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Studies on the activity of some novel derivatives of ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl)formate

Imidazo-triazine derivatives display a broad spectrum of biological activity. Till now all known imidazo[2,1-*c*][1,2,4]triazines have been prepared and tested as cardiovascular agents (9), bactericides (8) and central nervous system stimulants (2). The other derivatives of this heterocyclic ring system have been received as the Maillard reaction inhibitors. Nowadays it is considered that the Maillard reaction takes part in various diseases relating to diabetes and aging. Some of imidazo[2,1-*c*][1,2,4]triazines, known from the patent literature, have been designed for the treatment and /or prevention of various diabetes complications such as coronary disease, periphery circulatory disorder, renal disease, cerebrovascular disorder, diabetic neurosis, retinities, articular sclerosis or the diseases caused by aging such as senile cataract, atherosclerosis etc. by inhibiting the Maillard reaction (5).

Furthermore, imidazo-triazine derivatives reported herein contain in their molecules similar features (e.g., potential pharmacophore formations: the phenyl ring, the additional carbonyl group as the potential acceptor centre of hydrogen bond) to many morphine-like analgesics such as benzitramide, fentanyl, petidine and selective ligand of δ-opioid receptors (SNC-80). These similar features according to pharmacophore model introduced by Beckett with its further modifications can play an important role in expressing pharmacological activity, especially the analgesic action. The presence of carbonyl group in the structure of the obtained compounds can probably play a supporting role in binding with receptor due to the high negative potential present on the oxygen atom. The presented herein imidazo[2,1-*c*][1,2,4]triazine derivatives have no basic nitrogen atom. The lack of this atom could play a role in the receptor activation stage. It was also observed in the first potent naturally occurring nonnitrogenous KOR selective agonist – salvinorin A, the main active ingredient of Salvia divinorum, which has no action at the 5-HT_2 receptors, the principle molecular target responsible for the actions of classical for opioids side effects (4).

Previous studies concerning the synthesis and biological activity of imidazo[2,1-*c*][1,2,4]triazin-4(4*H*)-ones (10-12) have disclosed some compounds with various aryl substituents at the 8-position, and with benzyl, substituted benzyl, methoxycarbonylmethyl and 3-oxo group at position 3 to reveal a significant antinociceptive activity on the central nervous system in behavioral animal tests, and a low acute toxicity (LD_{50} in the range from over 1100 to over 2000 mg · kg^{-1} i.p.).

On the other hand, the definite derivative of 2-[4-oxo-8-(4-chlorophenyl)-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl]acetic acid revealed significant activity against all Gram-negative bacterial strains tested (13). Therefore, the newly obtained ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)formates were tested to exclude or confirm their expected antibacterial potency deduced from the literature data (8) and our previous studies (13) in order to limit or broaden their possible biological activity spectrum.

The title compounds were obtained by the two independent synthesis methods from appropriate 1-aryl-2-hydrazonoimidazolidines (1-aryl-2-hydrazinoimidazolines) by cyclocondensation reaction with diethyl 2-(hydroxyimino)malonate and diethyl 2-oxomalonate (14). The following compounds were examined in relation to bacterial strains:

I. Ethyl 1-[4-oxo-8-(4-methylphenyl)-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl]formate;
II. Ethyl 1-[4-oxo-8-(3-chlorophenyl)-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl]formate;
III. Ethyl 1-[4-oxo-8-(3,4-dichlorophenyl)-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl]formate.

Molecular structure of the synthesized compounds was confirmed by IR, ^{1}H-NMR, EI-MS spectra and elemental analysis. The purity of all the compounds synthesized was checked by thin-layer chromatography. TLC was carried out on commercial Merck SiO_2 60 F_{254} plates having fluorescence indicator; the spots were visualized with UV light $\lambda = 254$ nm. These compounds were characterized by solubility in dimethylformamide (I) or dimethylformamide/ methanol mixture (II, III) and dimethyl sulfoxide (I-III) (14).

MATERIALS AND METHODS

Assay of antibacterial activity *in vitro* Imidazo-triazines of types I–III were tested for their antibacterial activities by the disc-diffusion method by Kirby-Bauer, using Mueller-Hinton medium for bacteria. The majority of test microorganisms were obtained from clinical specimens of the Laboratory of Medical Microbiology Department, Medical University of Lublin. The assayed collection included 48 strains of the following Gram-positive bacteria: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes* and 16 strains of Gram-negative one – *Escherichia coli* (Table 1).

Table 1. Microorganism cultures used to microbiological screening

Group	Strain	Number of strains
Gram-positive bacteria	*Staphylococcus aureus*	21
	Staphylococcus epidermidis	15
	Streptococcus pyogenes	12
Gram-negative bacteria	*Escherichia coli*	16

In the disc-diffusion method, sterile paper discs (ϕ 5mm) impregnated with dissolved in dimethyl sulfoxide compound at the concentration of 100 $\mu g\ mL^{-1}$ were used. Discs containing DMSO were used as a solvent control. The microorganism cultures were spread over the Mueller-Hinton agar for the tested bacteria in Petri dishes. Then, the paper discs impregnated with the solutions of the compounds tested were placed on the surface of the media inoculated with the microorganism. The plates were incubated at 35°C/24 h for the microorganisms' cultures. After incubation, the growth inhibition zones around the discs were observed indicating that the examined compound inhibits

the growth of microorganisms (1, 6, 7). Each assay in this experiment was repeated three times. Ampicillin in a concentration of 200 μg mL^{-1} was used as a standard drug. Results are presented in Table 2.

Table 2. Antibacterial activities of the evaluated ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)formates (I-III) against the tested bacterial isolates using the disc-diffusion method

Comp.	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Streptococcus pyogenes*	*Escherichia coli*
I	-	-	+++	+
II	++	++	++	++
III	-	+	+	-
Standard	++	+	++	++

Results were interpreted in terms of the diameter of inhibition zone: (-): < 9 mm; (+): 10–15 mm; (++): 16–20 mm; (+++): > 20 mm

Standard: ampicillin at concentration of 200 μg mL^{-1}

Prediction of potential biological activity. Potential biological properties of the investigated ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)formates were evaluated using computer program PASS (Prediction of Activity Spectra for Substances); http://www.ibmh.msk.su/service.htm. This program predicts the biological activity spectrum for a compound on the basis of its structural formula. It estimates the probability of the molecule to be active (Pa) and inactive (Pi) for each type of activity from the biological activity spectrum. The most probable biological activities predicted by PASS program are shown in Table 3.

Table 3. The most probable (Pa>70%) types of biological activity of the investigated ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)formates predicted by PASS program

Comp.	Pa	Pi	Activity
I	95.5	0.1	GABA A receptor antagonist
	80.6	0.2	GABA receptor antagonist
	77.3	0.6	Cyclic GMP phosphodiesterase inhibitor
II	95.2	0.1	GABA A receptor antagonist
	80.9	0.2	GABA receptor antagonist
	78.0	0.5	Cyclic GMP phosphodiesterase inhibitor
III	95.3	0.1	GABA A receptor antagonist
	81.4	0.2	GABA receptor antagonist
	77.6	0.5	Cyclic GMP phosphodiesterase inhibitor

RESULTS AND DISCUSSION

Previously, it has been found that presented herein imidazo-triazines of types II and III have potential to reduce the growth of the uterus cancer cell line (SiHa, ECACC 85060701), colon

adenocarcinoma cell line (LS180, ECACC 87021202) and breast carcinoma cell line (T47D, ECACC 85102201). Compound II was the most active against SiHa cancer line, because its GI was 41 and 52 %, respectively for both examined concentrations (10 and 50 μg mL^{-1}). Compound III was found to be the most potent against LS180 and SiHa cancer lines, especially in a higher concentration (50 μg mL^{-1}). Its GI values were 50 and 46 % for these cancer lines, respectively. Based on the performed examination, the distinctly marked lower cytotoxicity of the tested compounds against normal cell lines and almost 2-times higher against cancer cell lines was ascertained. Taking into consideration the GI comparative study results concerning the influence of tested compounds on cancer and normal cell lines it can be expected the selective action of examined compounds. Also the anticancer activity of the tested compounds was found to be dose-dependent. These compounds were proved to demonstrate antiproliferative properties, justifying their further investigation as potential anticancer agents (14).

In connection with significant antiproliferative activity of the above mentioned compounds, it seemed worthwhile to carry out antibacterial assay to exclude or confirm their potential antimicrobial property, deduced from the previous studies and from the literature survey (8, 13).

Susceptibility of 48 Gram-positive and 16 Gram-negative bacterial strains to synthesized compounds was determined. The results are presented in Table 2. All the tested compounds in the examined concentration (100 μg·mL^{-1}) showed antibacterial activity against two (compounds I and III) or four (compound II) test bacteria. According to the data listed in Table 2, derivative II, containing the lipophilic, weak-electron withdrawing a chloro group at the 3-position of the phenyl ring revealed the broadest activity spectrum. Moreover, this compound was found to exhibit a comparable level of potency to that of ampicillin. Replacement of a 3-chloro group for either the lipophilic weak electron-donating 4-methyl one (I) or introduction of the additional substituent, e.g., a chlorine atom at the 4-position of the phenyl ring as in case of 3,4-dichloro derivative (III), generally resulted in reduction in the activity spectrum. However, replacement of a 3-chloro group of the phenyl ring by a 4-methyl one in I resulted in a significant increase of potency against *Streptococcus pyogenes* and decrease of effectiveness against *Escherichia coli*. It is noteworthy that this structural change in the molecule of compound I led to a complete loss of activity against *Staphylococcus aureus* and *Staphylococcus epidermidis*. However, the structural change in the molecule of compound III led to a complete loss of activity against *Staphylococcus aureus* and *Escherichia coli* and resulted in a decrease of antibacterial action against *Staphylococcus epidermidis* and *Streptococcus pyogenes*. The microbiological screening afforded to broaden the possible biological spectrum of activity of these biologically active compounds.

In conclusion, compound II in concentration of 100 μg·mL^{-1}inhibited the growth of all Gram-positive and Gram-negative bacterial strains tested. It was found to be effective on 21 strains of *Staphylococcus aureus*, 15 strains *Staphylococcus epidermidis*, 12 strains of *Streptococcus pyogenes* and 16 strains of *Escherichia coli*. Taking into account the significant antibacterial activity of imidazo-triazine II, the research on this field will be continued. It is likely to happen that its structural analogues should also be active.

Based on PASS program for all the compounds investigated (I–III) the most probable seemed to be GABA A receptor antagonistic activity (Pa about 95.3 %, and Pi 0.1 %). Also, the inhibition of cyclic guanosine monophosphate (cGMP) was predicted as highly possible (Pa in the range 77.3–78.0 %) for them. It follows from the literature data that substituted purinones as analogues of cGMP are potent phosphodiesterase (PDE) inhibitors *in vitro*. Besides, imidazo[5,1-*f*][1,2,4]triazin-4(3H)-ones are highly cGMP- PDE-5 selective and show IC_{50} values for PDE-3 and PDE-4 greater than 50 nM. In comparison to well known pyrazolo[4,3-*d*]pyrimidin-7-one phosphodiesterase inhibitors (for

instance Sildenafil) the imidazo-triazines reported in the literature indicate improved selectivity over PDE-1 and substantially improved PDE-5 inhibition (3).

Furthermore, both the investigated imidazo-triazines of types II and III showed Pa>50% in the categories of anticonvulsant and lipoprotein lipase inhibitors. Additionally, compound I demonstrated Pa>50 % in the category of growth factor antagonist and antihypoxic.

CONCLUSIONS

1. It can be concluded from the antibacterial assay that all the tested compounds (I–III) at the concentration of 100 $\mu g \cdot mL^{-1}$ had influence on the growth of two or four microorganisms tested.

2. Compound II was the most active of the series. It revealed antibacterial activity against all the tested strains of Gram-positive and Gram-negative bacteria. Moreover, its potency was comparable to that of ampicillin.

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SUMMARY

The bicyclic imidazo-triazine ring is the structural element of many synthetic biologically active compounds that have different pharmacological activity spectrum. Besides, it follows from the literature data that depending on the nature of substituent certain derivatives of imidazo-triazine may also show antibacterial properties. The purpose of this study was to confirm or exclude the potential antibacterial activity of biologically active novel derivatives of ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)formate deduced from the literature survey. Microbiological tests were conducted on 64 strains of bacteria. The examined compounds at the concentration of 100 μgmL^{-1} had influence on the growth of microorganisms tested. Also, the most probable biological properties of investigated imidazo[2,1-*c*][1,2,4]triazine derivatives were evaluated using computer PASS programme.

Badanie aktywności nowych pochodnych estru etylowego kwasu
1-(4-okso-8-arylo-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazyno-3-ylo)mrówkowego

Bicykliczny układ imidazo-triazyny jest obecny w strukturze syntetycznych, biologicznie czynnych związków wykazujących różnorodne działanie farmakologiczne. Ponadto z danych literatury wynika, że w zależności od podstawnika niektóre pochodne tego układu mogą wykazywać aktywność przeciwbakteryjną. Celem pracy jest potwierdzenie lub wykluczenie potencjalnej aktywności przeciwbakteryjnej nowych pochodnych estru etylowego kwasu 1-(4-okso-8-arylo-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazyno-3-ylo)mrówkowego. Testy aktywności przeciwdrobnoustrojowej przeprowadzono na 64 szczepach bakteryjnych. Przebadane związki w stężeniu 100 μgmL^{-1} hamowały wzrost testowanych drobnoustrojów. Ponadto określono najbardziej prawdopodobne typy aktywności badanych imidazo[2,1-*c*][1,2,4]triazyn przy zastosowaniu programu komputerowego PASS.