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2, 5-DISUBSTITUTED OXADIAZOLE AND IT'S DERIVATIVE INHIBIT PROTEIN TYROSINE PHOSPHATASE PTP1B, ENHANCING INSULIN RECEPTOR PHOSPHORYLATION

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ABSTRACT

Protein tyrosine phosphatase 1B (PTP1B) is known to be a key regulator of insulin sensitivity. A series of 2, 5-disubstituted oxadiazole were synthesized and screened for the PTP1B inhibition, insulin receptor phosphorylation and for its pharmacokinetic profiles. In Enzyme-based assay for PTP1B new chemical entity-9 (NCE-9), showed a potent inhibition of PTP1B with an $IC_{50}$of 0.46 µmol/L and no visible inhibitory activity for other PTP family members. NCE-9 also shows increased the insulin induced tyrosine phosphorylation of IRβ and boosted IR phosphorylation more potently. NCE-9 showed good in vivo pharmacokinetic profiles in C57BL/6J mice and SD rats. Overall, these studies suggest that NCE-9 inhibit PTP1B and enhance insulin receptor phosphorylation with good in vivo pharmacokinetic profile. It would be a new therapeutic candidate with potential for the treatment of type 2 diabetes.

KEYWORDS: NCE-9, Protein tyrosine phosphatase 1B, IRβ.

INTRODUCTION

The relationship between obesity and type 2 diabetes has a polygenetic component and is associated with insulin resistance.[1] Insulin resistance is evident in many tissues that are important for glucose homeostasis, including muscle, liver, fat and at the level of the central

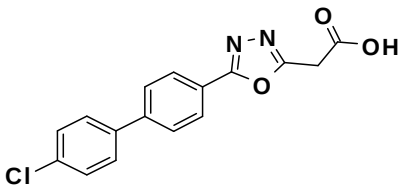
nervous system (CNS).[2,3] Metabolic insulin signal transduction occurs through activation of the insulin receptor, including autophosphorylation of tyrosine (Tyr) residues in the insulin-receptor activation loop.[4] This leads to recruitment of insulin receptor substrate (IRS) proteins, followed by activation of phosphatidylinositol 3-kinase (PI3K) and downstream protein kinase B (PKB; also known as AKT), and activation and subsequent translocation of the glucose transporter GLUT4.[5,6] This process is negatively regulated by PROTEIN TYROSINE PHOSPHATASES (PTPs), and is a general mechanism for downregulation of receptor tyrosine kinase (RTK) activity.[7] Several PTPs, including receptor protein tyrosine phosphatase-α (rPTP-α), leukocyte antigen-related tyrosine phosphatase (LAR), SH2-domain-containing phosphotyrosine phosphatase (SHP2) and protein tyrosine phosphatase 1B (PTP1B), have been implicated in modulating insulin signal transduction.[8] PTP1B seems to be a key regulator of insulin-receptor activity that acts at the insulin receptor and at downstream signaling components, such as IRS1.[9] Inhibition of PTP1B results in sensitization to insulin signaling and protection against diet induced obesity.[10] The protein tyrosine phosphatases (PTPs) constitute a family of closely related key regulatory enzymes that dephosphorylate phosphotyrosine residues in their protein substrates.[11] The PTP family can be divided into two major groups based on their substrate specificity: classical phosphotyrosine (pTyr)-specific PTPs (38 phosphatases belong to this subfamily) and dual specificity PTPs which recognize pTyr, phosphothreonine (pThr) and phosphoserine (pSer) residues.[12] The widely expressed PTP1B is the prototype for the super family of PTPs, and was the first mammalian PTP identified and purified to homogeneity.[13] It belongs to intracellular classical PTP family.[14] Full-length PTP1B contains 435 amino acids and constitutes the major cellular form. But a shorter 298- or 321-residue variant is widely used for biochemical investigations. PTP1B contains two aryl phosphate-binding sites, i.e. a high-affinity catalytic site and a low-affinity non-catalytic site. The latter is demarcated by Arg24 and Arg254.[15] Previous studies have revealed that the lack of PTP1B can enhance insulin sensitivity, improve glycaemic control, and resist to high fat diet-induced obesity. It is suggested that PTP1B inhibitors may enhance insulin sensitivity and act as effective therapeutics for type II diabetes, insulin resistance, as well as obesity. Therefore, PTP1B has been an attractive drug target for type II diabetes and obesity.[16] These advantages have initiated the search for some novel potential partial PTP1B inhibitor with lesser side effect. Prompted by these promising results, many groups have initiated PTP1B inhibitor programs targeting the active site.[17,18] However, it quickly became apparent that this was not an easy target. Although several potent inhibitors have been reported, obtaining compounds with the

appropriate pharmacokinetics for clinical development have been elusive. PTP1B displays a substrate preference for phosphotyrosine flanked on the N-terminus by acidic residues.[19,20] This is owing to the positive nature of the active site, which attracts these negatively charged substrates while they are repulsed from the rest of the otherwise negative surface of the phosphatase. Consequently, compounds that inhibit PTP1B have potential in therapeutic strategies for treatment of type 2 diabetes and obesity. However, no small molecule inhibitors of PTP1B are undergoing clinical trials in the context of diabetes and obesity, because of unsatisfactory efficacy or side effects. In an effort to search for novel PTP1B inhibitor, we screened a library of various structurally diverse synthetic compounds. Among active compounds identified, compounds with disubstituted oxadiazole structure was chosen based on the novelty and ease of derivative synthesis, and chemical modification of this molecule lead to the NCE-9 as a lead compound for novel PTP1B inhibitor (Fig. 1). NCE-9 chemically known as 5-Biphenyl-4-yl-[1,3,4,] oxadiazole-2-carboxylic acid. In this study, we characterized NCE's, for PTP1B inhibition, insulin receptor phosphorylation and for pharmacokinetic profile using biochemical & pharmacological assays.

MATERIALS AND METHODS

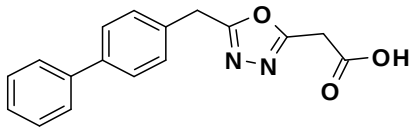
Compounds

All NCE's was synthesized at SNJBs Shriman Sureshdada Jain College of Pharmacy, Nasik, India. The compounds were suspended in 0.25% Tween-80+0.5% methyl cellulose solution for in vivo studies.



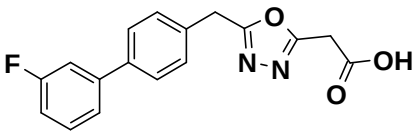
[5-(4'-Chloro-biphenyl-4-yl)-[1,3,4]oxadiazol-2-yl]-acetic acid

Fig. 1 Structure of NCE-3



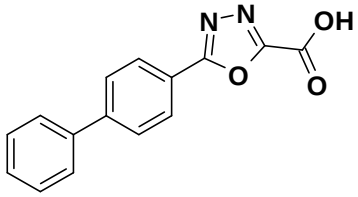
(5-Biphenyl-4-ylmethyl-[1,3,4]oxadi azol-2-yl)-acetic acid

Fig. 2 Structure of NCE-5



[5-(3'-Fluoro-biphenyl-4-ylmethyl)- [1,3,4]oxadiazol-2-yl]-acetic acid

Fig. 3 Structure of NCE-8



5-Biphenyl-4-yl-[1,3,4]oxadiazole-2 -carboxylic acid

Fig. 4 Structure of NCE-9

***In vitro* enzyme assays**

Enzyme-based assay for PTP1B

A colorimetric assay to measure inhibition against PTP1B was performed in 96-well plates. In this assay, the tested compounds were solubilized in DMSO and serially diluted for the concentrations ranging from 0.03 µM to 1000 µM. The assays were carried out in a final volume of 100 µL containing 50 mmol/L MOPS, pH 6.5, 2 mmol/L pNPP, 30 nmol/L GSTPTP1B and serially diluted compounds (2% DMSO). The catalysis of pNPP was continuously monitored on a SpectraMax 340 microplate reader at 405 nm for 2 min at 30°C. The IC50 value was calculated from the nonlinear curve fitting of the percent inhibition vs the inhibitor concentration [µM] using graph-pad prism software.[11]

Enzyme-based assay of PTP1s

A colorimetric assay in 96-well plate format was carried out for CDC25B (Cell signaling technology, 9525), SHP1 (Cell signaling technology, 3759), SHP2 (Invitrogen, 44558G), LAR (Sigma, SAB4200321) and PRL-3 (Invitrogen, 182324), by using O-methylfluorescein phosphate (OMFP) as substrate at concentration of 150 µmol to 300 µmol under optimum pH. NCE-9 was serially diluted for 10 point CRC with final concentration of 1% DMSO (Sigma, D2650). Prior to assay initiation NCE-9 and enzyme were preincubated for 3 min at 4°C. Assay was initiated by adding desired concentration of OMFP substrate. Product formation was monitored on Spectrmax 340 micoplate reader at 405 nm. The result was tabulated and expressed as IC50 Values. The reaction containing 1% DMSO at final volume was considered as positive control. The reaction carried in absence of OMFP as negative control. The reaction was carried for minimum of triplicate and results have been averaged. NCE-9 is potent against PTP1B (0.46 µmol/L) and selective over SHP1 and SHP2 and has moderate activity of Cdc25B and PRL3.[11]

Tyrosine phosphorylation of IRβ in CHO/hIR cells

The CHO/hIR cells (Gift from IISc, Bangalore) were cultured in DMEM F12 media with 10% FBS (GIBCO, 10082-139) and 5% Penicillin-Streptomycin (GIBCO, 10378016) at 37°C, 5% CO_2 as monolayer and used at 90% confluency. Cell were washed with PBS (Sigma, P4417) to remove traces of FBS and were trypsinized using Cellstripper (Cellgro, 25-056-CI). 30x103 cells/well were seeded and incubated overnight prior to compound treatment. The compound was treated at 10 point CRC for 2 hrs followed by treatment with 10 nM of insulin for 10 min. Cells treated with 0.5% DMSO were considered as negative

control and 1 mmol/L orthvanadate prepared in 0.5% DMSO used as positive control. The cells lysate was prepared by using cell lysis buffer (cell signaling technology). Protein concentration was estimated using Bradford method. Thus prepared lysate was subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane for western blotting. Blots were probed with specific anti-pTyr1162/1163 IR antibodies. The blots were normalized by β-actin.[11]

Animals

Male C57BL/6J mice were purchased from Laxmi Biofarms Pvt. Ltd. Ale Phata, Pune, India. The research work was conducted in accordance with the internationally accepted principles for laboratory animal use and care. The rats were housed under good hygienic conditions in the Animal house under standard conditions of temperature (24±1)^{o}C, relative humidity (65 ±10)% and 12 hrs light, 12 hrs dark cycle. The rats were fed with standard pellet diet and drinking water ad libitum. The animals were allowed to acclimatize to experimental conditions by housing them for 8-10 days prior to the experiments. All animal use was in compliance Experimental Animal Care issued by the Committee for Purpose of Control and Supervision of Experiments on Animal (CPCSEA).

***In vivo* pharmacokinetic profile of NSE's**

C57BL/6 mice (adult males, 20–25 g) fasted for 6 h were administered NCE's at 1 mg/kg for oral and 1 mg/kg for intravenous pharmacokinetic profile. Blood samples were collected at different time interval by retro-orbital puncture under light isoflurane anesthesia and separated plasma was stored at −80°C until used. Compound concentrations in plasma were determined by HPLC analysis and the pharmacokinetic parameters were calculated by a non-compartmental method with WinNolin professional Version 4.1.

***In vivo* pharmacokinetic profiling of NCE-9 has been done in SD rats.**

SD rats (adult males, 250-300 g) fasted for 6 h were administered NCE-9 at 1mg/kg for oral and 1mg/kg for intravenous pharmacokinetic profile. Blood samples were collected at different time intervals by retro orbital puncture and separated plasma was stored at -80^{o}C until used. Compound concentrations in plasma were determined by Liquid chromatography-Mass spectroscopy (LC-MS/MS) following protein precipitation with acetonitrile containing internal standard.

Analysis of NCE-9 concentration in plasma by using HPLC

HPLC operating conditions: The HPLC system was a Waters 2695 Alliance system (Waters, Milford, USA) equipped with performance PLUS inline degasser along with an auto-sampler was used to inject 50 µL aliquots of the processed samples on a Thermo Hypurity C18 column (250 x 4.6 mm, 5 µm; LGC Promochem India Limited, Bangalore, India), which was maintained at ambient room temperature (25 ± 1°C). An isocratic mobile phase comprising acetonitrile: water: 20 mM potassium dihydrogen phosphate pH 3.5):: 65:5:30 (v/v/v) delivered at a flow-rate of 1 ml/min with a total run time of 15 min. The eluate was monitored using an UV detector set at 254 nm was used for chromatographic resolution of NCE-9 and Internal Standard (IS). The retention times for NCE-9 & IS was 9.20 & 12.1 min respectively.

Sample preparation: Liquid-liquid extraction method was followed for extraction of NCE-9 from mice plasma. To an aliquot of 100 µL plasma sample, IS solution (10 µL of 20 µg/ml) was added and mixed for 15 sec on a cyclomixer (Remi Instruments, Mumbai, India). After the addition of 1.2 ml of ethyl acetate, the mixture was vortexed for 5 min; followed by centrifugation for 10 min at 14,000 rpm on a Centrifuge 5430R (Eppendorff, Germany) at 4°C. The organic layer (1.1 ml) was separated and evaporated to dryness at 50°C using a gentle stream of nitrogen (Turbovap, Zymark Kopkinton, MA, USA). The residue was reconstituted in 150 µL of the 40% acetonitrile and 50 µL was injected in HPLC system.

RESULTS AND DISCUSSION

In vitro enzyme assays

Enzyme-based assay for PTP-B1

Analyses of quantitative trait loci and mutations in the gene encoding PTP1B in humans support the notion that aberrant expression of PTP1B can contribute to diabetes and obesity.[21,22,23] Mice that lack PTP1B display enhanced sensitivity to insulin, with increased or prolonged tyrosine phosphorylation of IR in muscle and liver.[24,25] Interestingly, PTP1B_/_ mice are protected against weight gain and have significantly lower triglyceride levels when placed on a high-fat diet. This is unexpected because insulin is also an anabolic factor, and increased insulin sensitivity can result in increased weight gain. PTP1B was subsequently shown to bind and dephosphorylate JAK2, which is downstream of leptin.[26,27] Thus, the resistance to diet-induced obesity observed in PTP1B_/_ mice is likely to be associated with increased energy expenditure owing to enhanced leptin sensitivity. Recent

tissue-specific knockout results indicate that body weight, adiposity and leptin action can be regulated by neuronal PTP1B.[28] Inhibiting neuronal PTP1B would require drugs that penetrate the blood–brain barrier. Consistent with the above results, antisense-based oligonucleotides that target PTP1B have shown efficacy in type 2 diabetes and have entered phase 2 clinical trials.[29,30] In addition, small-molecule inhibitors of PTP1B can work synergistically with insulin to increase insulin signalling and augment insulin-stimulated glucose uptake.[31] Moreover, pretreatment of leptin-resistant rats with a potent and selective PTP1B inhibitor results in a marked improvement in leptin-dependent suppression of food intake.[32] We found that compound 3 and 9 showed better inhibitory activity with IC50 0.85 and 0.46 µmol/lit respectively against PTP1B. Introduction of carboxylic acid group in N position of the oxadiazole made impact on its activity. In the next step we evaluated the NCE-9 against panel of 6 members of PTPs family.

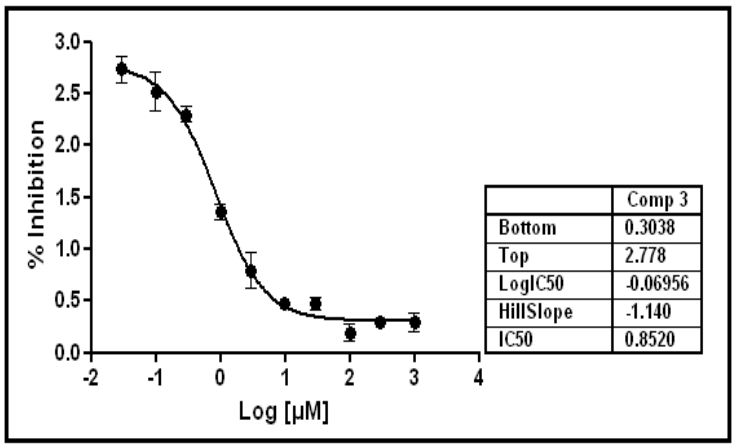


Fig 5 Effect of NCE-3 on Enzyme-based assay for PTP-1B inhibition

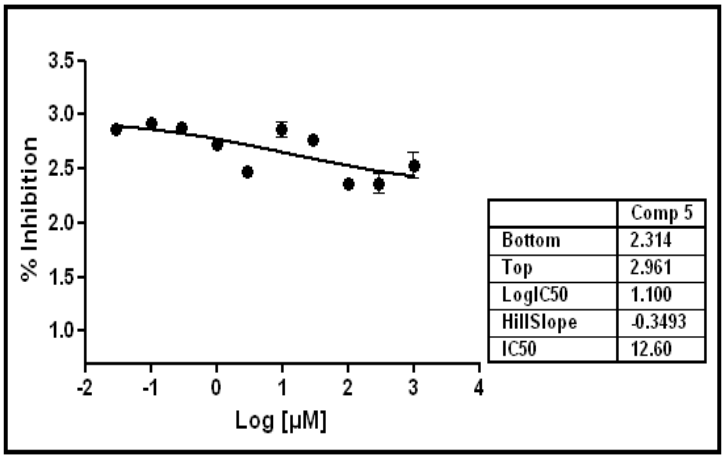


Fig. 6 Effect of NCE-5 on Enzyme-based assay for PTP-1B inhibition

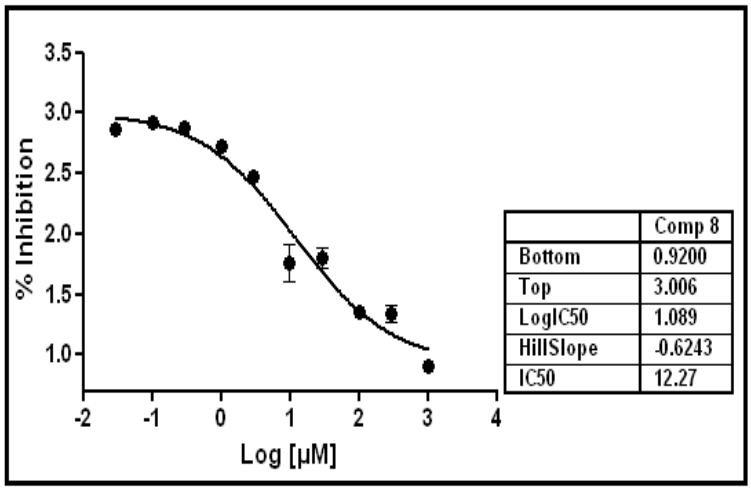


Fig. 7 Effect of NCE-8 on Enzyme-based assay for PTP-1B inhibition

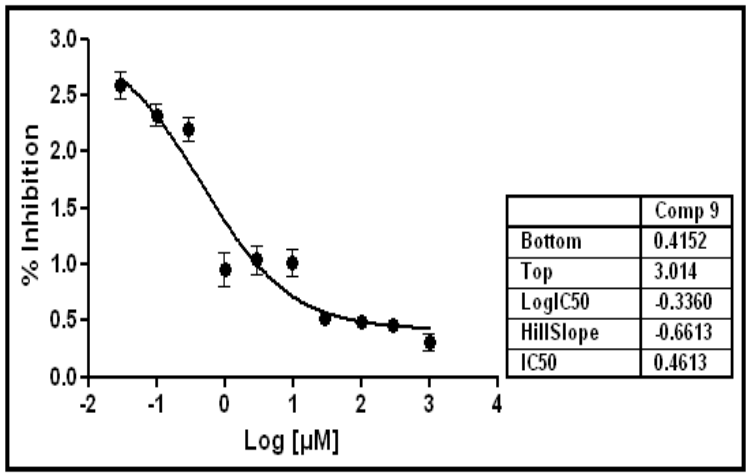


Fig. 8 Effect of NCE-9 on Enzyme-based assay for PTP-1B inhibition

Enzyme-based assay of PTP1s

Selectivity is one of the major issues in the development of PTP1B inhibitors as drugs. Because all PTPs share a high degree of structural conservation in the active site, the pTyr (phosphotyrosine)-binding pocket, designing inhibitors with both high affinity and selectivity for PTP1B poses a challenge. Fortunately, PTP substrate specificity studies have shown that pTyr alone is not sufficient for high-affinity binding, and residues flanking the pTyr are importantfor PTP substrate recognition.[33] The results indicate that there are subpockets adjacent to the PTP active site that can also be targeted for inhibitor development. These studies also provide a molecular basis for addressing and manipulating PTP inhibitor potency and specificity, and suggest a novel paradigm for the design of potent and specific PTP inhibitors; namely bidentate ligands that bind to both the active site and a unique adjacent

peripheral site. Consequently, unique PTP subpockets that border the active site can be targeted to enhance inhibitor affinity and selectivity. The rationale for the enhanced affinity of bidentate inhibitors is based on the principle of additivity of free energy of binding. The interaction of an inhibitor with two independent sites (e.g. a pTyr site and a unique peripheral site) in one PTP would confer exquisite specificity because other PTPs might not possess an identical second-site interaction. Based on this paradigm, several potent and selective PTP1B inhibitors have been developed. NCE-9 was screen against panel of 6 members of PTPase family (table 1). In contrast to the high selectivity of compound 9 against PTP1B, NCE-9 exhibited poor selectivity against several other therapeutically useful phosphates (i.e. SHP1, SHP2, NAR etc).

Table 1. Selectivity of NCE-9 for a panel of protein phosphates

Protein Phosphatases	IC50 (µmol/L)
PTP1B	0.46
LAR	> 30
Cdc25B	5
SHP1	> 30
SHO2	> 30
PRL3	8

Tyrosine phosphorylation of IRβ in CHO/hIR cells

Type 2 diabetes is associated with insulin resistance, possibly because of attenuated signaling from IR molecule. Data support the concept that PTP1B is a key negative regulator of insulin signal transduction. Because of termination of insulin signaling requires the dephosphorylation of IRβ and its downstream effector molecules, the deregulation of PTP1B through increased activity or expression can attenuate the insulin signal, resulting in insulin resistance.[34,35] Therefore reducing the activity of PTP1B, which dephosphorylates IRβ, would theoretically be expected to increase insulin sensitivity.[36,37,38,31] Overexpression of PTP1B in cell cultures decreases insulin-stimulated phosphorylation of IR and/or IRS-1, whereas reduction in the level of PTP1B, by antisense oligonucleotides or neutralizing antibodies, augments insulininitiated signalling.[29,37,39,40] NCE-9 increased the insulin induced tyrosine phosphorylation of IRβ and boosted IR phosphorylation more potently (1.1 µmol/L).

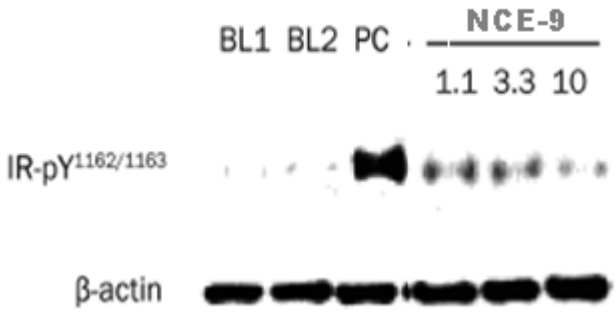


Fig. 9 Effects of NCE-9 on tyrosine phosphorylation of IRβ in CHO/hIR cells

In vivo pharmacokinetic profile of NCE's

Bioavailability is another important issue in the development of PTP1B-based small-molecule therapeutics. The active sites of PTPs have evolved to accommodate pTyr, which contains two negative charges at physiological pH. Consequently, most active-site-directed PTP inhibitors (non-hydrolyzable pTyr mimetics) reported to date possess a high charge density to serve as competitive inhibitors. Such molecules are generally not drug-like, with limited cell membrane permeability. Several strategies have been applied to improve the cell permeability and/or bioavailability of PTP1B inhibitors. In vivo pharmacokinetic study showed good oral bioavailability of NCE-3 and 9 with a favorable terminal elimination half-life when administered to C57BL/6J mice and NCE-9 in SD rats.

Table 2. Pharmacokinetic profile of NCE -3 in C57BL/6J

Pharmacokinetic parameters of NSE-3 in C57BL/6J		
Parameters	**I.V. (1 mg/kg)**	**P.O. (1 mg/kg)**
Cmax (ng/mL)	4151	3096
Tmax (h)	NA	0.5
AUC (0–24 h) (ng·h/mL)	9837	15418
Vss (L/kg)	0.2	NA
CL (mL/min/kg)	1.34	NA
T1/2 (h)	1.85	3.74
F%	NA	~100
Pharmacokinetic parameters of NSE-3 after i.v. (1 mg/kg) and p.o. (1 mg/kg) administration to male C57BL/6J mice (mean, N=3 animals/each time point)		

Table 3. Pharmacokinetic profile of NCE -5 in C57BL/6J

Pharmacokinetic parameters of NSE-5 in C57BL/6J		
Parameters	**I.V. (1 mg/kg)**	**P.O. (1 mg/kg)**
Cmax (ng/mL)	2097	580
Tmax (h)	NA	0.25
AUC (0–24 h) (ng·h/mL)	989	624
Vss (L/kg)	0.58	NA
CL (mL/min/kg)	16.6	NA
T1/2 (h)	2.82	2.95
F%	NA	~100
Pharmacokinetic parameters of NSE-5 after i.v. (1 mg/kg) and p.o. (1 mg/kg) administration to male C57BL/6J mice (mean, N=3 animals/each time point)		

Table 4. Pharmacokinetic profile of NCE -8 in C57BL/6J

Pharmacokinetic parameters of NSE-8 in C57BL/6J		
Parameters	**I.V. (1 mg/kg)**	**P.O. (1 mg/kg)**
Cmax (ng/mL)	3372	1587
Tmax (h)	NA	0.25
AUC (0–24 h) (ng·h/mL)	6554	4794
Vss (L/kg)	1.06	NA
CL (mL/min/kg)	2.36	NA
T1/2 (h)	7.25	4.19
F%	NA	68.8
Pharmacokinetic parameters of NSE-8 after i.v. (1 mg/kg) and p.o. (1 mg/kg) administration to male C57BL/6J mice (mean, N=3 animals/each time point)		

Table 5. Pharmacokinetic profile of NCE -9 in C57BL/6J

Pharmacokinetic parameters of NSE-9 in C57BL/6J		
Parameters	**I.V. (1 mg/kg)**	**P.O. (1 mg/kg)**
Cmax (ng/mL)	3039	2384
Tmax (h)	NA	0.25
AUC (0–24 h) (ng·h/mL)	3169	4139
Vss (L/kg)	0.89	NA
CL (mL/min/kg)	5.15	NA
T1/2 (h)	5.67	3.29
F%	NA	~100
Pharmacokinetic parameters of NSE-9 after i.v. (1 mg/kg) and p.o. (1 mg/kg) administration to male C57BL/6J mice (mean, N=3 animals/each time point)		

Table 6. Pharmacokinetic profile of NCE -9 in SD rat

Pharmacokinetic parameters of NSE-9 in SD rat		
Parameters	**I.V. (1 mg/kg)**	**P.O. (1 mg/kg)**
Cmax (ng/mL)	3473	3447
Tmax (h)	NA	0.25
AUC (0–24 h) (ng·h/mL)	7680	11983
Vss (L/kg)	0.41	NA
CL (mL/min/kg)	2.21	NA
T1/2 (h)	4.63	3.9
F%	NA	~100
Pharmacokinetic parameters of NSE-9 after i.v. (1 mg/kg) and p.o. (1 mg/kg) administration to male SD rat (mean±S.D., N=4 animals/route of administration)		

The present study was carried out to evaluate the effect of NCE's using appropriate in vitro and in vivo model with the aim of the discovery potent inhibitor of PTP1B with good oral bioavailability. NCE-9 exhibit potent inhibition of PTP1B in a dose dependent manner and increased the insulin induced tyrosine phosphorylation of IRβ and boosted IR phosphorylation more potently. In vivo pharmacokinetic study showed that good oral bioavailability of the compound with a favorable terminal elimination half-life when administered to C57BL/6J mice and SD rats. In summary, in searching for potent inhibitor of PTP1B with enhancing insulin receptor phosphorylation. We hope to optimize its structure further with the aim of increasing their potency and selectivity, as well as their efficiency in vivo model for diabetes and obesity.

CONCLUSION

These functional, biochemical, and molecular findings suggest of NCE-9 inhibit PTP1B and enhance insulin receptor phosphorylation with good in vivo pharmacokinetic profile However, further studies should be carried out to explore beneficial use of NCE-9 as PTP1B inhibitors in animal models of diabetes and obesity.

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