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Matijević-Sosa, Julija; Samaržija, Ita; Honović, Lorena; Jurišić, Blaženka

Source / Izvornik: **Acta Pharmaceutica, 2008, 58, 231 - 236**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

https://doi.org/10.2478/v10007-008-0010-7

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:163:622805

Acta Pharm. 58 (2008) 231–236
10.2478/v10007-008-0010-7

Short communication

N-Phthaloyl-glycine-hydroxamic acid as serum iron chelator in rats

JULIJA MATIJEVIĆ-SOSA[1,*]
ITA SAMARŽIJA[1]
LORENA HONOVIĆ[2]
BLAŽENKA JURIŠIĆ[3]

[1] *Faculty of Pharmacy and Biochemistry University of Zagreb, Zagreb, Croatia*

[2] *Clinical Laboratory, General Hospital Pula, Pula, Croatia*

[3] *Agency for Medicinal Products and Medicinal Devices of the Republic of Croatia, Zagreb, Croatia*

The aim of this study was to investigate the activity of *N*-phthaloyl-glycine-hydroxamic acid (Phth-Gly-HA) as a new iron chelator *in vivo* to be used in iron overload diseases. After intraperitoneal application of Phth-Gly-HA to male rats (1 mg kg^{-1} body mass) once a day for seven days, iron serum level decreased by 21%, whereas the iron value dropped by 32% in female rats (1.5 mg kg^{-1} body mass). The results indicate that the tested substance has the ability to bind serum iron by complexation. Besides transferrin iron release, mobilization of ferritin iron is also possible.

Keywords: *N*-phthaloyl-glycine-hydroxamic acid, iron chelators, iron overload

Accepted May 8, 2008

Major functions of iron, as an essential element for living organisms, are oxygen transport and its role in oxidative-reductive reactions that utilize its alternative ferrous--ferric states. Total body iron has to be strictly controlled because excess iron can be highly toxic, leading, through generation of reactive oxygen species (ROS), to iron overloading disorders (1, 2). Iron chelating therapy has long been the standard care for patients suffering from *Thalassemias* (anemias caused by mutation of globin genes) and other anemias, caused *e.g.* by renal dysfunction (lack of erythropoietin (EPO) production), and for patients receiving transfusion iron supplementation. Iron chelators are used to treat dialyzed patients; in kidney transplantation, cardiac diseases, malaria, iron poisoning, and can inhibit tumor cell growth. They are also used to stimulate EPO production (3–5).

Various structures of iron chelates have been investigated but the best known and in use for more than 40 years is desferrioxamine (DFO), despite its poor membrane permeability, short shelf life, difficulties in application and expensiveness. DFO is a natural hydroxamic acid (HA) of the microorganism *Streptomyces sp.* and belongs to sideropho-

* Correspondence, e-mail: jmatijevic@pharma.hr

res, compounds that are able to solubilize environmental iron hydroxides and, by forming a complex, transport iron into the cell (6, 7).

Some pharmacological activities of HA structures could be explained by iron chelating, *e.g.*, antibacterial activity due to inhibition of peptide deformylase as well as anti-inflammatory activity owing to inhibition of lypooxygenase (5-LO) (8–10).

In this work, we have studied the influence of *N*-phthaloyl-glycine-hydroxamic acid (Phth-Gly-HA) on iron levels in rat serum, with the goal of finding a potentially successful new chelator for iron overload diseases.

EXPERIMENTAL

Chemistry

N-phthaloyl-glycine-hydroxamic acid (Phth-Gly-HA) was synthesized in three steps according to a modified procedure (11). Glycine with phthalanhydride gave *N*-phthaloyl-glycine, which was by thionyl chloride converted to *N*-phthaloyl-glycine-chloride and finally, in the reaction with hydroxylamine hydrochloride *N*-phthaloyl-glycine-hydroxamic acid [$C_6H_4(CO)_2NCH_2CONHOH$] was prepared. Modifications were made in the third step of the synthesis. Higher excess of hydroxylamine, liberated from hydrochloride, towards relevant acyl-chloride was used, and the reaction was carried out by adding small portions of reactants alternatively, instead of all at the same time, to avoid possible decomposition of the product. In the course of synthesis, as well as separation by column chromatography and purification by crystallization, hydroxamic acid was detected due to intensive colour with $FeCl_3$ solution.

Animals, laboratory equipment and methods

Investigations of the chelating ability of the test compound under *in vivo* conditions were performed on Wistar rats of both genders (males weighing about 350 g and females 200–250 g). Care and treatment of experimental animals followed recommended guidelines (Approval by the Institutional Ethical Committee).

Three groups of male rats were formed. The first group (n = 10) served as control, the second (n = 4) was treated with the studied substance only once and the third group (n = 4) was treated for seven days, once a day, with the test substance.

Two groups of female rats were formed, the first (n = 3) as control and the second (n = 3) for the seven-day treatment.

The substance examined, Phth-Gly-HA, was prepared for application as a water solution in a concentration of 0.1 mg mL^{-1}. The same volume (3.5 mL per animal) of the test solution was injected intraperitoneally into both genders. The control group received 3.5 mL of physiological saline. The applied dose of Phth-Gly-HA (0.35 mg) was 1 mg kg^{-1} body mass of male rats and 1.5 mg kg^{-1} body mass of female rats.

Blood samples were taken from the tested animals after one day from male rats and after seven days from both genders. The level of serum iron was measured. Samples (up to 2 mL) from treated as well as from control animals were taken from *vena jugularis*

with a short surgical procedure under short anesthesia with diethyl ether. The blood was collected directly into the test tube with resin for serum separation. The blood samples were left about 30 min at room temperature (21 °C) and centrifuged 10 min at ~2000 rpm. Serum was separated from the sediment and analyzed by the TPTZ [2,4,6-tris(2-pyridyl)-s-triazine] method (12). Iron determination was performed by commercially available tests on an automated analyzer Olympus 600 (WB Saunders Company).

RESULTS AND DISCUSSION

The values of serum iron in rats before and after application of active substance are shown in Tables I and II. Using intraperitoneal application (1 mg kg^{-1}) of Phth-Gly-HA for seven days, once a day, the levels of serum iron decreased from average 37.8 μmol L^{-1} to 29.8 μmol L^{-1} for male rats (Table I), *i.e.*, they decreased by 21%. After treatment of female rats (1.5 mg kg^{-1}) for seven days, once a day, the serum iron decreased from 59.3 μmol L^{-1} to 38.3 μmol L^{-1} (Table II), *i.e.*, dropped by 35%. Interestingly, treatment of male rats for only one day led to quite a large decrease of iron (68% of the control) (Table I). The assumption that some regulatory and compensatory mechanisms are involved after a few days, which could inhibit further decrease of serum iron, could be taken into consideration. A feedback regulation of iron absorption into the gastro-intestinal tract, which is regulated by a mucosal receptor blocking further iron absorption after it is saturated, is possible (1).

The obtained results show that Phth-Gly-HA possesses serum iron chelation ability, mostly from transferrin iron. The main goal in designing new chelators is actually to mobilize stored iron, ferritin, as well. Still, the best chelator for medicinal purposes, DFO, can extract stored iron with difficulty. It is possible that Phth-Gly-HA could remove ferritin iron owing to the recent knowledge about the relationship between the corresponding structures and properties of numerous examined chelators (6).

DFO, as a hexadentate ligand with three hydroxamic groups, forms a 1:1 stable complex with the ferri ion, preventing ROS formation. Hexadentate chelators with bulky mass (600–900 Da) possess lower bioavailability. However, bidentate ligands such as Phth-Gly-HA and some other HAs with one hydroxamic moiety, or hydroxypyridones like deferiprone, form iron complexes at a ratio 3:1, which are less stable, less inert and

Table I. Serum iron in male rats after application of Phth-Gly-HA[a]

Number of male rats (*n*)	Treatment with Phth-Gly-HA (day)	Iron concentration (mean ± SD) (μmol L^{-1})	Iron value in relation to control (%)
10	Control	37.8 ± 2.4	–
4	1	25.8 ± 0.4	68
4	7	29.8 ± 2.4	79

[a] 1 mg kg^{-1} body mass for one day and seven days

Table II. Serum iron in female rats after application of Phth-Gly-HA[a]

Number of female rats (*n*)	Treatment with Phth-Gly-HA (day)	Iron concentration (mean ± SD) (μmol L^{-1})	Iron value in relation to control (%)
3	Control	59.3 ± 3.7	–
3	7	38.3 ± 1.8	65

[a] 1.5 mg kg^{-1} body mass for seven days

thus more soluble, which could help chelation of storage iron. For this reason, as well as owing to smaller size (100–250 Da), bidentate ligands have higher bioavailability that additionally grows with lipophilicity, based on hydrophobic interactions with metalloproteins. Further advantage of the small-size chelators is that no bidentate interaction is possible between ligand and porphyrin-bound iron. Iron redistribution from such complexes is still possible. Acetohydroxamic acid (75 Da), benzohydroxamic acids (137 Da) and deferiprone (139 Da) showed the ability of passing narrow ferritin channels leading to stored Fe(III) ions, which were chelated directly. However, some chelators have to reduce ferric ions to a more mobile ferro form before complexation and then oxidation to a stable ferri complex follows. The efficacy of chelation depends on a number of factors. Due to negatively charged ferritin channels, medium pH and chelator charge are important. The presence of urea, capable of broadening ferritin channels, strongly increases the effectiveness of chelation. There is still inadequate understanding of all the mechanisms of chelator entry to ferritin, mobilization of iron and exit of the formed complexes (13–16).

To avoid undesirable effects of particular commonly used chelators, recently combined drugs, *e.g.*, the hexadentate DFO and the bidentate deferiprone, have been used. Search for better human iron chelators still remains a challenge (6, 17).

CONCLUSIONS

Phth-Gly-HA decreases the level of serum iron in rats by complexation of transferrin iron and probably also part of ferritin iron. This assumption is based on monohydroxamic acid structure of the chelator, being a bidentate ligand of considerable lipophilicity due to its aromatic ring rather than to bulky mass (220 Da). According to these properties, Phth-Gly-HA should possess high bioavailability, which could be of therapeutic use.

Our hypothesis needs to be confirmed by further experiments. It will be interesting to know the portion of ferritin iron, examine the influence of this chelator on EPO production, determine the chelation capacity in dependence on pH values (differences beween physiological pH and pH under some pathological conditions), or the chelation efficacy with a catalyst such as urea. Its possible application in humans should be examined.

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S A Ž E T A K

N-ftaloil-glicin-hidroksamska kiselina kao kelator željeza u serumu štakora

JULIJA MATIJEVIĆ-SOSA, ITA SAMARŽIJA, LORENA HONOVIĆ i BLAŽENKA JURIŠIĆ

U cilju pronalaženja novog efikasnog kelatora koji bi mogao poslužiti u liječenju bolesti izazvanih viškom željeza, u ovom je radu ispitano djelovanje *N*-ftaloil-glicin-hidroksamske kiseline (Phth-Gly-HA) *in vivo*. Istraživan je utjecaj kelatora na razinu željeza u serumu štakora nakon intraperitonealne primjene vodene otopine Phth-Gly-HA (0,1 mg mL^{-1}) jednom dnevno tijekom 7 dana. Kontrolne su životinje primale fiziološku otopinu. Kod mužjaka injektiranje test supstancije (1 mg kg^{-1}) uzrokovalo je pad serumskog željeza za 21%. Kod ženki je nakon tretmana (1,5 mg kg^{-1}) izmjereno sniženje razine željeza za 35%. Rezultati pokazuju da ispitivana supstanca ima sposobnost kompleksiranja serumskog željeza, pretežno transferinskog, ali da postoji mogućnost mobilizacije željeza i iz feritinskih zaliha.

Ključne riječi: *N*-ftaloil-glicin-hidroksamska kiselina, kelatori željeza, bolesti uzrokovane viškom željeza

Farmaceutsko-biokemijski fakultet Sveučilišta u Zagrebu, Zagreb

Opća bolnica Pula

Agencija za lijekove i medicinske proizvode, Zagreb