Original Article
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Novel Indole derivative as first P-glycoprotein inhibitor from the skin of Indian toad (*Bufo melanostictus*)

Short title: Novel Indole derivative inhibits digoxin efflux

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Abstract
Objective: To study the inhibitory effect of Novel Indole Derivative (NID) from Indian toad skin (*Bufo melanostictus*) on P-glycoprotein.
Materials and methods: Dried Indian Toad skin was used to isolate NID with column chromatography, and its structure was elucidated by IR Spectra, ^{13}C NMR, ^{1}H NMR Spectra, and LC-MS. Female Wistar rats were used to determine LD50, *In vitro* permeability studies were done with the intestinal sac method, and *In vivo* pharmacokinetic studies were carried out to prove P-gp inhibition using the rat model.
Results: The NID has shown increased apparent permeability $P_{app}(x10^{-6}cm/sec)$ significantly ($p<0.001$) from 1.04±0.11 to 2.90±0.08 in ileum 1.44±0.14 to 3.92±0.13 in jejunum this *in vitro* results confirmed that P-gp inhibited, this was further confirmed by *in vivo* studies and found to observe the increased oral bioavailability of digoxin significantly in NID treated groups from 3.26±0.25 to 7.47±0.18 ng/mL, the volume of distribution decreased from 232.56±64.59 to 86.57±7.04 L/kg. AUC increased from 37.89±1.13 to 64.62±0.70 ng/mL/hr. This demonstrates NID increased the oral bioavailability of digoxin significantly.
Conclusion: Many compounds were isolated from Indian toad skin. This NID was not reported earlier. Results demonstrate NID increased the oral bioavailability of digoxin significantly. The isolated NID from Indian toad skin proved as a potent P-gp inhibitor in both *in vitro* and *in vivo* studies, and further studies needed to develop as a possible new drug candidate.
Keywords: Apparent permeability, Bioavailability, Novel Indole derivative, Permeability glycoprotein.

INTRODUCTION

Toxic animals are widely distributed throughout the globe [1, 2]. Venomous animals are recognized as a new emerging source of new drug discovery and therapeutics [3]. In recent years many new bioactive compounds from different toads were reported [4]. Toads belong to amphibians and anura family; toad skin and parotid glands play an essential role in the survival of amphibians from diverse conditions and predators [5 6]. Toads possess two types of glands beneath their skin, mucous glands, and granular glands. Mucous glands secrete thick mucus secretions, which are essential to keep toad skin moist [7]. Granular glands secrete acrid, toxic substances, which provides protection from predators [8]. This acrid, poisonous substance, when it comes intact, induces inflammation, irritation, and vomiting sensations in toad predators[9]. This glandular secretion chemically belongs to potent substances like steroids, alkaloids, peptides, proteins, and biogenic amines [10]. New drug discovery is a challenge many active compounds extracted from plants, animals, fungi, other sources. There is still to discover new compounds from the above sources[11]. Toad skin extracts have been widely used for treating many types of ailments in China and other countries as traditional alternative medicine. The chemical composition and pharmacological activities of toad skin are remaining unclear [12]. Permeability glycoprotein (P-gp) is an essential transporting protein present on the cell membrane that effluxes many xenobiotic substances like drug molecules out of cells [13]. P-gp has a significant impact on drug absorption, distribution, metabolism, excretion and associated with drug-drug interactions[14-16]. P-gp is over-expressed on the surface of cancer cells and prevents drug entry into the tumor due to rapid and prolonged efflux mechanism [17, 18]. P-gp induces resistance to anticancer drugs, which leads to therapeutic failure. There are many phytochemicals and drugs reported as P-gp inhibitors but associated with severe side effects [19, 20]. An alternate approach is needed to overcome this issue by exploring new compounds from new sources [21, 22], in this study toad skin extract studies for inhibitory action on P-gp. In this study, Digoxin (DIG) was used as probe substrate [23], and Verapamil (VER) [24] was taken as standard inhibitor. The isolated NID inhibited P-gp and enhanced the oral bioavailability of DIG *in vivo* studies.

EXPERIMENTAL

Sample Collection and Preparation

Adult live toads (45 to 50 g) were collected from the near places of Warangal and University surroundings. After collecting the toads, the skins were isolated carefully and shade dried at room temperature (27^0C); after complete dryness soaked in methanol for 30 days in an amber-colored bottle, the supernatant was collected, evaporated to dryness using Rota evaporator, in the end, dark brown solid mass methanolic extract (44g) was obtained. The methanol extract (ME) was extracted further with ethyl acetate; this ethyl acetate fraction (EAF) was collected. EAF was subjected to column chromatography on silica gel (100-200 mesh-Merck), eluted slowly in increasing polarity mixture of solvents like n-Hexane, chloroform, ethyl acetate, ethanol, methanol, and water to obtain different fractions. Total 5 fractions were collected; fraction-2 was obtained as a pale gray colored compound, which on TLC produced a single spot. Further purification was done with acetone and methanol [25, 26]. The final isolated compound yield was found to be 800mg.

Animals

Female and Male Wistar rats were procured from Vyas Enterprises, Hyderabad, acclimatized for ten days, then used housed in standard laboratory conditions [27]. All experimental animal protocols were approved by the ethics committee of IAEC (Ethical committee approval number: **IAEC/02/UCPSc/KU/2016)**.

Chemicals and other requirements

Acetonitrile (Merck-Mumbai), methanol (Merck-Mumbai), Ethyl acetate (Merck-Mumbai), Digoxin (Sigma Aldrich-Bangalore), Verapamil (Lupin Pvt Labs-Pune-India) Equipment used are n-Hexane (Merck-Mumbai), Chloroform (Merck- Mumbai), Ethanol (Merck- Mumbai), HPLC (Schimadzu, with phenominex C-18 column), Biofuge- centrifuge (Heraeus instrument- Germany), Chromatography column-Borosilicate made, TLC aluminum Plates-Sigma Aldrich, Ultra Sonicator (Ramsit Scientific equipement-Hyd) , Rotavapor-R-300 (Mumbai-India), Oral feeding needle, Syringe Filters-Minisart (Sartorius stedim Biotech-Germany).

Toxicity studies

According to the OECD-423 guidelines maximum, tolerated dose (MTD) was determined using 15 female Wistar rats. Rats were divided into 5 groups (n=3), control group treated with normal saline, second group given NID (5mg/kg.p.o), third group NID (50mg/kg.p.o), fourth group NID (300 mg/kg.p.o),fifth group NID (2000 mg/kg.p.o). Toxic effects were recorded for 14 days during the period observed for mortality, physiological parameters like body weight changes, food intake, water intake, and behavioral changes in each animal noted [28].

Characterization of NID using spectral data

Spectral analysis was done using liquid chromatography with mass spectrometry (LC-MS) analysis 2.6.1. The identification of components was done using mass spectral library. The ^{1}H spectra were recorded at 300 K on spectrometer operating at 600.13 MHz (14.1 T) using a 5-mm inverse probe equipped with a z-shielded gradient. NMR samples were prepared by dissolving extract in 500 µL of DMSO and 1 µL of DMF as internal standard, ^{13}C NMR spectral reports were made by comparison of the observed chemical shift values with the reported values. And IR spectrophotometer is used, and spectral data is used to find functional groups. Chem-Draw pro 8.0 (Perkin Elmer) used for structure assessment.

***In vitro* studies**

Intestinal sac study was performed according to the previously described methods [29]. Rats were grouped and sacrificed using anesthetic ether; the intestine was surgically removed, flushed with 50 mL of saline (5%). The small intestine was cut into two segments jejunum and ileum of equal length (5 cm). The probe drug (DIG 500 µg/mL) was dissolved in pH 7.4 isotonic Dulbecco's PBS (D-PBS) containing 25 mM glucose. Similarly DIG+VER (100 µg/mL), DIG+ID 2mg/mL and DIG+ID 4mg/mL loaded. And both ends of the sac were ligated tightly with surgical suture. The sacs were placed in a beaker containing 40 mL of D-PBS, containing 25 mM glucose. The medium was pre-warmed at 37 ˚C and pre-oxygenated with 5% CO_2/ 95% O_2 under bubbling with mixture gas, the transport of the DIG from apical to basolateral and basolateral to apical samples collected periodically for 120 minutes periodically, the collected samples stored at -20 ˚C until analysis. The samples were analyzed by high-performance liquid chromatography (HPLC).

Calculation of apparent permeability coefficient

The apparent permeability coefficient (P_{app}) of DIG was calculated from the following equation:

$$\mathrm{Papp} = \frac{\mathrm{dQ}}{\mathrm{dt}} \cdot \frac{1}{\mathrm{AC0}}$$

Where dQ/dt: Transport rate of the drug in the serosal medium, A: is the surface area of the intestinal sacs, and C_0: Initial concentration inside the sacs [30].

Samples preparation for intestinal sac samples analysis

Samples were extracted using a simple protein precipitation method by adding acetonitrile (200 µL) to samples (100 µL). Samples were vortexed for 10 minutes and centrifuged at 6000 rpm for 15 min. The resultant clean supernatant (20 µL) was injected and analyzed using HPLC. The mobile phase consists of Acetonitrile: Water 65:35. Flow rate: 1mL/min, Pressure: 115kg.f/cm^2, UV-detection at: 220 nm.

***In vivo* studies**

Male Wistar rats were used kept one week for acclimatization during the period supplied with normal *ad libatum* and free access for water. After one week they were divided into 4groups (n=6) first group treated with DIG (0.5mg/kg.p.o). Second group treated with DIG (0.5mg/kg.p.o)+VER (2mg/kg.p.o), third group treated with DIG (0.5mg/kg.p.o)+NID (2mg/kg.p.o) fourth group DIG (0.5mg/kg.p.o) + NID (4mg/kg.p.o). Blood sample were collected by picturing lateral tail vein[31] at 0,0.5,1,2,4,6,8,12 and 24h time points. Samples were centrifuged and supernatant extracted with acetonitrile precipitation method. Samples were stored at -4^{0}C until used for analyzed by HPLC [32].

RESULTS

Structure assessment using spectral analysis

Based on the spectral analysis using LC-MS, IR spectra, ^{13}C NMR and ^{1}H NMR δ (Table 1) values the structure of NID is elucidated (Figure 1).

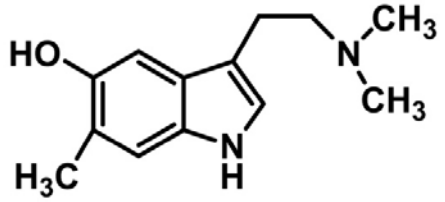

Figure 1. Chemical structure of NID

Table 1. ^{1}H NMR, ^{13}C Chemical shift in $CDCl_3$ data of NID

H^1	δppm	C^{13}	δc ppm
1	5.5(S.H)	1	------
2	6.92(S.H)	2	119
3	-----	3	120
4	6.9(S.H)	4	111
5	5.5(S.H)	5	151
6	2.6(S.H)	6	107
7	6.9(S.H)	7	125
8	2.15(δ 3H,J=7.4HZ)	8	123
9	2.4(δ3H, J=7.2HZ)	9	129
10	-----	10	103
11	2.6(S,3H)	11	15
12	2.9(S.3H)	12	66
	-----	13	24
	-----	14	47

Toxicity assessment and determination of MTD

The mortality was found in three animals at 50mg/kg treated groups, according to OECD-423 guidelines, comes under category-2 [33], (LD50 cut-off dose 25 mg/kg). At 5mg/kg, the animals remained alive after the administration of NID. Bodyweight slightly decreased in NID 5mg/kg, compared to control. Water intakes decreased somewhat in NIA 5mg/kg, compared to control, and locomotar activity was not changed significantly (Figure 2A-2D). The maximum tolerated dose found to be 25 mg/kg.

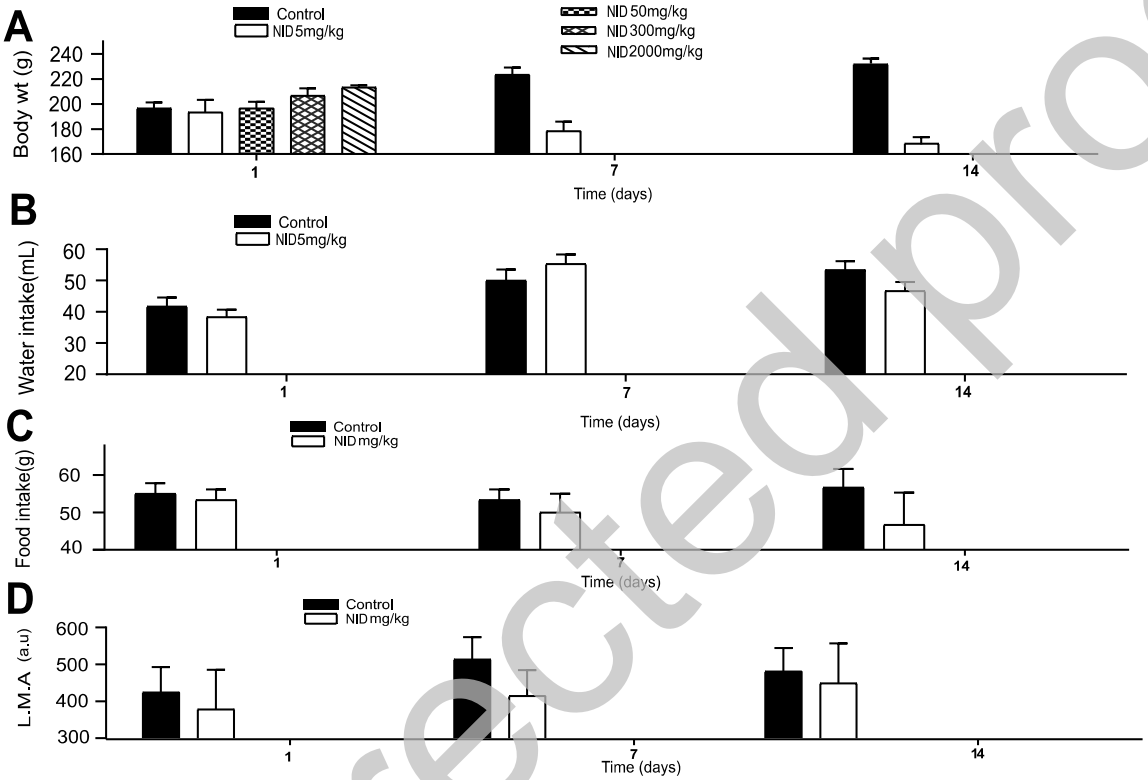

Figure 2. Toxicity studies of NID; NID: Novel Indole derivative, DIG: Digoxin, VER: Verapamil, a: Body weight, B: Food intake C: Water intake D: Locomotar activity.

***In vitro* studies**

The P_{app}(x10^{-6} cm/sec) significantly increased (P<0.001) in NID treated groups from 1.04±0.11 to 2.90±0.08 in the ileum, and 1.44±0.14 to 3.92±0.13 in jejunum compare to the control group (Table 2).

Table 2. *In vitro* apparent permeability studies

Apparent permeability	DIG	DIG+VER	DIG+NID 2mg/mL	DIG+NID 4mg/mL
Ileum	1.04±0.11	1.77±0.09**	2.42±0.12**	2.90±0.08**
Jejunum	1.44±0.14	2.00±0.17**	2.45±0.13**	3.92±0.13***

DIG: Digoxin VER: Verapamil NID: Novel Indole Derivative, data represents Mean±SD Values, one way ANOVA was used for statistical analysis significant difference**, P<0.01, P<0.001 in comparison with the control (DIG).**

***In vivo* studies**

The plasma drug concentration of DIG significantly increased in NID treated groups compared to control and positive control groups (Figure 3). C_{max} increased from 3.26±0.254 to 7.47±0.186 ng/mL, T_{max} decreased from 27.17±13.85 to 9.88±1.13 hrs, AUMC from 371.27±18.16 to 530.57±16.52 ng.h^2/mL, AUC increased from 37.89±1.132 to 64.62±0.70 ng.h^2/mL, CL from 6.09±0.24 to 7.87±0.22 L/h/Kg, Vd decreased from 232.56±64.59 to 86.57±7.049 L/Kg, MRT from 9.79±0.27 to 8.20±0.19 hr significantly. p<0.001) (Table 3).

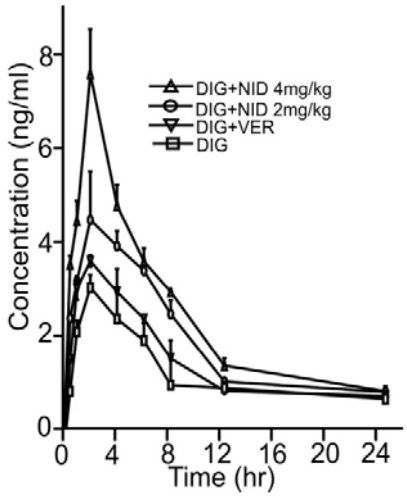

Figure 3. Effect of NID on Pharmacokinetics of DIG NID: Novel Indole derivative, DIG: Digoxin, VER: Verapamil, values mentioned in mean±SD (n=6)

Table 3. Effect of NID on Pharmacokinetic parameters of digoxin

Pk parameter	DIG	DIG+VER	DIG+NID 2mg/kg	DIG+ NID 4 mg/kg
C_{max} (ng/mL)	3.26±0.254	3.79±0.117***	4.59±0.097****	7.47±0.186****
T_{max} (hr)	27.17±13.85	12.512±0.447**	10.70±0.430***	9.88±1.137***
$T_{½}$ (hr)	2.00±0.00	2.00±0.00	2.00±0.00	2.00±0.00
AUMC (ng.h^2/mL)	371.27±18.16	398.61±9.00*	468.92±13.79****	530.57±16.52****
AUC $_{0-t}$ (ng/mL/hr)	37.89±1.132	43.01±0.43****	52.95±1. 31****	64.62±0.70****
CL (L/h/kg)	6.09±0.24	6.65±1.74	6.98±0.23	7.87±0.22**
K_{el} (h^{-1})	0.03±0.016	0.05±0.02***	0.065±0.003****	0.071±0.08****

Vd (L/kg)	232.56±64.59	141.99±12.94****	107.72±15.74****	86.57±7.04****
MRT (hr)	9.79±0.279	9.26±0.12***	8.85±0.10****	8.20±0.19****

Data represents Mean±SD values, one-way ANOVA was used for statistical analysis. Significant difference**, P<0.05, P<0.001, p < 0.0001) in comparison with the control (DIG).**

Analysis of data

All the pharmacokinetic parameters were analyzed by using Phoenix WinNonlin version 8.3 kinetic software. The statistical analysis was performed using one-way analysis of variance (ANOVA) and Graph Pad Prism version (8.0.2).

Discussion

In this study, we isolated a new compound from Indian toad skin, and spectral data accessed the structure of the compound. Some other studies reported different compounds from toad skin [34], but this compound was not reported earlier. The maximum tolerated dose was determined according to OECD guidelines and found 5 mg/kg. Clinically P-gp plays a significant role in drug absorption and drug entry into targeted cells; studies reported that p-gp expression was more in all types of cancers, inflammatory diseases, and diabetes mellitus [35, 36]. P-gp is over-expressed on the surface of many cancerous cells and prevents drug entry into the tumor, acts as the efflux pump, extrudes many anticancer drugs before they can reach the intended target [37]. More P-gp expression leads to more P-gp mediated drug efflux, which leads to decreased drug absorption, bioavailability, and therapeutic failure; there are many p-gp inhibitors discovered so far, but they non-selective to target molecule and low binding affinities [38]. P-gp inhibition by NID may show the new way, and beneficial cancer treatments to enhance anti-cancer drug bioavailability and facilitate drug entry into the targeted tumor by modulating P-gp mediated efflux. The isolated compound NIA can be used to improve the bioavailability of antidiabetic agents, which are P-gp substrates reported for low bioavailability due to efflux by P-gp. The isolated NID can be useful when co-administered with drugs like antihypertensive agents, antiviral agents, and anti-biotic agents useful to reduce multidrug resistance reported in their treatment also helps to enhance oral bioavailability and drug accumulation and related toxicity. *In vitro* studies proved that NID inhibited P-gp and enhanced the apparent permeability of DIG. Few more studies reported that VER increases the oral bioavailability of DIG up to 60% but reported side effects [39]; in our research, NID has shown better oral bioavailability of DIG In; a similar study reported that toad paratoid gland secretion inhibited P-gp and increased the bioavailability of substrate drug [40].

CONCLUSION

The isolated compound from Indian toad skin is confined as a Novel Indole Derivative (NID) and not reported earlier; the compound significantly inhibited P-gp mediated transportation. *In vivo* studies revealed that NID increased the oral bioavailability of DIG. Co-administration of a drug with potent molecules like NID can alter transporter function to improve drug bioavailability.

Institutional animal ethical committee number: IAEC/02/UCPSc/KU/2016

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Abbreviations:
ANOVA: Analysis of variance
AUC: Area under the curve
AUMC: Area under movement curve
CL: Clearance
C_{max}: Peak plasma Concentration
DIG: Digoxin
IAEC: Institutional Animal Ethical Committee
IICT: Indian Institute of Chemical Technology
IR Spectra: Infra-Red spectra
LC-MS: Liquid chromatography-mass spectroscopy
LD: Lethal dose
MTD: Maximum tolerated dose
NID: Novel indole derivative
NIT: National Institute of Nutrition
OECD: Organization for economic cooperation and development.
P_{app}: Apparent permeability
P-gp: Permeability glycoprotein
t1/2: Half life
T_{max}: Time maximum
VER: Verapamil
^{13} C NMR: Carbon 13 Nuclear Magnetic resonance
^{1}H NMR: Proton nuclear magnetic resonance

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