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## Synthesis, enzyme inhibition and anticancer investigations of unsymmetrical 1,3-disubstituted ureas

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Abstract: In this study, seventeen urea derivatives, including the five new derivatives *N*-mesityl-*N*'-(3-methylphenyl)urea (2), *N*-(3-methoxyphenyl)-*N*'-(3-methylphenyl)urea (4), *N*-mesityl-*N*'-(4-methylphenyl)urea (6), *N*-(1,3-benzothiazol-2-yl)-*N*'-(3-methylphenyl)urea (9) and *N*-(2-methylphenyl)-2-oxo-1-pyrrolidinecarboxamide (15), were synthesized by reacting *ortho*-, *meta*- and *para*-tolyl isocyanate with primary and secondary amines using a previously reported method. All the series 1–17 were subjected to urease,  $\beta$ -glucuronidase and snake venom phosphodiesterase enzyme inhibition assays. The ranges of inhibition of urease,  $\beta$ -glucuronidase and phosphodiesterase enzymes were 0.30–45.3, 4.9–44.9 and 1.2–46.4 %, respectively. Moreover, an effect of these compounds on a prostate cancer cell line was observed. The new compound *N*-(1,3-benzothiazol-2-yl)-*N*'-(3-methylphenyl)urea (9) showed *in vitro* anticancer activity with an *IC*<sub>50</sub> value of 78.28±1.2 µM. All the compounds were characterized by state of art spectroscopic techniques.

*Keywords:* amine;  $\beta$ -glucuronidase; disubstituted ureas; isocyanate; phosphodiesterase; urease.

## INTRODUCTION

Nitrogen-containing heterocyclic compounds play a significant role not only in the life science industry, but also in many other industrial fields related to special and fine chemistry. Among them, ureas represent an extensively used tremendous class of compounds with multi-focal applications in several fields.<sup>1</sup> A number of these compounds are reported to exhibit a wide spectrum of biological and pharmacological activities. In the last decade, much attention has been paid to the synthesis and application of such substituted urea derivatives.<sup>2,3</sup>



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Ureas have been synthesized by a number of methodologies basically employing substances such as phosgene. However, due to the harsh reaction conditions and the hazardous nature of phosgene, it has been promisingly substituted by safer compounds. Thus numerous substitutes of phosgene are well documented in the literature.<sup>4</sup> Additionally, their syntheses have been achieved by simpler and much more economical chemical substances such as carbon dioxide and carbon monoxide by the catalytic carbonation of amines.<sup>5,6</sup> Other methods involved the use of carbonates such as organic carbonate esters and ethylene carbonate for the mass scale production of N,N'-disubstituted ureas.<sup>7,8</sup>

Disubstituted ureas possess wide therapeutic activities, such as potent inhibitors of interleukin-8, anthelmintics, antimalarial, anti-HIV, diuretic, analgesic, antibacterial, antifungal, antimicrobial, algaecidal or antiperiphytic agents.<sup>9–12</sup> N,N'-Disubstituted ureas, amides and carbamates are reported as new powerful and stable inhibitors of soluble epoxide hydrolase, both in vivo and in vitro.13 They were determined to be useful for the treatment of hypertension, Ravnaud syndrome, respiratory distress syndrome, inflammation, diabetic complications, arthritis and renal diseases.<sup>14</sup>

A urease is an enzyme that decomposes urea to ammonia and carbonic acid and provides nitrogen to an organism.<sup>15,16</sup> On the other hand, bacterial ureases cause different pharmacological problems, ranging from the development of infectious stones, pathogenesis of encephalopathy, pyelonephritis, urinary catheter encrustation and hepatic coma to peptic ulceration.<sup>15–20</sup>

 $\beta$ -Glucuronidase is an exoglycosidase enzyme and its activity reflects liver enzyme loss during cell turnover in humans.<sup>21,22</sup> In the cell, it is present in lysosomes, although high levels are present in necrotic areas of large tumours. Sly syndrome is an inherited disease characterized by a deficiency of glucuronidase.<sup>23</sup> It was reported that in certain diseases, e.g., cancer, hepatic diseases, inflammatory joint and AIDS, the activity of  $\beta$ -glucuronidase is increased.<sup>21</sup>

A phosphdiesterase (PDE) is an enzyme that participates in cell functions to maintain intracellular levels of cyclic adenosine and cyclic guanosine monophosphate by hydrolyzing cyclic nucleotides.<sup>24</sup> Nucleotide pyrophosphatase/phosphodiesterase (NPP) is a class of enzyme comprised of seven isoforms among them three are well known ectoenzymes as NPP-1 (PC-1), NPP-2 (autotaxin) and NPP-3 (B10; gp130 (RB13-6)) in mammalian. They are widely distributed in mammalian intestinal mucosa, liver cells and serum, snake venom and in various plants.<sup>25</sup> The calcification of bones and tissues is regulated by the NPP-1 enzyme. In humans, chondrocalcinosis and idiopathic infantile arterial calcification result from an over or under expression of NPP-1, respectively.<sup>26,27</sup> The potent phosphodiesterase (PDE) inhibitor drug sildenafil therapeutically obtained great world-wide success for the treatment of erectile dysfunction. Selective PDE inhibitors are highly beneficial in the treatment of various diseases, e.g., PDE-II

inhibitors in sepsis; PDE-IV inhibitors in allergic rhinitis, asthma, psoriasis, multiple sclerosis, chronic obstructive pulmonary disease (COPD), Alzheimer disease, schizophrenia and depression and PDE-V inhibitors in cardiovascular disease, female sexual dysfunction and pulmonary hypertension.<sup>24</sup>

Therefore approaches based on inhibition of these enzymes always remain a need of world for the treatment of diseases arising from disturbed level of these enzymes.

The huge volume of research on the synthesis and anticancer activities of urea derivatives was previously reviewed. Many aromatic urea and thiourea derivatives, such as benzoylureas, N-(2-chloroethyl)-N'-phenylureas, primaquine derivatives, 3-(haloacylamino)benzoylurea and N-(benzothiazol-2-yl)-N-morpho-linourea were synthesized and considered extensively for anticancer activity against murine leukaemia, colon carcinoma, haematoma, lymphoma, melanoma, breast cancer and prostate cancer cell lines of patients.<sup>28–31</sup>

In view of the large volume of literature, it is clear that the discovery of novel biologically active compounds for the treatment of malignancy is very important. A challenge still exists to design even better inhibitors with improved therapeutic effects. In continuation of ongoing studies on the discovery of new enzyme inhibitors and anticancer agents, the synthesis of 1,3-disubstituted ureas by a simple one pot reaction of tolyl isocyanates with selective amines is described herein. The resulting compounds were evaluated for their inhibitory activities on urease,  $\beta$ -glucuronidase and snake venom phosphodiesterase enzymes and their cytotoxicity against prostate cancer cell lines.

## EXPERIMENTAL

Melting points were taken on Gallenkamp melting point apparatus. Thin layer chromatograpy was performed on pre-coated silica gel plates (Kieselgel 60 F<sub>254</sub>, Merk, Germany) and the spots were visualized under UV radiation (Dual range, Merk Millipore, UK) at 254 and 365 nm. The IR spectra were taken on a Thermo Nicolet Avatar 370 DTGS FT-IR spectrometer. The EI-MS measurements were performed on a MAT-312 and a JEOLJMS-HX 110 instrument. The <sup>1</sup>H-NMR spectra were recorded on a Bruker Avance 400 MHz instrument,  $\delta$  in ppm related to SiMe<sub>4</sub> (0 ppm) as internal standard. The <sup>13</sup>C-NMR spectra were taken on a Bruker Avance 125 MHz instrument. Elemental analyses were realized on a Perkin Elmer 2400 CHN elemental analyzer. All used solvents were of reagent grade. Yields, physical, analytical and spectral data for the prepared ureas are given in the Supplementary material to this paper.

## General procedure for the synthesis of the 1,3-disubstituted ureas 1–17

The respective amine (3.18 mmol) was taken in 5–10 mL of 1,4-dioxane and then approximately 1.60 mmol of *ortho-*, *meta-* or *para-*tolyl isocyanate was added dropwise. The reactions are completed in 30–60 min. After completion of the reaction, the mixture was cooled with ice to afford white crystals of the title compound.<sup>32</sup>

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#### Urease inhibition assay

Reaction mixtures containing 1 unit of urease enzyme isolated from jack-bean and 55  $\mu$ L of buffers containing 100 mM urea were incubated with 5  $\mu$ L of 1 mM test compound for 15 min in 96-well plates at 30 °C. The ammonia production, which in turn quantifies the urease activity, was measured by the indophenol method. For this purpose, 70  $\mu$ L of alkali (0.5 % w/v NaOH and 0.1 % active chloride NaOCl) and 45  $\mu$ L of phenol reagent (1 % w/v phenol and 0.005 % w/v sodium nitroprusside) were poured into each well. After 50 min, the increase in absorbance at a wavelength of 630 nm was measured by a microplate reader (Molecular Device, USA). All reactions were run in triplicate in a total volume of 200  $\mu$ L and the change in absorbance per min were processed using Soft-Max Pro software (Molecular Device, USA).<sup>33</sup>

#### β-Glucuronidase assay

The  $\beta$ -glucuronidase activity was determined by a spectrophotometric method that involved the measurement of the absorbance of *p*-nitrophenol formed from the substrate at 405 nm. The reaction mixture consisting of 185 µL of 0.1 M acetate buffer (pH 7.00), 10 µL of enzyme solution and 5 µL of test compound (0.4 mM) solution was incubated for 30 min at 37 °C. The plates were read on a multiplate reader SpectraMax plus 384 (Molecular Devices, U.S.A.) after the addition of 50 µL of 0.4 mM *p*-nitrophenyl- $\beta$ -D-glucuronide at 405 nm. All assays were performed in triplicate.<sup>34</sup>

## Phosphodiesterase-I inhibition assay

Phosphodiesterase I inhibition assay was performed using snake venom according to a previously reported method with minute variations. Briefly, 33 mM Tris–HC1 buffer of pH 8.8 (97  $\mu$ L), 30 mM Mg acetate with an enzyme concentration of 0.000742 U well<sup>-1</sup> and 0.33 mM bis-(*p*-nitrophenyl) phosphate (Sigma N-3002, 60  $\mu$ L) as substrate were taken. EDTA with an *IC*<sub>50</sub>±SD of 274±0.007  $\mu$ M was used as the positive control.

After a pre-incubation period of 30 min, the enzyme with the test samples was observed spectrophotometrically for enzyme activity on a microtitre plate reader at 37 °C by following the rate of change in OD min<sup>-1</sup> at 410 nm of the *p*-nitrophenol released from *p*-nitrophenyl phosphate. All assays were processed in triplicate.<sup>35</sup>

#### Anticancer assay

Anticancer activity of compounds was evaluated in 96-well flat-bottomed micro plates by using the standard reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. For this purpose, PC3 cells (prostate cancer) were cultured in Dulbecco-modified Eagle medium, containing 5 % of foetal bovine serum (FBS), 100  $\mu$ g mL<sup>-1</sup> of streptomycin and 100 IU mL<sup>-1</sup> of penicillin in 25 cm<sup>3</sup> flasks, in an incubator at 37 °C under a 5 % carbon dioxide atmosphere. The exponentially growing cells were counted with a haemocytometer and diluted to a concentration of 1×10<sup>5</sup> cells mL<sup>-1</sup>. The diluted culture was then introduced into 96-well plates (100  $\mu$ L well<sup>-1</sup>) and incubated overnight. After incubation, medium was separated and 200  $\mu$ L of fresh medium was added with various concentrations of the compounds in the range 1–100  $\mu$ M. After 72 h, 50  $\mu$ L MTT (2 mg mL<sup>-1</sup>) was added to each well and incubated further for 4 h. Subsequently, 100  $\mu$ L of DMSO was added to each well. The MTT was reduced to formazan within viable cells and its absorbance was measured at 570 nm using a microplate ELISA reader (Spectra Max plus, Molecular Devices, CA, USA).<sup>36</sup>

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#### **RESULTS AND DISCUSSION**

## Chemistry

In a typical reaction *ortho-*, *meta-* or *para-*tolyl isocyanate was treated with a primary or secondary amine in 1,4-dioxane at room temperature (Scheme 1). The product formation and progress of reaction was observed by TLC. The difference in retardation factor ( $R_f$ ) values of the reaction mixture and the starting pure reactants indicated completion of the reaction. After completion of reaction, the mixture was cooled by the addition of a few cubes of crushed ice. As a result, the solid product precipitated. The obtained solid was filtered and washed several times with cold distilled water to remove excess amine and to obtain the pure product<sup>34</sup> (Table I).



Scheme 1. Synthetic route for 1,3-disubstituted ureas.

Cmpd.	–R	-R'	-R"	Cmpd.	-R	-R'	-R''
1	<i>m</i> -CH <sub>3</sub>	N	_	10	<i>m</i> -CH <sub>3</sub>	CH <sub>3</sub>	Н
2	<i>m</i> -CH <sub>3</sub>	CH <sub>3</sub> ————————————————————————————————————	Н	11	<i>p</i> -CH <sub>3</sub>	- s	Η
3	<i>m</i> -CH <sub>3</sub>	CH—CH <sub>3</sub>	Н	12	o-CH <sub>3</sub>	CH <sub>3</sub> 	Н

 TABLE I. Synthesis of 1,3-disubstituted urea derivatives (1–17)





The structures of the compounds were confirmed by mass, NMR and IR spectroscopy, and CHN elemental analysis. Their melting points were also recorded. The compounds were evaluated for their inhibition of different enzymes and anticancer activities.

## Biology

Enzyme inhibition screening. In vitro screening is one of the initial phases in drug discovery and many drug molecules are enzyme inhibitors; hence, their discovery and improvement is an active area of research in biochemistry and pharmacology. Thus in this study, compounds 1–17 were synthesized and randomly checked in three enzyme-inhibition assays (urease,  $\beta$ -glucoranidase and phosphodiestrase-I). All compounds showed various range of % inhibition against these enzymes (Table II).

In the series, compounds 1-4, 9 and 10 are *m*-tolyl urea derivatives having various R' groups. It was found that compound 4 with an *m*-methoxy group in the phenyl ring of R' exhibited 53.2 % urease inhibition and was the most potent of these compounds, which exhibited an activity of less than 50 %. For compounds

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**5–8**, **11** and **17**, a similar result was observed, *i.e.*, that the *para* tolyl compound **8** having an *m*-methoxy group in phenyl ring of R' was the most potent with a 59.1 % urease inhibition. Compound **6** with a 2,4,6-trimethylphenyl group was also active (45.3 % inhibition). Of the *o*-tolyl derivatives **12–16**, again compound **14** with an *m*-methoxy in the phenyl group of R' exhibited the greatest inhibition (47.5 %). The other compounds had an activity in the range of 0.3 to 45.3 % inhibition. Comparing compounds **4**, **8** and **14** with same R' group, compound **8**, which is a *p*-tolyl urea, exhibited the highest urease inhibition.

TABLE II. Enzyme inhibitions ( $\pm SD$ , %) and anticancer activities ( $IC_{50}\pm SD$ ,  $\mu$ M) of synthesized urea derivatives (1–17); PC3 = prostate cancer cell lines; – = no inhibition; SD = = standard deviation;  $IC_{50}$  = concentration at 50 % inhibition

Cmpd.	Urease	$\beta$ -Glucuronidase	Phosphodiesterase	Anticancer (PC3)
1	37.70±0.09	26.90±0.02	46.40±0.02	>100
2	21.70±0.04	4.90±0.01	_	>100
3	$0.30 \pm 0.05$	_	32.00±0.01	>100
4	53.20±0.03	40.30±0.01	4.80±0.01	>100
5	19.90±0.04	2.30±0.02	_	>100
6	45.30±0.02	20.40±0.01	_	>100
7	32.50±0.01	_	_	>100
8	$59.10 \pm 0.01$	37.80±0.01	_	>100
9	$40.70 \pm 0.01$	16.50±0.01	_	78.3±1.2
10	$6.90 \pm 0.04$	$28.70\pm0.02$	37.80±0.01	>100
11	$12.20\pm0.02$	20.60±0.01	_	>100
12	4.00±0.03	8.50±0.01	_	>100
13	30.20±0.02	6.00±0.03	_	>100
14	$47.50 \pm 0.02$	44.90±0.01	2.10±0.01	>100
15	$23.70 \pm 0.01$	$19.60 \pm 0.01$	$1.20\pm0.01$	>100
16	$12.90 \pm 0.02$	7.20±0.02	6.50±0.01	>100
17	_	24.50±0.02	9.800±0.003	>100
Standard	Thiourea	D-Sacchuric acid,	EDTA	Doxorubicin
	$98.20 \pm 0.01$	1,4-lactone, 97.1±1.2	90.10±0.01	0.91±0.12

The high activity of the *m*-methoxyphenyl moiety-containing compounds may arise because of the mutual effect of the electronegative oxygen of the methoxy group and the nitrogens and oxygen of the carbamide residue, which may increase the ligand–chelator ability of the compounds to form octahedral complexes with the nickel ions of urease enzyme. Moreover, the presence of bulky groups around the active nitrogen in other compounds decreased the activity of enzyme and the *m*-methoxyphenyl was the least bulky group in this series, which makes it easier for the urease to enter the active substrate binding site. In contrast, certain urea derivatives with *m*-methyl- and *m*-methoxyphenyl groups were previously reported in the literature as potent inhibitor of urease.<sup>37,38</sup>

However, in the inhibition studies of  $\beta$ -glucuronidase, compounds 1, 2, 4–6 and 8–17 showed % inhibition in the range 4.9 to 44.9 %, *i.e.*, no significant activity, while the other compounds gave negative results towards  $\beta$ -glucuronidase in the enzyme inhibition assay.

In the phosphodiestrase enzyme inhibition studies, the range of % inhibition was 1.2 to 46.4 %, *i.e.*, none of the prepared urea derivatives showed significant activity, except compound **1**, N-(3-methylphenyl)-2-oxo-1-pyrrolidinecarbox-amide, that exhibited low activity with a percentage inhibition value of 46.4 %, while its *ortho* and *meta* derivatives were nearly inactive towards phosphodiesterase-I (Table II).

Anticancer screening against PC3 cell lines. To explore further their biological activity against prostate cancer cell lines (PC3), all the compounds were tested. Unfortunately, only one urea derivative, N-(1,3-benzothiazol-2-yl)-N'-(3-methylphenyl)urea (9) showed better cytotoxicity activity with an  $IC_{50}$  value of 78.3, as compared to our standard doxorubicin having an  $IC_{50}\pm SD$  value of 0.91±0.12  $\mu$ M (Table II).

## CONCLUSIONS

In this study, attention was focused on the synthesis and biological activities of 1,3-disubstitued ureas. Thus, seventeen urea derivatives, including five new derivatives **2**, **4**, **6**, **9** and **15**, were synthesized. The % inhibition for urease,  $\beta$ -glucuronidase and phosphodiesterase enzymes were in the range 0.3–45.3, 4.9–44.9 and 1.2–46.4 %, respectively. In the *in vitro* anticancer study, the new compound *N*-(1,3-benzothiazol-2-yl)-*N*'-(3-methylphenyl)urea showed significant anticancer activity with an *IC*<sub>50</sub> value of 78.28±1.2  $\mu$ M against PC3 cell lines.

## SUPPLEMENTARY MATERIAL

Yields, physical, analytical and spectral data for the prepared ureas are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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#### ИЗВОД

# СИНТЕЗА, ЕНЗИМСКА ИНХИБИЦИЈА И ИСПИТИВАЊЕ АНТИТУМОРСКЕ АКТИВНОСТИ НЕСИМЕТРИЧНИХ 1,3-ДИСУПСТИТУИСАНИХ УРЕА

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Током истраживања синтетисано је седамнаест деривата, укључујући и нова једињења: *N*-мезитил-*N*-(3-метилфенил)уреа (**2**), *N*-(3-метилфенил)-*N*-(3-метоксифенил)- уреа (4), *N*-мезитил-*N*-(4-метилфенил)уреа (6), *N*-(1,3-бензотиазол-2-ил)-*N*-(3-метил -фенил)уреа (9) и *N*-(2-метилфенил)-2-оксо-1-пиролидинкарбоксамид (15) реакцијом *орто-, мета-* и *пара*-толил-изоцијаната са примарним и секундарним аминима. Испитана је инхибиторна активност добијених деривати према уреази, *β*-глукуронидази и фосфодиестерази змијског отрова. Утрвђено је да је опсег инхибиције према уреази, *β*-глукуронидази и фосфодиестерази ѕмијског отрова. 0,3–45,3, 4,9–44,9 и 1,2–46,4 %, редом. Осим тога, испатан је и ефекат синтетисаних једињења према ћелијској линији рака простате. Нов дериват *N*-(1,3-бензотиазол-2-ил)-*N*-(3-метилфенил)уреа (9) показује *in vitro* активност *IC*<sub>50</sub> од 78,3±1,2  $\mu$ М. Сва синтетисана једињења окаратактерисана су стандардним спектроскопским техникама.

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