydrogen bond with DNA, respectively (Fig. 5A). On the other hand, NI241 forms three hydrogen bonds with Tyr60, His144, and Asp242 of p50; consequently, Tyr60 of p50 could not interact with DNA (Fig. 5B). Since Tyr60 in DBR of p50 is important for the DNA binding,[22–25] the interaction of NI241 with Tyr60 appears to be significantly associated with the inhibition of p50 DNA binding activity. Although His144 interacts with DNA,[23] His144 and Asp242 do not exist in the DBR of p50.[26,27] Furthermore, in this docking mode, these two amino acid residues do not form hydrogen bonds with DNA (Fig 5A).

To evaluate the binding specificities between NI241 analogues and p50, we performed the docking studies of NI241 analogues to DBR of p50 in a manner similar to NI241 using GLIDE 4.0.[33]

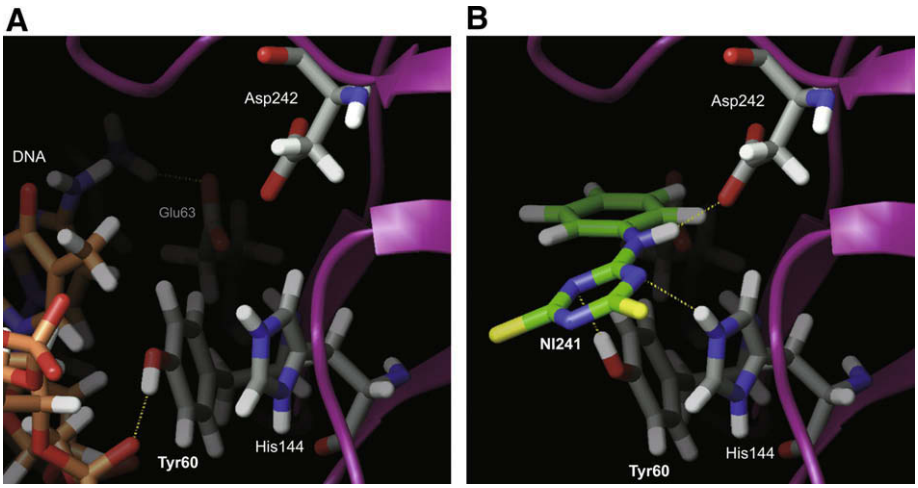

Figure 5. A predicted binding mode of NI241 on p50 with DNA. The binding mode was obtained from docking simulations. (A) The crystal structure of p50 (maroon) in complex with DNA (coral). (B) A predicted binding mode of NI241 (green) with p50. Nitrogen, oxygen, hydrogen, and chlorine atoms are illustrated in blue, red, white, and yellow, respectively. The hydrogen bonds are depicted as yellow dashed lines.

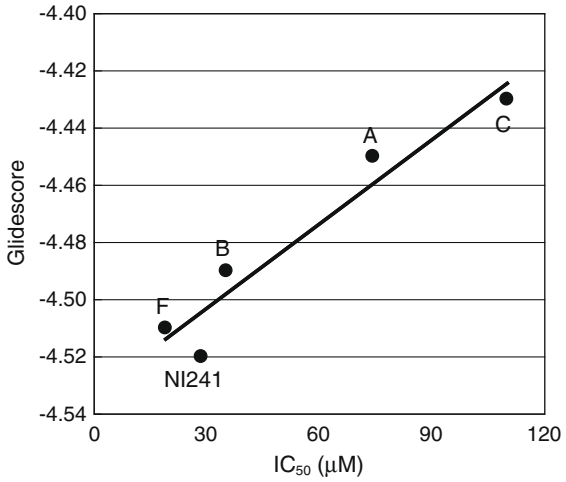

Figure 6. The correlation between Glidescore and IC_{50} value of NI241 and its analogues. Predicting the binding affinity and rank-ordering NI241, compound A, B, C, and F were implemented by Glidescore. The correlation between Glidescore and IC_{50} value of them (R^2 value) was calculated to be 0.93.

NI241 analogues were ranked based on Glidescore whereby the more negative values correspond to greater predicted binding affinities. As shown in Figure 6, a correlation was observed between Glidescore and IC_{50} values of NI241 analogues ($R^2 = 0.93$) except for compound D and E which have a nitro group of the *N*-phenyl group. The Glidescore of these two compounds were lower than those of the other analogues including NI241 (data not shown). Since the inhibitory effects of these compounds which have a nitro group were weaker than that of other NI241 analogues, the nitro group may interfere the accurate calculation of the binding affinities. Nevertheless, these results indicate the specific binding of NI241 and its analogues to the unique pocket on p50 constructed by hot amino acid residues of Tyr60, His144, and Asp242.

3. Discussion

NF-κB affects a multitude of cellular and organismal processes and is considered a good potential drug target.[12,13,28–30] In the present study, we searched our chemical library to identify small molecules that directly inhibit the DNA binding of NF-κB. We identified one inhibitory molecule, NI241 (Fig. 1), by evaluating the inhibitory effect using FCS and EMSA. This compound was found to be a dose-dependent inhibition of NF-κB with IC_{50} value of 28.6 μM (Fig. 2).

When we examined the inhibitory effects of NI241 analogues (Fig. 3) on the DNA binding of p50 by FCS and a two-component fit analysis (Fig. 4), all analogues inhibited the DNA binding of p50 in dose-dependent manners. Interestingly, the inhibitory effects were altered by differences in the functional groups. The analogues that have a functional group at the C4-position of the *N*-phenyl group mediated weaker inhibition than NI241. Notably, the inhibitory effect of compound D, which has a nitro group at this position, was the weakest, and its IC_{50} value was >300 μM (Fig. 4, left panel). Compound E, which has a nitro group at the C3-position, showed stronger inhibition than compound D; but, its inhibitory effect was weaker than that of NI241. The IC_{50} of the compound F, which has a methyl group at the C3 position, was 19.0 μM, indicating that its inhibitory effect was the strongest among these NI241 analogues (Fig. 4, right panel).

A functional group at the C4-position of the *N*-phenyl group reduced the inhibitory effect of NI241 on the DNA binding of p50. When the position of the nitro group is shifted from C4 to C3, the inhibitory effect is increased. Since these reduced inhibitory effects were not dependent on the functional groups at the C4-position, NI241 analogues, which have functional groups at the C4-position, may interfere with the DNA binding activity of p50 due to steric hindrance. Thus, the introduction of a functional group at other positions may be appropriate to the optimization of NI241. Indeed, compound F, which has a methyl group at the C3-position of the *N*-phenyl group, showed stronger inhibition than NI241. As for the effects of a nitro group, compounds D and E showed weaker inhibition than other analogues. The nitro group is an electron-withdrawing group and reduces the electron density of the phenyl ring. This property may significantly reduce the inhibitory effects of compounds D and E on the DNA binding of p50. In particular, compound D may be influenced by both steric hindrance and the nitro group, and it showed the weakest inhibition among the NI241 analogues examined. Taken together, the identification of optimal substituents and positions of the *N*-phenyl group is an important point for the optimization of NI241 NF-κB inhibitors.

Inhibitors that directly interfere with the DNA binding activity of proteins block the DNA binding sites by interfering with the side chains of the amino acid residues, by affecting the protein conformation, or by providing steric hindrances. In the binding mode obtained from our docking simulations, NI241 forms a hydrogen bond with the hydroxy group of Tyr60 in p50 (Fig. 5B). Tyr60 in DBR is one of the amino acid residues implicated in the DNA binding of p50,[22–25] and the hydroxy group of Tyr60 forms a hydrogen bond with DNA (Fig. 5A). When NI241 analogues were docked into DBR of p50 as well as NI241, a good correlation was observed between Glidescore and IC_{50} value of NI241, compound A, B, C and F ($R^2 = 0.93$, Fig. 6). This implies that the binding conformation and binding model of the NI241 to DBR of p50 are reasonable and reliable. Thus, the predicted binding mode suggests that NI241 may specifically interfere with the DNA binding of p50 by forming a hydrogen bond to Tyr60 of p50. This hydrogen bond may be important for the specific inhibitory effect of NI241 on the DNA binding of p50.

The inhibitory effect of NI241 was mediated by the substituents and their positions on the *N*-phenyl group. Computational analyses predicted that a hydrogen bond between NI241 and Tyr60 of p50 is important for the inhibitory effect. In conclusion, NI241 is a small seed molecule that will potentially lead to the development of more potent and specific NF-κB inhibitors. Determining the mechanism by which NI241 interacts with NF-κB will allow us to design novel NF-κB inhibitors by rational modifications based on its structure.

4. Experimental

4.1. Materials

All chemicals were purchased from ASINEX and dissolved in dimethyl sulfoxide. We used recombinant human GST-NFκB p50 subunit (Δ434-969) (GST-hp50) supplied by Upstate. 5′-Alexa 647-labeled κB single-stranded oligonucleotide, which corresponds to the binding sites of NF-κB, was purchased from Japan Bio Services (Saitama, Japan).

4.2. Fluorescence correlation spectroscopy (FCS) analysis

The following Alexa647-labeled, double-stranded oligonucleotide containing a κB element was used: 5′-Alexa647-AGTTGA*GG GGACTTTCC*CAGGC-3′ (sense) and 5′-GCCTG*GGAAAGTCCCC*TCA ACT-3′ (antisense); the *underlined* sequences show the κB element.

GST-hp50 (12 ng) was used for FCS analysis. Reaction mixtures containing binding buffer (15 mM Tris–HCl (pH 7.5), 75 mM NaCl, 1.5 mM EDTA, 1.5 mM dithiothreitol, 7.52% glycerol, and 0.3% Nonidet P-40) and GST-hp50 were stood on ice for 10 min, and then NI241 analogue (0–300 μM) was added to the mixtures and incubated at room temperature. After 30 min, 1 nM Alexa647-labeled NF-κB probe was added to the mixtures and further incubated at room temperature for 30 min. The 30 μl of the mixture was loaded onto a 384-well, glass-bottomed microplate, and FCS measurement was performed by the single-molecule fluorescence detection system, MF20 (Olympus).[20,21] All experiments were performed under identical conditions with a data acquisition time of 30 s per measurement, and measurements were repeated five times per sample.

4.3. Electrophoretic mobility shift assay (EMSA)

NF-κB probe (5′-AGTTGA*GGGGACTTTCC*CAGGC-3′) was phosphorylated with T4 polynucleotide kinase in the presence of [γ-^{32}P] ATP and was purified using a QIAquick Nucleotide Removal kit (Qiagen). GST-hp50 (12 ng) was used for EMSA. Reaction mixtures containing binding buffer (15 mM Tris–HCl (pH 7.5), 75 mM NaCl, 1.5 mM EDTA, 1.5 mM dithiothreitol, 7.52% glycerol, and 0.3% Nonidet P-40), 1 μg of poly(dI-dC)·(dI-dC) and GST-hp50 were stood on ice for 10 min, and then NI241 (0–1 mM) was added to the mixtures and incubated at room temperature. After 30 min, 1 μM ^{32}P-labeled NF-κB probe was added to the mixtures and further incubated at room temperature for 30 min. The 20 μl of the mixture was loaded onto a native 5% polyacrylamide gel prepared in 0.5 × TBE and electrophoresed at 170 CV for 2.5 h. The gel was dried after electrophoresis and exposed to an imaging plate. This imaging plate was analyzed by BAS-1800 (Fujifilm).

4.4. Computational molecular modeling and docking

The NI241 structure was built, manipulated, and adjusted for chemical correctness using MAESTRO 7.5.[31] Geometry minimization was performed on NI241 using the MMFFs force field and the Polak-Ribiere Conjugate Gradient (PRCG) with MACROMODEL 9.1.[32] The 3-D structure of NF-κB p50 was obtained from the protein data bank (PDB code: 1NFK). All water molecules were removed and just one p50 subunit was used for modeling. The optimized NI241 was docked into the crystal structure of p50. The docking simulations were performed with GLIDE 4.0.[33] The interpretation of the docking results is based on this fitness function in the form of Glidescore. To evaluate the binding specificities between NI241 analogues and DBR of p50, the docking studies of NI241 analogues were performed with GLIDE 4.0.[33] The optimized NI241 analogues as well as NI241 were docked into the crystal structure of p50. The energy evaluations of these compounds were done with Glidescore and single best pose is generated as the output for a particular analogue. A correlation was calculated between Glidescore and IC_{50} value of NI241 analogues via a linear regression analysis.

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