Uniwersytet Jagielloński

Collegium Medicum

Dominik Bakalarz

Molecular mechanisms of novel carbon monoxide and hydrogen sulfide donors activity enhancing physiological gastric mucosal integrity against acute drug-induced gastrotoxicity

Molekularne mechanizmy działania nowych donorów tlenku węgla i siarkowodoru wzmacniające fizjologiczną integralność błony śluzowej żołądka w redukcji ostrej gastrotoksyczności polekowej

Praca doktorska

Promotor: Dr hab. n. med. Marcin Magierowski
Promotor pomocniczy: Dr n. med. Edyta Korbut

Pracę wykonano w Pracowni Inżynierii Komórkowej i Diagnostyki Izotopowej, w Katedrze Fizjologii Collegium Medicum Uniwersytetu Jagiellońskiego w Krakowie

Kierownik Pracowni: Dr hab. n. med. Marcin Magierowski
Kierownik Katedry: Prof. dr hab. n. med. Tomasz Brzozowski

Kraków, rok 2021

Pragnę serdecznie podziękować
mojemu promotorowi oraz opiekunowi naukowemu
Dr hab. Marcinowi Magierowskiemu
za opiekę merytoryczną oraz nieocenione wsparcie
w trakcie realizacji niniejszej pracy doktorskiej

Składam serdeczne podziękowania mojemu
promotorowi pomocniczemu ***Dr Edycie Korbut***
za poświęcony czas oraz cenne wskazówki
w trakcie realizacji badań

Szczególne wyrazy wdzięczności kieruję również
do ***Prof. dr hab. Tomasza Brzozowskiego****,*
Kierownika Katedry Fizjologii UJCM w Krakowie,
za przekazaną wiedzę oraz wszelką okazaną życzliwość

Wyrazy wdzięczności kieruję do ***Prof. Binghe Wang'a***
z Georgia State University w Atlancie (USA)
za serdeczne przyjęcie mojej osoby podczas stażu
naukowego w Jego jednostce oraz za owocną współpracę naukową

Chciałbym także bardzo serdecznie podziękować Koleżankom i Kolegom
z naszego zespołu badawczego za cenne dyskusje i pomoc w realizacji badań
jak również pozostałym Współautorom załączonych publikacji

Pracę tę dedykuję moim Rodzicom

Fragment pracy dotyczący molekularnego mechanizmu działania donoru tlenku węgla, jest tematycznie powiązany i jest częściowo wynikiem realizacji grantu badawczego finansowanego przez Narodowe Centrum Nauki (UMO-2019/33/B/NZ4/00616).

SPIS TREŚCI

1. WYKAZ PUBLIKACJI STANOWIĄCYCH PRACĘ DOKTORSKĄ..... 7
2. STRESZCZENIE W JĘZYKU POLSKIM……..... 8
3. STRESZCZENIE W JĘZYKU ANGIELSKIM (ABSTRACT)……. 14
4. PUBLIKACJE……. 20
5. OŚWIADCZENIA WSPÓŁAUTORÓW……... 58

1. WYKAZ PUBLIKACJI STANOWIĄCYCH PRACĘ DOKTORSKĄ

1. **Bakalarz D**, Surmiak M, Yang X, Wójcik D, Korbut E, Śliwowski Z, Ginter G, Buszewicz G, Brzozowski T, Cieszkowski J, Głowacka U, Magierowska K, Pan Z, Wang B, Magierowski M. Organic Carbon Monoxide Prodrug, BW-CO-111, in Protection against Chemically-Induced Gastric Mucosal Damage. Acta Pharm Sin B. 2021 Feb;11(2):456-475. doi: 10.1016/j.apsb.2020.08.005; Punktacja ministerialna: 140 pkt. *Impact Factor* (IF) za 2019 r.: 7.097 (Q1 wg Web of Sience), **5-letni IF: 7.865**

2. **Bakalarz D**, Korbut E, Yuan Z, Yu B, Wójcik D, Danielak A, Magierowska K, Kwiecień S, Brzozowski T, Marcinkowska M, Wang B, Magierowski1 M. Novel Hydrogen Sulfide (H_2S)-Releasing BW-HS-101 and Its Non-H_2S Releasing Derivative in Modulation of Microscopic and Molecular Parameters of Gastric Mucosal Barrier. Int J Mol Sci. 2021 May; 22(10): 5211. doi: 10.3390/ijms22105211; Punktacja ministerialna: 140 pkt. IF za 2019 r.: 4.556 (Q1 wg Web of Sience), **5-letni IF: 4.653**

2. STRESZCZENIE W JĘZYKU POLSKIM

W świetle dotychczas przeprowadzonych badań naukowych udowodniono, że tlenek węgla (CO) oraz siarkowodór (H_2S) jako gazowe mediatory, produkowane i uwalniane endogennie w tkankach organizmu, posiadają wielokierunkową aktywność biologiczną i uczestniczą w wielu procesach fizjologicznych i patologicznych. Dlatego też, rola tych molekuł w organizmie jest równie ważna jak ich bliźniaczego mediatora – tlenku azotu (NO). Wykazano, że CO i H_2S mogą działać ochronnie w obrębie błony śluzowej przewodu pokarmowego, modulując odpowiedź zapalną oraz szereg funkcji na poziomie molekularnym i czynnościowym. Szlaki enzymatyczne zaangażowane w endogenną biosyntezę tych gazowych mediatorów są istotnymi składowymi fizjologicznej bariery ochronnej błony śluzowej przewodu pokarmowego warunkującymi utrzymanie jej integralności podczas ekspozycji na ostry stres, ischemię z reperfuzją, bądź też dożołądkową (i.g.) aplikację czynników chemicznych, takich jak etanol i leki. Należy zaznaczyć, że korzystna bioaktywność CO i H_2S nie jest ograniczona wyłącznie do przewodu pokarmowego, gdyż obserwuje się ją również w układzie krążenia, gdzie molekuły te działają m.in. naczyniorozszerzająco oraz hamują agregację płytek krwi i adherencję leukocytów do śródbłonka, a także w układzie nerwowym, gdzie działają neuromodulująco, antyoksydacyjne i cytoprotekcyjnie.

Biosynteza CO w organizmie odbywa się w wyniku oksydatywnej degradacji hemu, katalizowanej przez oksygenazy hemowe (HMOX), głównie izoformę indukowaną stanem zapalnym HMOX-1 oraz konstytutywną HMOX-2. CO jest jednym z produktów rozpadu hemu, obok biliwerdyny i jonów Fe^{2+}. Natomiast H_2S powstaje głównie w wyniku metabolizmu L-cysteiny, przy udziale dwóch enzymów, γ–liazy cystationinowej (CTH) oraz β-syntazy cystationinowej (CBS), dla których kofaktorem jest fosforan-5-pirydoksalu (witamina B6). H_2S powstaje dodatkowo z 3-merkaptopirogronianu, wskutek aktywności enzymatycznej siarkotransferazy 3-merkaptopirogronianu (MPST). H_2S może być generowany również na drodze przemian metabolicznych egzogennych związków, np. polisiarczków zawartych w czosnku i dostarczanych wraz z pożywieniem, bądź z siarczanów przy udziale bakterii jelitowych w okrężnicy. Biorąc pod uwagę istotność działania tych molekuł w fizjologii i patofizjologii, w ciągu ostatnich lat zidentyfikowano lub opracowano szereg donorów farmakologicznych, które uwalniają CO lub H_2S.

Droga podania środka farmakologicznego jest istotnym czynnikiem mającym wpływ na aktywność biologiczną oraz dalszą biodystrybucję w organizmie. Wcześniejsze prace koncentrowały się na aplikacji CO drogą inhalacyjną. W późniejszych badaniach zauważono,

że CO ma wysokie powinowactwo do szeregu metali przejściowych, tworząc z nimi związki kompleksowe – karbonylki, które przyciągnęły wiele uwagi badaczy jako potencjalne donory CO. Jednym z pierwszych takich związków był tetrakarbonylek niklu ($Ni(CO)_4$), nad którym już w 1891 r. McKendrick i Snodgrass prowadzili badania, jednak związek ten okazał się być wysoce toksyczny. Kolejny przełom nastąpił, gdy grupa naukowców pod kierownictwem Motterliniego opracowała alternatywne donory CO (z j.ang. *Carbon Monoxide Releasing Molecules,* CORM), zawierające w swojej strukturze takie metale jak ruten, mangan, molibden i żelazo. Substancje te okazały się na tyle mało toksyczne, że znalazły zastosowane w badaniach na poziomie komórkowym *in vitro* oraz na modelach zwierzęcych *in vivo*. Jednym z takich związków jest dimer trikarbonylodichlororutenu (CORM-2), którego chroniczna aplikacja m.in. przyspieszała gojenie się przewlekłych wrzodów błony śluzowej żołądka. Jednakże, CORM-2 posiada w swojej strukturze potencjalnie cytotoksyczny ruten, który jak pokazały ostatnie badania wykazuje tendencję do wiązania się z grupami sulfhydrylowymi w białkach. Dlatego też, kliniczne zastosowanie tej substancji w przyszłości wydaje się ograniczone, pomimo wykazanego w warunkach laboratoryjnych potencjału prewencyjnego oraz terapeutycznego względem uszkodzeń błony śluzowej przewodu pokarmowego oraz zmian patologicznych obejmujących inne układy organizmu.

W warunkach eksperymentalnych, na przełomie lat stosowano różne donory H_2S, w tym np. wodorosiarczek sodu (NaHS). Ta nieorganiczna sól, w badaniach na zwierzętach zapobiegała gastrotoksyczności indukowanej przez niesteroidowe leki przeciwzapalne (NLPZ) oraz zwiększała sekrecję jonów wodorowęglanowych (HCO_3^-) w dwunastnicy, co przyczyniało się do zachowania integralności błony śluzowej przewodu pokarmowego. Innym prostym związkiem uwalniającym H_2S pochodzenia naturalnego jest zawarty w czosnku disiarczek diallilu (DADS). Wykazano, że DADS może m.in. hamować proliferację ludzkich komórek raka jelita grubego z linii HT29. W celu weryfikacji efektu biologicznego H_2S, stosowano również prekursor biosyntezy tej molekuły, L-cysteinę oraz odczynnik Lawessona. Wśród wielu doniesień, zaobserwowano, że dożołądkowa aplikacja tych substancji, m.in. przyspieszyła gojenie przewlekłych wrzodów żołądka.

Na poziomie czynnościowym wykazano, że CO i H_2S uwalniane z odpowiednich donorów wpływają na regulację mikrokrążenia żołądka, motoryki przewodu pokarmowego oraz wydzielania żołądkowego i jelitowego. Natomiast molekularny mechanizm działania tych molekuł w obrębie błony śluzowej żołądka wynikał z modulacji szlaków komórkowych regulowanych m.in. przez czynniki jądrowe NRF-2, NF-κB czy indukowany hipoksją czynnik HIF-1α.

W świetle uzyskanych wyników badań, przeciwzapalne i przeciwoksydacyjne działanie H_2S oraz CO jest zależne od stężenia i dynamiki uwalniania tych molekuł w tkance. Ze względu na obecność potencjalnie szkodliwych metali (np. rutenu w strukturze CORM-2), czy też szybki wyrzut H_2S w przypadku NaHS, powyżej wymienione związki nie dawały realnych szans na implementację tego typu rozwiązań w warunkach klinicznych oraz dalszy rozwój stopnia zaawansowania badań podstawowych w dziedzinie nauk medycznych. Dlatego też, z biegiem lat opracowywano i syntetyzowano kolejne donory H_2S oraz CO, o różnych strukturach i właściwościach chemicznych. Szczególnie istotne było uzyskanie kontrolowanego i powolnego uwalniania H_2S lub CO. Jednym z pierwszych takich donorów H_2S był związek GYY4137, którego uprzednia aplikacja m.in. zmniejszała uszkodzenia błony śluzowej żołądka wywołane ischemią z reperfuzją w modelu eksperymentalnym. Innym syntetycznym donorem H_2S jest związek AP-39, który cechuje się zdolnością uwalniania H_2S wewnątrz mitochondrium. Donor ten wykazuje m.in. działanie kardioprotekcyjne w warunkach laboratoryjnych. Niewątpliwie kamieniem milowym w badaniach nad donorami H_2S było zsyntetyzowanie pochodnych NLPZ, posiadających w swej strukturze komponentę uwalniającą H_2S, takich jak diklofenak i kwas acetylosalicylowy (ASA) sprzężone z desmetyloanetolotritionem (odpowiednio, ATB-337 i ACS14) lub naproksen sprzężony z 4-hydroksytiobenzamidem (ATB-346). ATB-346 w badaniach przedklinicznych cechował się znacząco zredukowaną gastrotoksycznością będącą głównym efektem ubocznym farmakoterapii NLPZ i obiecująco przechodzi II fazę badań klinicznych. Z tego względu, udoskonalanie i wdrażanie nowych bezpiecznych proleków uwalniających CO i H_2S w celu uzyskania efektu ochronnego i terapeutycznego jest szczególnie istotne dla dalszego rozwoju nauk medycznych, w tym szczególnie fizjologii i farmakologii przewodu pokarmowego na poziomie poznawczym i translacyjnym.

Dlatego też, celem niniejszej pracy było określenie molekularnego mechanizmu działania nowej grupy proleków uwalniających CO lub H_2S w utrzymaniu fizjologicznej integralności błony śluzowej żołądka warunkującej redukcję gastrotoksyczności indukowanej aplikacją ASA jako leku z grupy NLPZ lub porównawczo nekrotyzującego czynnika chemicznego, jakim jest etanol. Badania przeprowadzono w ramach współpracy z Prof. Binghe Wang'iem z Georgia State University w Atlancie (USA), który opracował nową klasę związków uwalniających gazowe mediatory, w tym donor CO o akronimie BW-CO-111 oraz donor H_2S o akronimie BW-HS-101. BW-CO-111 nie posiada w swojej strukturze metalu i charakteryzuje się samoistnym wyrzutem CO w roztworze wodnym, z czasem półtrwania wynoszącym około 12 min., natomiast BW-HS-101 cechuje się uwalnianiem H_2S indukowanym enzymatycznie

pod wpływem esteraz, z czasem półtrwania wynoszącym około 13 min.

Materiał biologiczny do analizy mikroskopowej oraz do badań biochemicznych i molekularnych pozyskano w ramach eksperymentów *in vivo*, które przeprowadzono na szczurach rasy Wistar. Zwierzętom zaaplikowano drogą dożołądkową (i.g.):

i) rozcieńczony dimetylosulfotlenek (DMSO) z wodą (w stosunku 1:9) jako placebo,

ii) BW-CO-111 (0.02-5 mg/kg),

iii) BW-CP-111 (0.1 mg/kg) jako kontrola strukturalna dla BW-CO-111, która nie uwalnia CO,

iv) BW-HS-101 (0.5-50 μmol/kg),

v) BW-iHS-101 (50 μmol/kg) jako kontrola strukturalna dla BW-HS-101, która nie uwalnia H_2S,

vi) nieaktywny metabolit BW-HS-101/BW-iHS-101, określony roboczo jako lakton (50 μmol/kg).

Analizę mikroskopową, biochemiczną i molekularną przeprowadzono również w grupach, które były traktowane donorem H_2S w kombinacji z inhibitorem syntazy NO (NOS), NG-nitro-L-argininą (L-NNA, 20 mg/kg dootrzewnowo (i.p.)), oraz inhibitorem HMOX, protoporfiryną cynkową IX (ZnPP, 10 mg/kg i.p.) w celu oceny interakcji BW-HS-101 ze szlakiem biosyntezy endogennego NO oraz CO.

Po podaniu ww. związków, zwierzęta otrzymały standardowo ASA (125 mg/kg i.g.) lub 75% etanol (i.g.) w celu wywołania modelowych uszkodzeń błony śluzowej żołądka. Następnie, po wprowadzeniu zwierząt w stan znieczulenia ogólnego dokonano pomiaru poziomu żołądkowego przepływu krwi (z j. ang. *gastric blood flow*, GBF) z użyciem przepływomierza laserowego, pobrano krew (surowica) z żyły próżnej dolnej. Po wypreparowywaniu żołądka i ocenie makroskopowej powierzchni modelowych uszkodzeń pobrano bioptaty do badań histopatologicznych oraz do dalszych analiz biochemicznych i molekularnych.

Odpowiednio utrwalone i wybarwione preparaty błony śluzowej żołądka poddano ocenie mikroskopowej z zastosowaniem odpowiedniej skali stopnia uszkodzeń modelowych.

W pobranych próbkach błony śluzowej żołądka oznaczono:

1) ekspresję mRNA dla HMOX-1, HMOX-2, cyklooksygenazy (COX)-1 oraz COX-2, indukowalnej NOS (iNOS), aneksyny-A1 (ANXA1), transformującego czynnika wzrostu (TGF)-β1, receptora dla TGF-β (TGFBR)1, TGFBR2, TGFBR3, interleukiny (IL)-1β, supresora sygnalizacji cytokin 3 (SOCS3), receptora dla IL-1 (IL1-R)1, IL1-R2, receptora dla czynnika martwicy nowotworów (TNF-R)2 oraz podjednostki A kompleksu dehydrogenazy

bursztynianowej i β-aktyny (ACTB) jako genów referencyjnych metodą *real-time* PCR,

2) ekspresję na poziomie białka dla HMOX-1, HMOX-2, COX-1, COX-2 oraz czynnika jądrowego NRF-2, a także ACTB i dehydrogenazy aldehydu 3-fosfoglicerynowego (GAPDH) jako białek referencyjnych metodą Western Blot,

3) stężenia odpowiednich biomarkerów pro- i przeciwzapalnych, takich jak IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, TNF-α, nabłonkowy czynnik wzrostu (EGF), czynnik stymulujący tworzenie kolonii granulocytów i makrofagów (GM-CSF), czynnik wzrostu śródbłonka naczyniowego (VEGF) techniką multipleksowania z użyciem platformy Luminex,

4) poziom 8-hydroksydeoksyguanozyny (8-OHdG) oraz poziom prostaglandyny (PG)E_2 metodą immunoenzymatyczną (ELISA),

5) poziom endogennego CO - metodą chromatografii gazowej z metanizerem i detektorem płomieniowo-jonizacyjnym (GC-O/FID).

Ponadto, w surowicy oznaczono stężenia tych biomarkerów pro- i przeciwzapalnych na poziomie systemowym, takich jak IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF-α, interferon (IFN)-γ, GM-CSF, VEGF techniką multipleksowania z odczytem fluorescencji.

Dokonano także pomiaru uwalniania CO ze związku BW-CO-111 *in vitro* w obecności soku żołądkowego metodą GC-O/FID oraz przeprowadzono konwersję chemiczną związku BW-HS-101/BW-iHS-101 celem uzyskania metabolitu tych związków jakim jest lakton do dalszej implementacji w modelach eksperymentalnych.

Zaobserwowano, że nowe donory CO i H_2S, tj. odpowiednio BW-CO-111 oraz BW-HS-101 zależnie od dawki hamowały rozwój uszkodzeń błony śluzowej żołądka indukowanych aplikacją ASA lub 75% etanolu, czemu towarzyszył statystycznie znamienny wzrost GBF. Dawki 0.1 mg/kg dla BW-CO-111 oraz 50 μmol/kg dla BW-HS-101 redukowały poziom uszkodzeń na poziomie mikro i makroskopowym o co najmniej 50%, dlatego też zostały wybrane do dalszych analiz. BW-CP-111 oraz lakton nie wykazały działania gastroprotekcyjnego oraz nie wpływały na analizowane szlaki molekularne. Co ciekawe, w pierwotnym założeniu nieaktywny analog BW-iHS-101, również wykazywał na poziomie mikroskopowym działanie ochronne, podobnie jak BW-HS-101, co może mieć związek ze zbliżoną strukturą chemiczną tych substancji. Jednak mechanizm molekularny działania BW-iHS-101 oraz BW-HS-101 był odmienny.

Aplikacja BW-CO-111 (0.1 mg/kg) zwiększyła biodostępność CO w błonie śluzowej żołądka. Gastroprotekcji BW-CO-111, względem wybranych uszkodzeń modelowych, towarzyszyło utrzymanie indukowanego aplikacją ASA wzrostu ekspresji mRNA dla HMOX-1 z jednoczesnym spadkiem ekspresji dla prozapalnych iNOS oraz COX-2 w błonie

śluzowej żołądka oraz utrzymanie wzrostu poziomu białka dla TGF-β na poziomie systemowym. Wysokie dawki ASA obniżały ekspresję mRNA dla przeciwzapalnej ANXA1 czego nie zaobserwowano po aplikacji BW-CO-111. Ponadto, BW-CO-111, obniżył również stężenia jedenastu biomarkerów zapalnych w surowicy (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF-α, IFN-γ i GM-CSF) w modelu etanolowym. Ochronnemu działaniu BW-CO-111 względem uszkodzeń indukowanych 75% etanolem towarzyszył wzrost stężenia gastroprotekcyjnej PGE_2 w błonie śluzowej żołądka.

Gastroprotekcyjnemu działaniu BW-HS-101 towarzyszył spadek poziomu stężenia markera oksydacji DNA, 8-OHdG w błonie śluzowej żołądka. Związki BW-HS-101 i BW-iHS-101 nie miały wpływu na wytwarzanie PGE_2 w błonie śluzowej żołądka, ale wykazywały działanie przeciwzapalne na poziomie ogólnoustrojowym obserwowane jako spadek stężenia w surowicy TNF-α i VEGFA. Co istotne, uwalniający H_2S związek BW-HS-101, ale nie BW-iHS-101 wpływał na wzrost ekspresji mRNA dla przeciwzapalnego SOCS3 i HMOX-1 oraz efektywniej redukował stężenie markerów zapalnych w błonie śluzowej żołądka z gastropatią indukowaną aplikacją ASA. Farmakologiczne zahamowanie aktywności biosyntezy NO przez aplikację L-NNA znacząco obniżało działanie ochronne BW-HS-101, czemu również towarzyszył spadek GBF.

Podsumowując, przeprowadzone badania wykazały, że H_2S oraz CO uwalniane z nowych donorów, takich jak odpowiednio BW-HS-101 oraz BW-CO-111 po aplikacji dożołądkowej wzmacniają fizjologiczną barierę ochronną błony śluzowej górnego odcinka przewodu pokarmowego. Tym samym, związki te wykazują działanie gastroprotekcyjne względem ostrych uszkodzeń błony śluzowej żołądka wynikających z bezpośredniej ekspozycji na czynnik chemiczny jakim jest etanol oraz redukują gastrotoksyczność indukowaną aplikacją ASA jako modelowego leku z grupy NLPZ. Molekularny mechanizm działania gastroprotekcyjnego H_2S uwalnianego z BW-HS-101 jest zależny od biosyntezy endogennego NO oraz modulacji aktywności przeciwzapalnego szlaku SOCS3, przy możliwym udziale ścieżki CO/HMOX-1. Molekularny mechanizm gastroprotekcyjnego działania BW-CO-111 może być mediowany przez aktywność szlaku TGF-β oraz ANXA1. Ochronne działanie CO uwalnianego z BW-CO-111 na śluzówkę żołądka wynika częściowo z utrzymania poziomu ochronnej PGE_2. Zarówno BW-HS-101 jak i BW-CO-111 wykazały działanie przeciwzapalne na poziomie systemowym jak i bezpośrednio w obrębie śluzówki żołądka.

SŁOWA KLUCZOWE: BW-CO-111, BW-HS-101, gazowe mediatory, gastrotoksyczność polekowa, bariera śluzówkowa żołądka

3. STRESZCZENIE W JĘZYKU ANGIELSKIM (ABSTRACT)

A growing body of scientific evidence showed that carbon monoxide (CO) and hydrogen sulfide (H_2S) are important gaseous mediators produced and released endogenously in mammalian tissues. Both molecules exert multidirectional biological activity and participate in many physiological and pathological processes. Therefore, these molecules play an important role in the organisms, similarly to the third sibling gaseous mediator, nitric oxide (NO). It has been shown that CO and H_2S can protect gastrointestinal (GI) mucosa by modulation of inflammatory response and several processes at the molecular and functional levels. The enzymatic pathways involved in the endogenous biosynthesis of these gaseous mediators are key components of the physiological protective barrier of the GI mucosa taking part in the maintenance of its integrity during exposure to acute stress, ischemia/reperfusion or intragastric (i.g.) application of chemical agents such as ethanol and drugs. It should be noted that the beneficial bioactivity of CO and H_2S is not limited to the GI tract since it was also observed within cardiovascular system, where these molecules exert vasodilatory effect or inhibit the aggregation of platelets and adherence of leukocytes to the endothelium, as well as within central nervous system where they act as neuromodulator, antioxidant and cytoprotective factors.

CO biosynthesis in mammalian tissues is based on oxidative degradation of heme, catalyzed by heme oxygenases (HMOX), mainly inflammation-sensitive and inducible HMOX-1 as well as constitutive HMOX-2. CO is one of the heme degradation products, next to biliverdin and Fe^{2+} ions. Whereas H_2S is generated endogenously mainly as a result of L-cysteine metabolism by enzymatic activity of cystathionine γ-lyase (CTH) and cystathionine β-synthase (CBS), with pyridoxal-5-phosphate (vitamin B6) as a cofactor. H_2S is additionally formed from 3-mercaptopyruvate, due to the enzymatic activity of 3-mercaptopyruvate sulfur transferase (MPST) and can also be generated through the metabolism of exogenous compounds e.g., from polysulfides derived from garlic and food or from sulphates by the activity of colonic microbiota. Considering the important contribution of these molecules in physiology and pathophysiology, many CO- or H_2S-releasing pharmacological donors have been identified or developed in recent years.

The route of administration of a pharmacological agent is an important factor affecting its biological activity and further biodistribution. Previously published data were based on the inhalational CO application. Nevertheless, it was noted that CO has a high affinity to transition metals and ability to form complexes with them, namely carbonyls. These compounds attracted

attention as potential CO donors. McKendrick and Snodgrass were focused on the one of the first compounds from this class - nickel tetracarbonyl ($Ni(CO)_4$). However, this compound was shown to be highly toxic. Furthermore, Motterlini *et al.* developed alternative group of metal-based CO-releasing compounds (Carbon Monoxide Releasing Molecules, CORM), containing ruthenium (Ru), manganese, molybdenum, and iron in their structure. These CO-donors were shown to have sufficiently low toxicity to be implemented as pharmacological tools in the research area involving *in vitro* cellular and *in vivo* animal models. One of these compounds is tricarbonyldichlororuthenium (II) dimer (CORM-2) which was reported e.g., to accelerate the healing of chronic gastric ulcers. However, CORM-2 contains in its structure potentially cytotoxic Ru which tends to bind to sulfhydryl groups in proteins. Therefore, despite the preventive and therapeutic potential of CORM-2 demonstrated experimentally within GI mucosa and within other systems, the possible clinical implementation of this compound seems to be limited.

On the other hand, under experimental conditions, various H_2S donors were used over the years, including for example, sodium hydrosulfide (NaHS). This inorganic salt was shown to decrease gastrotoxicity induced by non-steroidal anti-inflammatory drugs (NSAIDs), to increase the secretion of bicarbonate ions (HCO_3^-) in the duodenum, and to enhance the maintenance GI mucosal integrity in animal studies. Another simple H_2S liberating compound of natural origin is the diallyl disulfide (DADS) derived from garlic. Among others, DADS was reported to inhibit the proliferation of HT29 human colon cancer cells. Additionally, L-cysteine as the precursor of H_2S biosynthesis and Lawesson's reagent were used to verify the biological effect of this gaseous mediator. Among many reports, it has been observed that intragastric (i.g.) application of these compounds accelerated the healing of chronic gastric ulcers.

At the functional level, it has been shown that CO and H_2S released from chemical donors affect gastric microcirculation, GI motility and secretion. The molecular mechanism of action of these molecules within gastric mucosa involves the modulation of cellular pathways such as nuclear factors NRF-2, NF-κB or the hypoxia-inducible factor (HIF)-1α.

Based on previously published data, anti-inflammatory and anti-oxidative activity of H_2S and CO depends on the concentration and the release dynamics of these molecules in the tissue. Due to the presence of potentially cytotoxic metals (e.g., Ru in CORM-2 structure), or the rapid release of H_2S from NaHS, the above-mentioned compounds do not seem to be further implemented in clinical pharmacology and to significantly increase the advancement of basic research in the field of medical sciences. Therefore, over the recent years, a variety of H_2S and CO donors with different structures and chemical properties were developed and synthesized.

It was particularly important to obtain a pharmacological tool with controlled and slow release of H_2S or CO. One of the first H_2S slow-releasing donors was GYY4137. This compound was reported to reduce ischemia/reperfusion-induced gastric mucosal damage in experimental model. Another synthetic H_2S donor, AP-39 is capable to target mitochondria. This compound was shown to exert e.g., cardioprotective activity. Importantly, a milestone in the research on the role of H_2S and its donors in biomedicine was the development of novel derivatives of NSAIDs with an H_2S-releasing moiety in their structure. This includes e.g., diclofenac and acetylsalicylic acid (ASA) coupled with desmethylanethol trithione (ATB-337 and ACS14, respectively) or naproxen coupled with 4-hydroxytibenzamide (ATB-346). In preclinical studies, ATB-346 was shown to have significantly reduced gastrotoxicity being the main adverse effect of NSAIDs pharmacotherapy. Moreover, ATB-346 passed Phase II clinical trials. Thus, the development and implementation of novel, safe CO and H_2S releasing prodrugs as a protective and therapeutic solutions is important for the further development of medical sciences, including particularly GI physiology and pharmacology at the cognitive and translational level.

Therefore, the aim of this study was to evaluate the molecular mechanism of action of a novel group of CO- or H_2S-releasing prodrugs in the maintenance of the physiological gastric mucosal integrity determining the reduction of gastrotoxicity induced by i.g. administration of acetylsalicylic acid (ASA) as a representative NSAID or comparatively, by ethanol as necrotizing chemical agent. This study was performed in collaboration with Prof. Binghe Wang from Georgia State University in Atlanta (USA), who developed a new class of organic gaseous mediators-releasing prodrugs, including CO donor with the acronym BW-CO-111 and H_2S donor with the acronym BW-HS-101. Importantly, BW-CO-111 does not contain metal in its structure and is characterized by a spontaneous CO release in aqueous solution with a half-life of approximately 12 minutes. BW-HS-101 is characterized by an enzymatically induced H_2S release by esterase activity, with a half-life of approximately 13 minutes.

Biological samples for microscopic evaluation as well as for biochemical and molecular analysis was collected within *in vivo* experiments on male Wistar rats. The animals were administered i.g. with following chemicals:

i) dimethyl sulfoxide (DMSO) and water (1:9 ratio) as placebo,

ii) BW-CO-111 (0.02-5 mg/kg),

iii) BW-CP-111 (0.1 mg/kg) as a structural control for BW-CO-111 without ability to release CO,

iv) BW-HS-101 (0.5-50 μmol/kg),

v) BW-iHS-101 (50 μmol/kg) as a structural control for BW-HS-101 without ability to release H_2S,

vi) inactive metabolite of BW-HS-101/BW-iHS-101, operatively designated as a lactone (50 μmol/kg).

Microscopic, biochemical, and molecular analyses were also performed within the groups that were treated with the above-mentioned H_2S donor applied in combination with the NO synthase inhibitor (NOS), NG-nitro-L-arginine (L-NNA, 20 mg/kg intraperitoneally (i.p.)), and the inhibitor of HMOX, zinc Protoporphyrin IX (ZnPP, 10 mg/kg i.p.) to evaluate the interaction of BW-HS-101 with the endogenous NO and CO biosynthesis pathways.

After treatments, animals were administered with ASA (125 mg/kg i.g.) or 75% ethanol (i.g.) to induce experimental model of gastric mucosal damage. Next, under general anaesthesia, the level of gastric blood flow (GBF) was measured using a laser flowmeter, blood (serum) samples were collected from *vena cava*. Stomach was isolated and macroscopic evaluation of gastric mucosal lesions was performed. Gastric tissue samples were collected for histopathological examination and further biochemical and molecular analyses.

Appropriately fixed and stained gastric tissue sections were assessed microscopically based on the scale evaluating the degree of gastric damage.

The following molecular analyses were performed based on gastric mucosal biopsies:

1) mRNA expression for HMOX-1, HMOX-2, cyclooxygenase (COX)-1 and COX-2, inducible NOS (iNOS), annexin-A1 (ANXA1), transforming growth factor (TGF)-β1, TGF-receptor β (TGFBR)1, TGFBR2, TGFBR3, interleukin (IL)-1β, suppressor of cytokine signalling 3 (SOCS3), IL-1 receptor (IL1-R)1, IL1-R2, tumor necrosis factor receptor (TNF-R)2 and the succinate dehydrogenase complex flavoprotein subunit A (SDHA) and β-actin (ACTB) as reference genes by real-time PCR,

2) protein level for HMOX-1, HMOX-2, COX-1, COX-2, and nuclear factor NRF-2, as well as ACTB and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference proteins by Western Blot,

3) concentrations of appropriate pro- and anti-inflammatory biomarkers, such as IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, TNF-α, epithelial growth factor (EGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF) by Luminex multiplex microbeads fluorescent assay,

4) the level of 8-hydroxydeoxyguanosine (8-OHdG) and the level of prostaglandin (PG)E_2 by enzyme-linked immunosorbent assay (ELISA),

5) endogenous CO level by gas chromatography with methanizer and flame ionization detector (GC-O/FID).

In addition, serum concentrations of pro-and anti-inflammatory biomarkers, such as IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF-α, interferon (IFN) -γ, GM-CSF, VEGF were measured at the systemic level by multiplex microbeads fluorescent assay.

Measurement of CO release from BW-CO-111 *in vitro* in the presence of stimulated gastric fluid was performed by GC-O/FID. Chemical conversion of BW-HS-101/BW-iHS-101 was also performed *in vitro* to obtain a metabolite of these compounds (lactone), for further implementation in experimental models.

Novel CO and H_2S prodrugs, BW-CO-111 and BW-HS-101, respectively, dose-dependently inhibited the development of gastric mucosal damage induced by the i.g. administration of ASA or 75% ethanol. This effect was accompanied by increased GBF level. The doses of 0.1 mg/kg for BW-CO-111 and 50 μmol/kg for BW-HS-101 reduced the level of macro- and/or microscopic gastric damage by at least 50%. Therefore, these doses were selected for further analyses. BW-CP-111 and lactone did not show any gastroprotective effect and did not affect selected molecular pathways. Surprisingly, the chemical analogue without ability to release H_2S, BW-iHS-101 also showed a protective effect at the microscopic level, in similar extent to BW-HS-101. This effect may be related to the similar chemical structure of these substances. However, the molecular mechanism of action of BW-iHS-101 and BW-HS-101 was different.

Administration of BW-CO-111 (0.1 mg/kg) increased the bioavailability of CO in gastric mucosa. BW-CO-111-mediated gastroprotection against chemically induced lesions was accompanied by the maintenance of ASA-induced increase in gastric mucosal mRNA expression for anti-inflammatory HMOX-1 with a simultaneous decrease in the expression of pro-inflammatory iNOS and COX-2 and maintenance of the increased systemic TGF-β protein level. ASA downregulated mRNA expression for the anti-inflammatory ANXA1, which was not observed after pre-treatment with BW-CO-111. Moreover, BW-CO-111 also decreased serum content of eleven inflammatory biomarkers (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF-α, IFN-γ and GM-CSF) in samples of rats exposed to ethanol. Preventive effect of BW-CO-111 against the GI damage induced by 75% ethanol was accompanied by an increase in cytoprotective PGE_2 gastric mucosal concentration.

BW-HS-101 gastroprotection was accompanied by a decrease in the gastric mucosal concentration of the DNA oxidation marker, 8-OHdG. Both, BW-HS-101 and BW-iHS-101 had no effect on the gastric mucosal production of PGE_2 but exerted anti-inflammatory effect

at the systemic level reflected by decreased TNF-α and VEGFA serum concentrations. Importantly, the H_2S-releasing BW-HS-101 but not BW-iHS-101, increased the mRNA expression for anti-inflammatory SOCS3 and HMOX-1 and more effectively reduced the concentration of inflammatory markers within the gastric mucosa with ASA-induced gastropathy. Pharmacological inhibition of NO biosynthesis pathway activity significantly decreased GBF and the protective effect of BW-HS-101.

In conclusion, this study reported that H_2S and CO released from novel organic prodrugs, such as BW-HS-101 and BW-CO-111, respectively, after i.g. administration enhance the physiological protective mucosal barrier within upper GI tract. Thus, these gaseous mediators-releasing compounds exert gastroprotective effects against acute gastric mucosal damage induced by direct exposure to ethanol as the chemical agent and importantly to reduce gastric toxicity induced by the administration of ASA, as a representative drug from the NSAIDs group. The molecular mechanism of the gastroprotective action of BW-HS-101-derived H_2S is dependent on the biosynthesis of endogenous NO and the modulation of the activity of the anti-inflammatory SOCS3 signalling, possibly mediated at least in part by the CO/HMOX-1 pathway. The molecular mechanism of BW-CO-111-mediated gastroprotection may involve the co-activity of the TGF-β and ANXA1 pathways. The protective effect of BW-CO-111-derived CO on the gastric mucosa in part due to the maintenance of protective PGE_2 level. Both prodrugs, BW-HS-101 and BW-CO-111 showed anti-inflammatory effects at the systemic level and directly within the gastric mucosa.

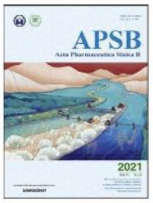
KEY-WORDS: BW-CO-111, BW-HS-101, gaseous mediators, drug-induced gastric toxicity, gastric mucosal barrier

Acta Pharmaceutica Sinica B 2021;11(2):456–475

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com



ORIGINAL ARTICLE

Organic carbon monoxide prodrug, BW-CO-111, in protection against chemically-induced gastric mucosal damage

Dominik Bakalarz[a,b], Marcin Surmiak[a,c], Xiaoxiao Yang[d], Dagmara Wójcik[a], Edyta Korbut[a], Zbigniew Śliwowski[a], Grzegorz Ginter[a], Grzegorz Buszewicz[e], Tomasz Brzozowski[a], Jakub Cieszkowski[a], Urszula Głowacka[a], Katarzyna Magierowska[a], Zhixiang Pan[d], Binghe Wang[d,*], Marcin Magierowski[a,*]

[a]*Department of Physiology, Jagiellonian University Medical College, Cracow 31-531, Poland*
[b]*Department of Forensic Toxicology, Institute of Forensic Research, Cracow 31-033, Poland*
[c]*Department of Internal Medicine, Jagiellonian University Medical College, Cracow 31-531, Poland*
[d]*Department of Chemistry and Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA 30303, USA*
[e]*Department of Forensic Medicine, Medical University of Lublin, Lublin 20-093, Poland*

Received 31 March 2020; received in revised form 18 May 2020; accepted 17 July 2020

KEY WORDS

Gastroprotection;
Carbon monoxide;
Prodrug;
Anti-inflammation;
NSAID;
Ethanol;
Gastric mucosal damage

Abstract Metal-based carbon monoxide (CO)-releasing molecules have been shown to exert anti-inflammatory and anti-oxidative properties maintaining gastric mucosal integrity. We are interested in further development of metal-free CO-based therapeutics for oral administration. Thus, we examine the protective effect of representative CO prodrug, BW-CO-111, in rat models of gastric damage induced by necrotic ethanol or aspirin, a representative non-steroidal anti-inflammatory drug. Treatment effectiveness was assessed by measuring the microscopic/macroscopic gastric damage area and gastric blood flow by laser flowmetry. Gastric mucosal mRNA and/or protein expressions of HMOX1, HMOX2, nuclear factor erythroid 2-related factor 2, COX1, COX2, *iNos*, *Anxa1* and serum contents of TGFB1, TGFB2, IL1B, IL2, IL4, IL5, IL6, IL10, IL12, tumor necrosis factor α, interferon γ, and GM-CSF were determined. CO content in gastric mucosa was assessed by gas chromatography. Pretreatment with BW-CO-111 (0.1 mg/kg, i.g.) increased gastric mucosal content of CO and reduced gastric lesions area in both models followed by increased GBF. These protective effects of the CO prodrug were supported by changes in

*Corresponding authors.
E-mail addresses: wang@gsu.edu (Binghe Wang), m.magierowski@uj.edu.pl (Marcin Magierowski).
Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2020.08.005

expressions of molecular biomarkers. However, because the pathomechanisms of gastric damage differ between topical administration of ethanol and aspirin, the possible protective and anti-inflammatory mechanisms of BW-CO-111 may be somewhat different in these models.

1. Introduction

Carbon monoxide (CO) is an endogenous signaling molecule produced by heme oxygenase (HMOX)-mediated heme degradation and has been shown to exhibit cytoprotective effects among others[1]. Taking into account its molecular activity and ability to modulate a variety of intracellular pathways under physiological and pathological conditions, CO is a sibling gasotransmitter to endogenously produced hydrogen sulfide (H_2S) and nitric oxide (NO)[2]. CO has been shown to modulate synaptic plasticity and tissue regeneration within central nervous system and also to affect gastric mucosal integrity and to modulate duodenal secretion within gastrointestinal (GI) tract[2–5]. Morevoer, CO producing HMOX1 is known to be induced by oxidation and inflammation and is considered as a part of self-defense feedback mechanism activated within particular tissues in response to the exposure to various stressors[6–8]. Importantly, CO has a relatively high toxicity threshold reaching up to 10% of carboxyhemoglobin (COHb) concentration in blood[9]. Therefore, the last two decades have seen much effort in developing CO as a therapeutic agent[10–13] for treating inflammatory conditions and in offering cytoprotection in various organs including kidney[14–18], heart[19–21], liver[22–24], brain[25–27] and GI[2,28–38] among others[10,11]. Along this line, the issue of route of administration is a critical pharmaceutical factor. Earlier work was largely focused on using inhaled CO gas as the choice of administration. However, for a broad range of potential applications, it is desirable to develop non-inhalation forms of delivery. CO is known to have high affinity for a range of transition metals; thus metal-immobilized carbonyls attracted much attention for the experimental delivery of CO. As early as in 1891, McKendrick and Snodgrass[39] experimented nickel tetracarbonyl and found it to be an antipyretic. However, nickel tetracarbonyl is highly toxic. Motterlini and others[11,12,40–43] developed alternatives as metal-based CO-releasing molecules (CO-RMs) using metals such as ruthenium, manganese, molybdenum and iron, which are sufficiently benign to allow for biological and pharmacological studies at the cellular level *in vitro* and in animal models. Aiming at improving delivery properties and minimizing metal-related toxicity, modified CO-RMs have been reported including enzyme-controlled release[44,45] and encapsulated metal-based CO-RMs[46–50], as well as photosensitive organic (metal-free) CO-RMs[51–56] and oral forms of CO in a solution[57]. Most recently, we have been working on developing metal-free organic CO prodrugs belonging to different structural classes[10,58–60] with tunable CO release rates[58,60–63]. Some of these prodrugs are capable of triggered release (pH-[62], esterase-[61,64,65], and ROS-sensitive[66]), mitochondrion-targeting[22], employing dual-triggers[60,64], and delivering more than one payload using a single prodrug[60,61,64,67].

Previously, ruthenium-based CORM-2 and CO have been studied for their gastroprotective and ulcer healing effects[30,31,33]. However, recent studies of ruthenium-based CO-RMS have led some to ask for "a major reappraisal of the biological effects of CORM-2 and related CORMs"[68] because of ruthenium's ability to undergo chemical reactions with thiols[68–70] and other molecules or proteins[71–74]. Recently, organic CO prodrugs have been shown to be effective in treating systemic inflammation, experimental colitis, chemically induced liver injury, and ischemia reperfusion kidney injury[17,22,64,75]. It is worth mentioning that endogenously produced CO due to activity of HMOX/nuclear factor erythroid 2-related factor 2 (NRF2) pathway has been shown to play beneficial roles in the self-defensive response to noxious agents[2,35,37,76,77]. Herein, we describe our effort of studying for the first time an organic CO prodrug for its gastroprotection of injuries induced by aspirin, a classic NSAID or a chemical agent known to evoke necrotizing injury to the stomach such as ethanol.

Thus, in this study we aimed to investigate for the first time if pretreatment with a novel CO prodrug, BW-CO-111[62], prevents gastric mucosa against aspirin- or necrotic, ethanol-induced gastric damage on the micro- and macroscopic levels and if this effect is accompanied by the alterations in gastric blood flow (GBF). We focused on possible effects of BW-CO-111 on modulation of inflammation by screening serum concentrations of 11 inflammatory markers and transforming growth factor β (TGFB). Possible modulation of HMOX1/HMOX2 and NRF2 pathways by this CO prodrug has also been investigated. Alterations in gastric mucosal mRNA and/or protein expression for proinflammatory inducible nitric oxide synthase (*iNos*) and anti-inflammatory annexin-A1 (*Anxa1*), TGFB1 receptors (*Tgfbr1*, *Tgfbr2*, and *Tgfbr3*), and physiological gastroprotective barrier components, prostaglandin (PG)E_2-producing enzymes, cyclooxygenase (COX1 and COX2) were also assessed.

2. Materials and methods

2.1. Experimental design

Fifty male Wistar rats with average weight of 220−300 g were used in the study. Animals were fasted for 24 h with free access to tap water before each experiment. All procedures were approved by the I Local Ethical Committee for Care and Use of Experimental Animals, held by Faculty of Pharmacy, Jagiellonian University Medical College in Cracow (Decision No.: 311/2019; Date: 17 July 2019). Experiments were run with implications for replacement, refinement or reduction (the 3Rs) principle. Animal studies are reported in compliance with the ARRIVE guidelines.

Rats were randomly assigned to the appropriate experimental groups (5 rats each) and were pretreated i.g. by orogastric tube with 1 mL of 1) dimethyl sulfoxide (DMSO)/H_2O (1:9) as vehicle, 2) CO prodrug BW-CO-111 (0.02−5 mg/kg) or 3) BW-CP-111, the product after CO release from BW-CO-111, applied at a dose of

0.1 mg/kg, which is the equivalent of the effective dose of BW-CO-111 capable of reducing ethanol-induced injury area by more than 50%[62]. After 30 min, animals were administered i.g. with 1 mL of 75% ethanol or 1.5 mL of ASA (125 mg/kg dissolved in 0.2 mol/L HCl), based on previously implemented and described experimental models of drugs-induced or necrotic gastric mucosal injuries[35,78]. BW-CO-111 was selected due to its half-live for CO release[62]. In a separate series of experiments, animals were pretreated i.g. with vehicle or BW-CO-111 (0.1 mg/kg) and 30 min later gastric mucosal biopsies were collected for determination of gastric mucosal CO content as described below.

All compounds and chemicals were purchased from Sigma–Aldrich (Schnelldorf, Germany) unless otherwise stated. BW-CO-111 and BW-CP-111 were synthesized following procedures described previously[62].

2.2. *GBF determination, macro- and microscopic gastric damage assessment and sample collection*

One hour after administration of ethanol or aspirin (ASA), under isoflurane anesthesia, the abdomen was opened for the GBF measurement by laser flowmetry, as described previously[38]. Briefly, the GBF was determined in the oxyntic part of the gastric mucosa not involving ethanol- or aspirin-induced mucosal damage using laser flowmeter (Laserflo, model BPM 403A, Blood Perfusion Monitor, Vasamedics, St. Paul, MN, USA). Average values of three measurements were expressed in mL/min per 100 g of gastric tissue. Blood samples were collected from the *vena cava* and separated serum was stored at −80 °C until further analysis[38,79]. Next, the stomach was excised, opened along the greater curvature and the area of gastric damage was determined planimetrically and expressed in mm^2 [35]. Gastric mucosal samples were scraped off on ice, snap-frozen in liquid nitrogen and stored at −80 °C until further analysis[35]. Gastric mucosal biopsies were collected as described previously for determination of CO content[80].

For microscopic analysis, the gastric tissue sections were excised and fixed in 10% buffered formalin, pH = 7.4. Samples were dehydrated by passing them through a series of alcohols with incremental concentrations, equilibrated in xylene for 10–15 min and embedded in paraffin; paraffin blocks were cut into about 4 μm sections using a microtome. The prepared specimens were stained with haematoxylin/eosin (H&E). Tissue slides were evaluated using a light microscope (AxioVert A1, Carl Zeiss, Oberkochen, Germany)[80]. Digital documentation of histological slides was obtained using above mentioned microscope and ZEN Pro 2.3 software (Carl Zeiss, Oberkochen, Germany)[80].

2.3. *Determination of gastric mucosal mRNA fold changes by real-time PCR*

Gastric mucosal mRNA expression fold change for *Hmox1*, *Hmox2*, *iNos*, *Cox1*, *Cox2*, *Anxa1* and *Tgfb1* was assessed by real time PCR, as described previously[80]. Briefly, total RNA was isolated using commercially available kit with spin-columns (GeneMATRIX-Universal RNA Purification Kit, EURx, Gdansk, Poland) according to manufacturer's protocols. Reversed transcription (RT) was performed using PrimeScript™RTMasterMix (Perfect Real Time Takara Bio Inc., Kyoto, Japan). RNA concentration was measured using Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). For each RT reaction, total RNA concentration was adjusted to (1 μg) per sample. Samples from healthy (intact) gastric mucosa were further used as reference control during calculations. Expression of mRNA for *Hmox1*, *Hmox2*, *iNos*, *Cox1*, *Cox2*, *Anxa1*, *Il1b* and succinate dehydrogenase complex, subunit A (*Sdha*) and β-actin (*Actb*) as reference genes was determined using specific primers[35,80]. To determine *Tgfbr1*, 5′-ACTCCCAACTACAGAAAAGCA-3′ forward and 5′-AAGGGCGATCTAGTGAGGGA-3′ reverse primers were used. To determine *Tgfbr2*, 5′-CCCCCGTTTGGTTCCAGAGT-3′ forward and 5′-CGGTCTCTCAGCACGTTGTC-3′ reverse primers were used. To determine *Tgfbr3*, 5′-GCTCCCAACAGTATCGG CTT-3′ forward and 5′-GCCTGTAGCCATTGTCCAGT-3′ reverse primers were used.

PCR reaction was run using thermal cycler Quant Studio 3 (Thermo Fisher Scientific) and SYBR Green I dye including kit [SG qPCR Master Mix (2 ×), EURx]. To maintain the same PCR reaction efficiency in all analyzed samples, the same amount of cDNA per each well was used. After reaction, melting curve for each sample, its technical replicates and for appropriate negative control were analyzed to exclude the data derived from potentially unintended products. Results were analyzed using the $-\Delta\Delta$Ct method[81].

2.4. *Determination of proteins expression in gastric mucosa by Western blot*

Protein expression for HMOX1, HMOX2, NRF2, COX1 and COX2 in gastric mucosa was determined using Western blot as described previously[79]. Rabbit monoclonal anti-HMOX1 (ab68477, Abcam, Cambridge, UK) in dilution of 1:1000, rabbit polyclonal anti-COX1 (13393-1-AP, Proteintech, Manchester, UK) in dilution of 1:1000, rabbit polyclonal anti-NRF2 (163936-1-AP, Proteintech) in dilution of 1:500, rabbit polyclonal anti-COX-2 (ab 15191, Abcam) in dilution of 1:1000, rabbit polyclonal anti-HMOX-2 (14817-1AP, Proteintech) in dilution of 1:1000 and rabbit monoclonal anti-GAPDH, (2118, Cell Signaling Technology, Danvers, MA, USA) in dilution of 1:2000 were used as primary antibodies. Protein expression was visualized using horseradish peroxidase-linked secondary anti-rabbit IgG antibody (7074, Cell Signaling Technology) or anti-mouse IgG antibody (7076, Cell Signaling Technology) in dilution of 1:2000 where appropriate. All primary and secondary antibodies were diluted in 5% non-fat milk, except anti-NRF2 antibody, which was diluted in 5% BSA.

Chemiluminescence was developed using WesternSure® ECL Substrate (LI-COR, Lincoln, NE, USA) or WesternBright Quantum (Advansta, Menlo Park, CA, USA) and was measured using C-DiGit® Blot Scanner (LI-COR). The intensity of bands was determined and analyzed using Image Studio 4.0 software (LI-COR). The expression of each protein of interest was determined using 5 samples per experimental group and obtained values were normalized to the expression of ACTB or GAPDH as loading controls[78,79].

2.5. *Luminex microbeads fluorescent assays*

Determination of serum concentrations of interleukin IL1B, IL2, IL4, IL5, IL6, IL10, IL12, IL13, interferon γ (IFNG), tumor necrosis factor α (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) was performed using Luminex microbeads fluorescent assay (Bio-Rad, Hercules, CA, USA) and Luminex MAGPIX system (Luminex Corp., Austin, TX, USA). Results were calculated from calibration curves and expressed in pg/mL[73].

2.6. Determination of CO content in gastric mucosa by gas chromatography (GC)

CO concentration in the gastric mucosa biopsies was determined as reported previously but using modified GC-based method described in detail elsewhere and briefly below [80].

Sample preparation: 10 mL of water were added to the tissue fragments (about 400−600 mg) and homogenized by sonication (Sonoplus, BANDELIN Electronic GmbH & Co. KG, Berlin, Germany). The volume of 2.5 mL of homogenate was pipetted into two 10 mL headspace vials (2 test samples). To obtain calibration samples, about 5 mL of the remaining volume of the homogenate was saturated with CO for 20 min (100% saturation CO). The CO used to saturate the calibration samples was obtained by reacting concentrated sulfuric acid with 80% formic acid. Unbound CO was removed by flushing with nitrogen for 3 min. Calibration solutions with CO saturation 1.25%, 2.5%, 5% and 10% were prepared from 100% saturated homogenates. 2.5 mL of each calibration solution was pipetted into headspace vials (4 calibration samples). The vials were then sealed with an aluminum cap and silicon Teflon septum. Each vial was gently flushed with helium for 30 s and then 1.5 mL of 20% potassium hexacyanoferrate was added with a syringe.

GC/O-FID (flame ionization detector)-headspace analysis: for the GC/O-FID-headspace analysis, a Thermo Trace GC Ultra (Thermo Electron Corp., Waltham, MA, USA) equipped with O-FID detector (FID with jet nickel microcatalytic methanizer) was used. The jet nickel microcatalyzer converts CO to methane at 330 °C, which increases the sensitivity of CO detection. The system was equipped with a Thermo TriPlus HS autosampler (Thermo Electron Corp.). The prepared samples were mixed and incubated at 70 °C for 8 min in autosampler agitator to the achieve complete CO liberation. 200 μL of gas-phase of each sample were injected with an autosampler gas-tight syringe (heated at 72 °C). Split/splitess injector (200 °C) with closed split was used. GC separation was performed with HP-Molesieve column (Agilent Technologies, Santa Clara, CA, USA; 30 m/0.53 mm ID/0.25 μm) at constant flow 15 mL/min of helium as a carrier gas. The temperature program consisted of the following steps: 60 °C for 2 min followed by 120 °C for 2 min achieved by a heating rate 60 °C/min.

2.7. Determination of CO release from BW-CO-111 in vitro by GC

GC was performed on an Agilent 7820 GC (Agilent Technologies) equipped with a thermal conductivity detector (TCD) and a packed molecular sieve column Carboxen 1000, 15 ft × 2.1 mm (RESTEK, Bellefonte, PA, USA); helium was used as the carrier gas. Oven temperature was programed as follows: 35 °C in 5 min, then to 225 °C at a rate of 20 °C/min, and then hold for 5.5 min. TCD detector was set at 125 °C. Calibration curve was generated by injecting a series of varying volumes of pure CO gas taken from a gas sampling bag into a 6-mL head-space vial pre-sealed with 2.8 mL phosphate buffered saline (PBS)/DMSO (1:6, *v:v*) medium, which was the same volume used in the CO release experiment from the prodrug. After incubation at 37 °C overnight, 250 μL of the head-space gas was injected to the GC. The peak area of CO was normalized with the peak area of oxygen as the internal standard (from the air in the head space). The ratio between CO and O_2 was plotted against the injected pure CO molar quantity to give the calibration curve.

2.8. Determination of CO release yield and profile of BW-CO-111 in PBS and simulated gastric fluid (SGF)

Phosphate buffered saline (PBS) was purchased from Corning (Corning, NY, USA) and SGF was made by dissolving NaCl (0.2%, *w/v*; Sigma−Aldrich, St. Louis, MO, USA) in pH 1.2 hydrochloric acid (HCl, Sigma−Aldrich) solution. DMSO was purchased form Sigma−Aldrich. About 2 mg of BW-CO-111 was used by dissolving in a mixed solvent with PBS (or SGF)/DMSO (1:6, *v:v*) to give a solution of about 1 mmol/L. Specifically, approximately 2 mg of BW-CO-111 was weighed into a 6-mL headspace vial (total volume 5.8 mL). After dissolving in 2.4 mL DMSO, 0.4 mL of PBS or SGF was added and the cap was sealed instantly. For the release yield determination, the vial was incubated at 37 °C for 6 h and 250 μL of headspace gas was injected into GC. For the release profile determination, 250 μL of headspace gas was taken from the vial at different time points (10−180 min) and injected into GC. After each sampling, 250 μL of air was injected into the vial to balance the pressure. The ratio between the calculated CO amount and the molar quantity of BW-CO-111 was used to determine the release yield and the release profile. The experiments were conducted in triplicate and the results are reported as mean ± SD.

2.9. Statistical analysis

Results were analyzed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Results are presented as mean ± SEM. Statistical analysis was conducted using Student's *t*-test or ANOVA with Dunnett's multiple comparison *post hoc* test if more than two experimental groups were compared. The group size for each experimental group was of $n = 5$ and $P < 0.05$ was considered as statistically significant.

3. Results

3.1. CO release from BW-CO-111 in vitro and CO content in gastric mucosa after pretreatment with this prodrug

Fig. 1A shows the reaction of CO release from BW-CO-111 to BW-CP-111[62]. *In vitro* CO release profile was tested with gas chromatography. CO calibration curve and the regression formula is shown in Fig. 1B and the goodness of fitting is $r^2 = 0.9994$. All data points of CO/O_2 ratio for the tested samples fell within the range of the calibration levels (0.625−9.821 μmol). As shown in Table 1 and Fig. 1C, BW-CO-111 released stoichiometric amount of CO under both acidic and neutral conditions. The release kinetics gave a half-life of 24.1 ± 4.0 min in the PBS/DMSO mixture and 17.1 ± 0.7 min in the SGF/DMSO mixture. The difference in release half-life likely reflects the solvent-sensitive nature of the controlling Diels−Alder reaction[58]. Fig. 1D shows that CO content in gastric mucosa was significantly increased 45 min after i.g. administration of BW-CO-111 (0.1 mg/kg) as compared with vehicle treated group ($P < 0.05$).

3.2. Gastroprotective effect of pretreatment with BW-CO-111 against ethanol- or aspirin-induced damage and possible modulation of gastric microcirculation

Fig. 2A shows that pretreatment with BW-CO-111 applied i.g. at a dose of 0.1 and 0.5 mg/kg, but not at a dose 0.02 or 5 mg/kg,

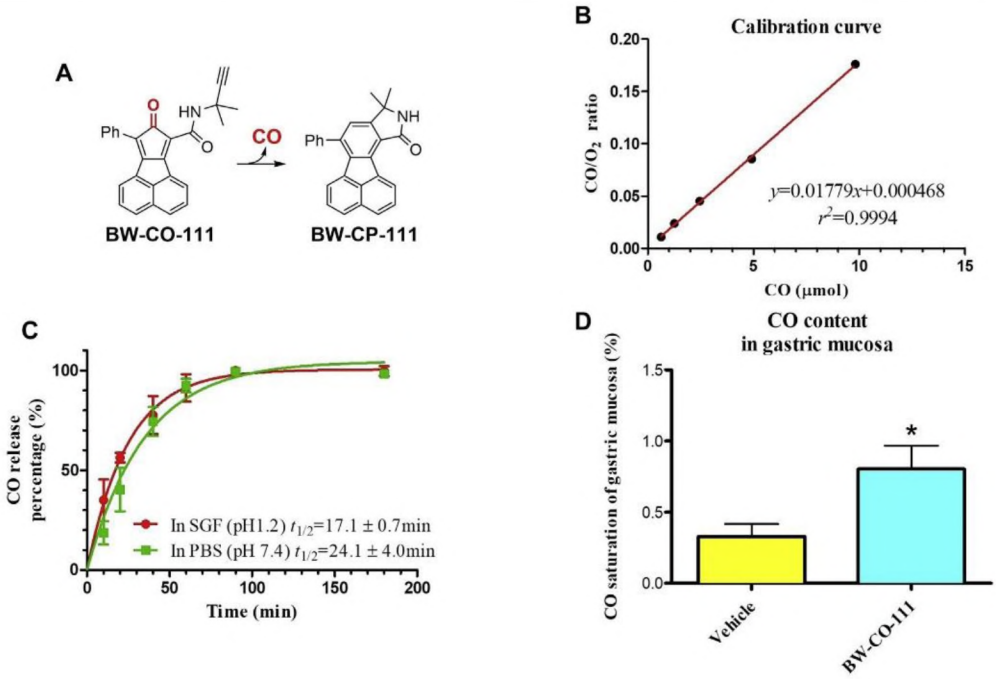


Figure 1 Chemical structures of CO prodrug BW-CO-111 and BW-CP-111 (A). Calibration curve for CO release from BW-CO-111 *in vitro* (B). Percentage of CO release *in vitro* and half-life at pH 7.4 and 1.2 for BW-CO-111 (C). Results are reported as mean ± SD. Gastric mucosal CO contents in gastric mucosa with or without pretreatment with BW-CO-111 (0.1 mg/kg, i.g.) 45 min prior to sampling (D). Results are mean ± SEM of 5 rats per group. $^*P < 0.05$ compared with intact.

Table 1 CO release yield of BW-CO-111.

Solution	Release yield (%)
SGF (pH 1.2; HCl:DMSO = 1:6)	103.0 ± 1.9
PBS (pH 7.4; buffer:DMSO = 1:6)	100.7 ± 1.7

Results are reported as mean ± SD.

significantly decreased ethanol-induced gastric damage area ($P < 0.05$). However, the dose of 0.1 mg/kg i.g. decreased gastric lesion area by more than 50% (Fig. 2A). The negative control BW-CP-111 (0.1 mg/kg, i.g.) did not significantly affect ethanol-induced gastric lesions area (Fig. 2A). Fig. 2B shows that pretreatment with BW-CO-111 (0.1 mg/kg, i.g.) significantly decreased aspirin-induced gastric lesion area ($P < 0.05$).

In rats administered with ethanol or aspirin, the GBF was significantly decreased as compared with intact gastric mucosa ($P < 0.05$, Table 2). Pretreatment with BW-CO-111 (0.1 mg/kg, i.g.) but not with BW-CP-111 (0.1 mg/kg, i.g.) significantly elevated GBF in gastric mucosa administered with ethanol and aspirin as compared with rats pretreated with vehicle ($P < 0.05$, Table 2).

Fig. 3A and B shows macroscopic and microscopic appearance of gastric injury in rats administered with 75% ethanol (A) or aspirin (B) and pretreated i.g. with vehicle or BW-CO-111 (0.1 mg/kg). In rats pretreated with vehicle, i.g. administration of 75% ethanol or aspirin (125 mg/kg) resulted in macroscopic hemorrhagic erosions and microscopic necrotic damage penetrating into gastric mucosa with notable leukocytes infiltration into submucosal layer with accompanying desquamation and necrosis of epithelium surface (Fig. 3A and B). Pretreatment with BW-CO-111 (0.1 mg/kg, i.g.) reduced the depth and surface area of the necrotic layer (Fig. 3A and B).

3.3. Alterations in Hmox1/HMOX1 and Hmox2/HMOX2 mRNA/protein expression in gastric mucosa compromised by EtOH after pretreatment with BW-CO-111

Administration of 75% ethanol significantly increased *Hmox1* but not *Hmox2*mRNA expression in gastric mucosa as compared with rats without any treatments (Fig. 4A and B, $P < 0.05$). Fig. 4A and B shows that gastric mucosal mRNA expression for *Hmox1* and *Hmox2* was not significantly affected in rats pretreated with BW-CO-111 followed by treatment with 75% ethanol 30 min later. Gastric mucosal protein expression for HMOX1, HMOX2 and NRF2 were not significantly affected in rats pretreated i.g. with BW-CO-111 (0.1 mg/kg) as compared with vehicle (Fig. 4C–E).

3.4. Modulation of gastroprotective PGE_2 content and Cox1/COX1 and Cox2/COX2 mRNA/protein expression in gastric mucosa compromised by EtOH after pretreatment with BW-CO-111

Fig. 5A and B shows that gastric mucosal mRNA expression for *Cox2* but not *Cox1* was significantly upregulated in rats administered with 75% ethanol as compared with rats without any treatments ($P < 0.05$). In rats pretreated with BW-CO-111

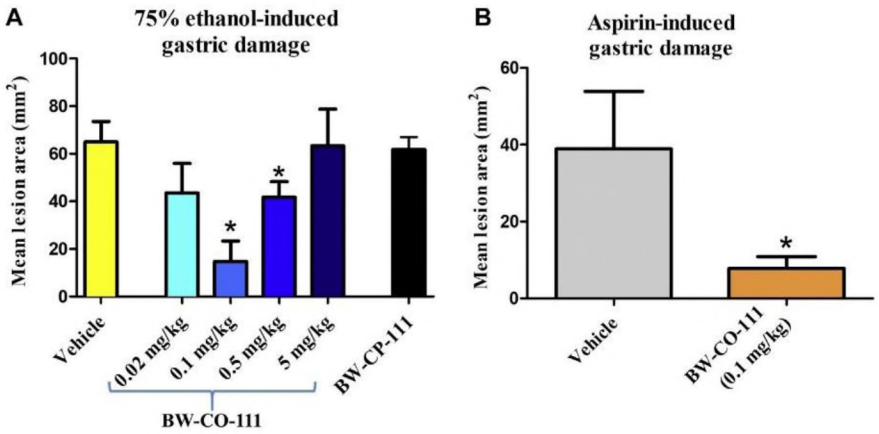


Figure 2 Ethanol- (A) and aspirin-induced (B) gastric lesion areas in rats pretreated i.g. with vehicle, CO prodrug BW-CO-111 (0.02−5 mg/kg) or BW-CP-111 (0.1 mg/kg). Results are mean ± SEM of 5 rats per group. *$P < 0.05$ compared with the vehicle-control group.

followed by ethanol administration 30 min later, the gastric mucosal *Cox1* and *Cox2* mRNA expression was not significantly affected as compared with vehicle-treated animals (Fig. 5A and B). The gastric mucosal protein expression for COX1 and COX2 was not significantly affected in rats pretreated i.g. with BW-CO-111 (0.1 mg/kg) as compared with vehicle (Fig. 5C−E). Fig. 5F shows that PGE_2 concentration in gastric mucosa was significantly decreased after exposure to ethanol as compared with intact ($P < 0.05$). Pretreatment with BW-CO-111 (0.1 mg/kg, i.g.) significantly increased PGE_2 content as compared with vehicle in animals exposed to ethanol ($P < 0.05$).

3.5. *Involvement of anti-inflammatory Anxa1 and TGFB pathway in the gastroprotective effect of pretreatment with BW-CO-111 in gastric mucosa compromised by EtOH*

Fig. 6A shows that gastric mucosal mRNA expression for *iNos* was significantly upregulated in rats treated with 75% ethanol after vehicle-pretreatment 30 min earlier as compared with animals without any treatments ($P < 0.05$). BW-CO-111 (0.1 mg/kg, i.g.) significantly decreased *iNos* mRNA expression fold changes as compared with vehicle-control group ($P < 0.05$, Fig. 6A). Fig. 6B shows that gastric mucosal mRNA expression of *Anxa1* was significantly downregulated in rats administered i.g. with 75% ethanol and pretreated i.g. with BW-CO-111 (0.1 mg/kg) as compared with vehicle ($P < 0.05$). Fig. 6C−E shows that gastric mucosal mRNA expressions for *Tgfbr1*, *Tgfbr2* and *Tgfbr3* were not significantly altered in rats administered i.g. with 75% ethanol and pretreated with vehicle as compared with healthy gastric mucosa ($P < 0.05$). Pretreatment with BW-CO-111 upregulated *Tgfbr2* and *Tgfbr3* but not *Tgfbr1* mRNA expression in gastric mucosa as compared with vehicle ($P < 0.05$, Fig. 6C−E). Fig. 6F and G shows respectively that TGFB1 and TGFB2 serum concentrations in rats pretreated with vehicle and administered with ethanol were significantly increased as compared with intact animals ($P < 0.05$). BW-CO-111 (0.1 mg/kg, i.g.) did not affect TGFB1 and TGFB2 serum concentration in rats administered with ethanol as compared with vehicle-pretreated group ($P < 0.05$, Fig. 6F and G). TGFB3 serum concentration was not significantly changed after administration of ethanol in rats with or without pretreatment with BW-CO-111 (Fig. 6H).

Gastric mucosal mRNA expression of anti-inflammatory *Anxa1* and pro-inflammatory *iNos* and *Il1b* was not significantly downregulated in rats pretreated with BW-CP-111 (0.1 mg/kg, i.g.) as compared with vehicle-pretreatment (Supporting Information Fig. S1A−S1C). Gastric mucosal mRNA expression of *iNos* and *Il1b* but not of *Anxa1* in 75% ethanol treated rats was significantly upregulated as compared with intact gastric mucosa ($P < 0.05$, Fig. S1A−S1C).

Table 2 Gastric blood flow (GBF) levels in gastric mucosa of rats pretreated i.g. with vehicle, CO prodrug BW-CO-111 (0.1 mg/kg) or BW-CP-111 (0.1 mg/kg) 45 min prior to treatment with 75% ethanol (EtOH) or aspirin (ASA, 125 mg/kg).

Experimental group	GBF (mL/min per 100 g of gastric tissue)
Intact	41.80 ± 0.8602
Vehicle + EtOH	27.40 ± 1.631*
BW-CO-111 + EtOH	34.00 ± 1.140**
BW-CP-111 + EtOH	29.20 ± 0.6633
Vehicle + ASA	26.60 ± 1.503*
BW-CO-111 + ASA	32.40 ± 1.536$^{\#}$

Intact refers to the values obtained in healthy gastric mucosa without ethanol or aspirin-induced gastric damage. Results are mean ± SEM of 5 rats per group. *$P < 0.05$ compared with the respective values in intact gastric mucosa. **$P < 0.05$ compared with the respective values in vehicle-control group administered with EtOH. $^{\#}P < 0.05$ compared with the respective values in vehicle-control group administered with ASA.

3.6. *The effect of pretreatment with BW-CO-111 on systemic inflammatory response in rats administered with EtOH*

Fig. 7 shows that administration of 75% ethanol significantly increased serum concentration of IL1B (A), IL2 (B), IL4 (C), IL5 (D), IL6 (E), IL10 (F), IL12 (G), IL13 (H), TNF (I), IFNG (J), and GM-CSF (K) as compared with intact rats ($P < 0.05$). Fig. 7 shows that pretreatment with BW-CO-111 before administration of 75% ethanol significantly decreased serum concentration of

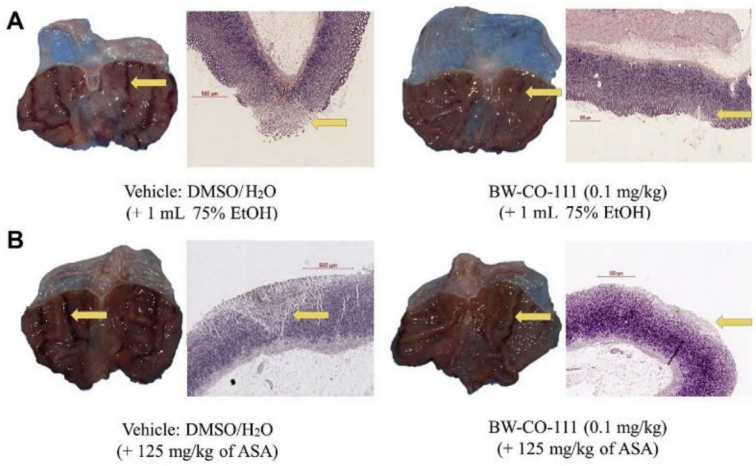


Figure 3 Macroscopic and microscopic appearance of randomly selected representative gastric mucosa of rats pretreated with vehicle or BW-CO-111 (0.1 mg/kg) and exposed to 75% ethanol (EtOH) (A) or aspirin (ASA) (B). Histological slides were stained with H/E. Yellow arrows indicate the macroscopic and microscopic gastric lesions.

IL1B (A), IL2 (B), IL4 (C), IL5 (D), IL6 (E), IL10 (F), IL12 (G), IL13 (H), TNF (I), IFNG (J), and GM-CSF (K) as compared with vehicle-control group ($P < 0.05$).

3.7. Alterations in Hmox1/HMOX1 and Hmox2/HMOX2 mRNA/protein expression in gastric mucosa compromised by aspirin after pretreatment with BW-CO-111

Fig. 8A shows that mRNA expression for *Hmox1* in gastric mucosa but not that for *Hmox2*, was significantly increased as compared with expressions of *Hmox1* and *Hmox2* mRNA recorded in healthy animals without any treatments ($P < 0.05$). Fig. 8A and B shows that gastric mucosal mRNA expression for *Hmox1* and *Hmox2* was not significantly affected in rats pretreated with BW-CO-111 as compared with vehicle in rats administered 30 min later with aspirin (125 mg/kg, i.g.). Gastric mucosal protein expression for HMOX1, HMOX2 and NRF2 were not significantly affected in rats pretreated i.g. with BW-CO-111 (0.1 mg/kg) as compared with vehicle (Fig. 8C–E).

3.8. Modulation of gastroprotective PGE_2 content and Cox1/COX1 and Cox2/COX2 mRNA/protein expression in gastric mucosa compromised by aspirin after pretreatment with BW-CO-111

Fig. 9A and B shows that gastric mucosal *Cox2* but not *Cox1* mRNA expression was significantly increased by aspirin treatment as compared with animals without any treatments ($P < 0.05$). Fig. 9A and B shows that gastric mucosal mRNA expression for *Cox2* but not mRNA expression of *Cox1* was significantly downregulated in rats pretreated with BW-CO-111 and administered 30 min later with aspirin ($P < 0.05$). Gastric mucosal protein expression for COX1 and COX2 was not significantly affected in rats pretreated i.g. with BW-CO-111 (0.1 mg/kg) as compared with vehicle (Fig. 9C and D). PGE_2 concentration in gastric mucosa was significantly decreased after administration of aspirin as compared with the respective values observed in intact gastric mucosa ($P < 0.05$, Fig. 9F). BW-CO-111 (0.1 mg/kg, i.g.) did not significantly affect PGE_2 concentration in gastric mucosa administered with aspirin as compared with vehicle pretreatment (Fig. 9F).

3.9. Involvement of anti-inflammatory Anxa1 and TGFB pathway in the protective effect of pretreatment with BW-CO-111 in gastric mucosa compromised by aspirin

Fig. 10A shows that gastric mucosal mRNA expression for *iNos* was significantly upregulated in vehicle-pretreated rats administered 30 min later with aspirin (125 mg/kg, i.g., $P < 0.05$). BW-CO-111 (0.1 mg/kg, i.g.) significantly decreased *iNos* mRNA expression fold changes as compared with vehicle-control group ($P < 0.05$, Fig. 10A). In rats administered with aspirin and pretreated i.g. with BW-CO-111 (0.1 mg/kg), the gastric mucosal mRNA expression for *Anxa1* was significantly upregulated as compared with vehicle ($P < 0.05$). Fig. 10C–E shows that mRNA expression for *Tgfbr3* but not *Tgfbr1* or *Tgfbr2* mRNA expression was significantly upregulated in gastric mucosa exposed to aspirin ($P < 0.05$). Pretreatment with BW-CO-111 before aspirin administration, did not affect mRNA expression for *Tgfbr1*, *Tgfbr2* or *Tgfbr3* in gastric mucosa as compared with vehicle ($P < 0.05$, Fig. 10C–E). As presented in Fig. 10F–H, TGFB1 and TGFB2 but not TGFB3 serum concentrations is significantly increased in rats pretreated with vehicle and administered with aspirin as compared with intact animals ($P < 0.05$). BW-CO-111 (0.1 mg/kg, i.g.) did not affect TGFB1, TGFB2 and TGFB3 serum concentrations in rats administered with aspirin as compared with vehicle-pretreated group ($P < 0.05$, Fig. 10F–H).

3.10. The effect of pretreatment with BW-CO-111 on systemic inflammatory response in rats administered with aspirin

The administration of aspirin (125 mg/kg, i.g.) significantly increased serum concentration of IL1B (A), IL2 (B), IL4 (C), IL5 (D), IL6 (E), IL10 (F), IL12 (G), IL13 (H), TNF (I), IFNG (J), GM-CSF (K) as compared with the values of these cytokines measured in intact rats ($P < 0.05$) (Fig. 11). Pretreatment with BW-CO-111 (0.1 mg/kg, i.g.) before administration of aspirin did

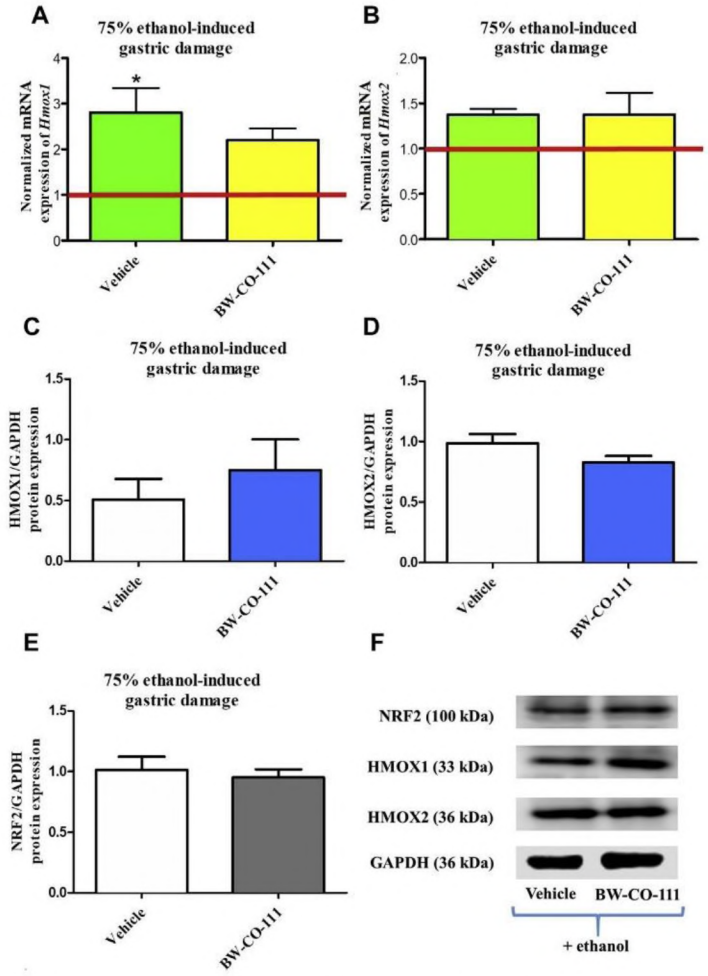


Figure 4 *Hmox1* (A), *Hmox2* (B) mRNA and HMOX1 (C, F), HMOX2 (D, F), NRF2 (E, F) proteins expression in gastric mucosa of rats pretreated i.g. with vehicle or BW-CO-111 (0.1 mg/kg) followed by 75% ethanol administration 30 min later. Results are mean ± SEM of 5 rats per experimental group. Red line indicates baseline value of mRNA expression in healthy gastric mucosa without any treatments. $*P < 0.05$ compared with healthy gastric mucosa.

not significantly affect serum concentration of IL1B (A), IL2 (B), IL4 (C), IL5 (D), IL6 (E), IL10 (F), IL12 (G), IL13 (H), TNF (I), IFNG (J), GM-CSF (K) as compared with vehicle-control group (Fig. 11).

4. Discussion

Gastric mucosa is permanently exposed to exogenous noxious factors derived orally, very often with food, such as chemical necrotizing irritants including ethanol or drugs[82–85]. Clinical use of NSAIDs such as aspirin is very often associated with serious side effects including induction of the hemorrhagic gastric erosions and microbleedings[86,87]. In spite of that, NSAIDs are still widely used because of its effectiveness in modulation of inflammation, fever and pain intensity[86,87]. Proton pomp inhibitors (PPI) are commonly used in clinics to prevent NSAIDs-induced GI complications. However, it has been observed that the prolonged treatment with PPI resulting in achlorhydria may lead to small intestinal bacterial overgrowth[88].

On the other hand, endogenous gaseous mediators, such as hydrogen sulfide (H_2S), CO or nitric oxide (NO) have been shown to be involved in the maintenance of physiological GI tract integrity and mucosal defense[89]. Moreover, H_2S-releasing pharmacological tools can prevent ethanol- or drugs-induced gastric damage and were effective in acceleration of gastric ulcer healing[36,79,90–92]. Interestingly, novel gaseous mediators-releasing hybrids of NSAIDs, such as H_2S-releasing derivative of naproxen, ATB-346 passed successfully the phase 2 of clinical trial[93]. Similarly to H_2S, ruthenium containing CO-releasing CORM-2 is capable to prevent gastric mucosa against oxidative- or necrotic-gastric damage[78,80]. However, the utility of metal-complexes with attached CO-ligands in CO signaling research is debatable[94].

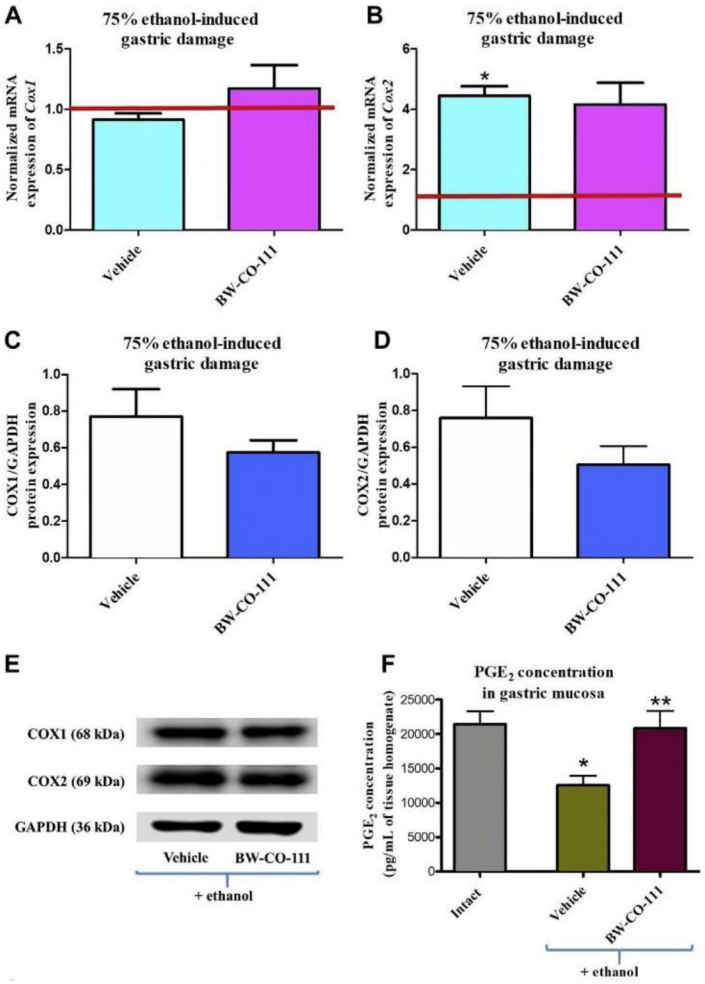


Figure 5 *Cox1* (A), *Cox2* (B) mRNA, COX1 (C, E), COX2 (D, E) proteins expression and prostaglandin E_2 content (PGE_2, F) in gastric mucosa of rats pretreated i.g. with vehicle or BW-CO-111 (0.1 mg/kg) and administered 30 min later with 75% ethanol. Results are mean ± SEM of 5 rats per experimental group. Red line indicates baseline value of mRNA expression in healthy gastric mucosa without any treatments (Intact). $*P < 0.05$ compared with healthy gastric mucosa; $**P < 0.05$ compared with vehicle.

In this study, we have demonstrated for the first time that novel organic and metal-free CO prodrug BW-CO-111 applied i.g. at a dose of 0.1 mg/kg protected the gastric mucosa against necrotic, ethanol- and aspirin-induced mucosal damage reducing by more than 50% the area of gastric lesions at the micro- and macroscopic levels as observed using well-known animal models[35,78]. This acute aspirin-induced GI injury has been previously reported to reflect topical and systemic actions of acidified form of this NSAID, including the reduced production of cytoprotective PGE_2 and the accompanying fall in gastric microcirculation[95,96]. It has been reported previously that CO can exert vasodilatory action possibly mediated by soluble guanylyl cyclase activity[35,97,98]. In our study, the pretreatment with BW-CO-111 counteracted the decrease in GBF in gastric mucosa compromised by ethanol or aspirin. In contrast, the pretreatment with BW-CP-111, a release product control of BW-CO-111, without ability to release CO, was not effective in gastric mucosal protection and GBF modulation. Moreover, prior administration of BW-CP-111 did not decrease pro-inflammatory markers mRNA expression upregulated in chemically damaged gastric mucosa exposed to 75% ethanol. These observations confirm that beneficial effects of BW-CO-111 could be due to its ability to release CO.

The chemical characterization and CO release kinetics for BW-CO-111 has been described in detail previously[62]. CO release is stoichiometric with a $t_{1/2}$ of approximately 17 min at pH 1.2 and 24 min at pH 7.4 as we have demonstrated in this study. BW-CO-111 applied i.g. at a dose of 0.1 mg/kg increased CO content in gastric mucosa as we observed by direct measurement of CO concentration in gastric tissue. This amount of CO was adequate to prevent gastric mucosa against ethanol- or aspirin-induced damage.

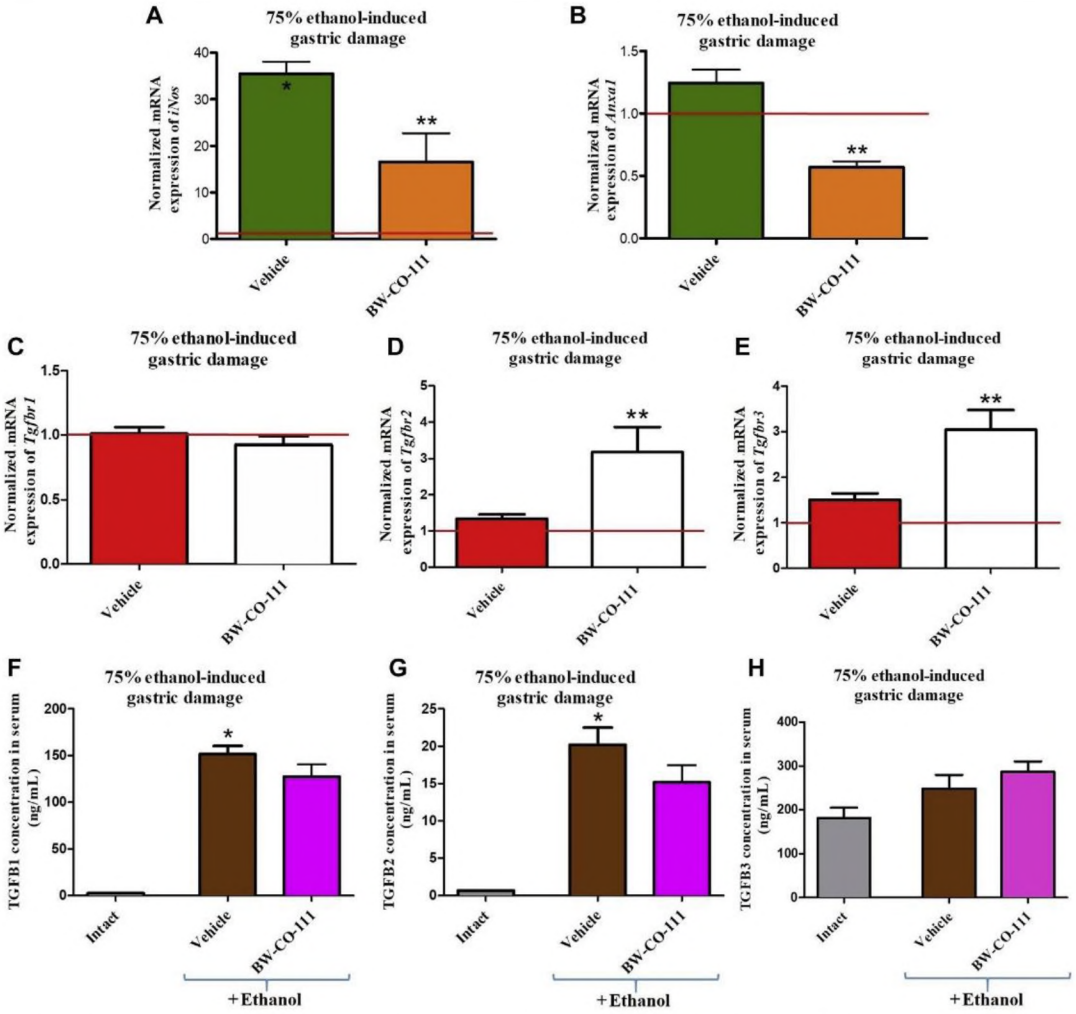


Figure 6 Expression of *iNos* (A), *Anxa1* (B) and *Tgfb* receptor 1 (*Tgfbr1*) (C), *Tgfbr2* (D), *Tgfbr3* (E) mRNA in gastric mucosa and alterations in serum concentration of TGFB1 (F), TGFB2 (G), TGFB3 (H) in rats pretreated i.g. with vehicle or BW-CO-111 (0.1 mg/kg, i.g.) 30 min before administration of 75% ethanol. Intact refers to healthy rats which were not treated with ethanol. Results are mean ± SEM of 5 samples per each experimental group with statistical significance marked only if 2-fold up- or downregulation was reached. Results are expressed as fold change of normalized gastric mucosal *iNos*, *Anxa1*, *Tgfbr1*, *Tgfbr2* and *Tgfbr3* mRNA expression. Red line indicates baseline value of mRNA expression in healthy gastric mucosa without any treatments. $^{*}P < 0.05$ compared with healthy gastric mucosa; $^{**}P < 0.05$ compared with respective values obtained in vehicle-pretreated group.

Interestingly, ruthenium containing CORM-2 was effective at a dose of 5 mg/kg i.g [35,78]. Thus, because of the difference in $t_{1/2}$ for CO release, the novel CO-donating compound BW-CO-111 appears to act as a promising and safe pharmacological alternative for further studies related to gastric disorders. This notion is supported by our present finding that intragastric treatment with BW-CO-111 exerted gastroprotection at a dose of 0.1 and 0.5 mg/kg and BW-CP-111 did not increase cytotoxic effect of aspirin or ethanol within gastric epithelium. Additionally, BW-CP-111 did not further elevate gastric mucosal mRNA expression for pro-inflammatory *iNos* or *Il1b* upregulated by ethanol. These observations not only provide the evidence that the beneficial effects of BW-CO-111 is due to its ability to release CO but also indicate that this compound is not cytotoxic, at least in this experimental model. Thus, this aspect seems to require further toxicological studies to be fully confirmed. Interestingly, BW-CO-111 applied at a dose of 5 mg/kg was not observed to prevent gastric mucosa against ethanol-induced damage. This is in pair with previously published data showing that CO-releasing CORM-2 was also not effective or even cytotoxic when applied at higher doses [78,99–101]. Such results are consistent with the biphasic nature of the dose–response curves for compounds with pleotropic effects, especially gasotransmitters such as

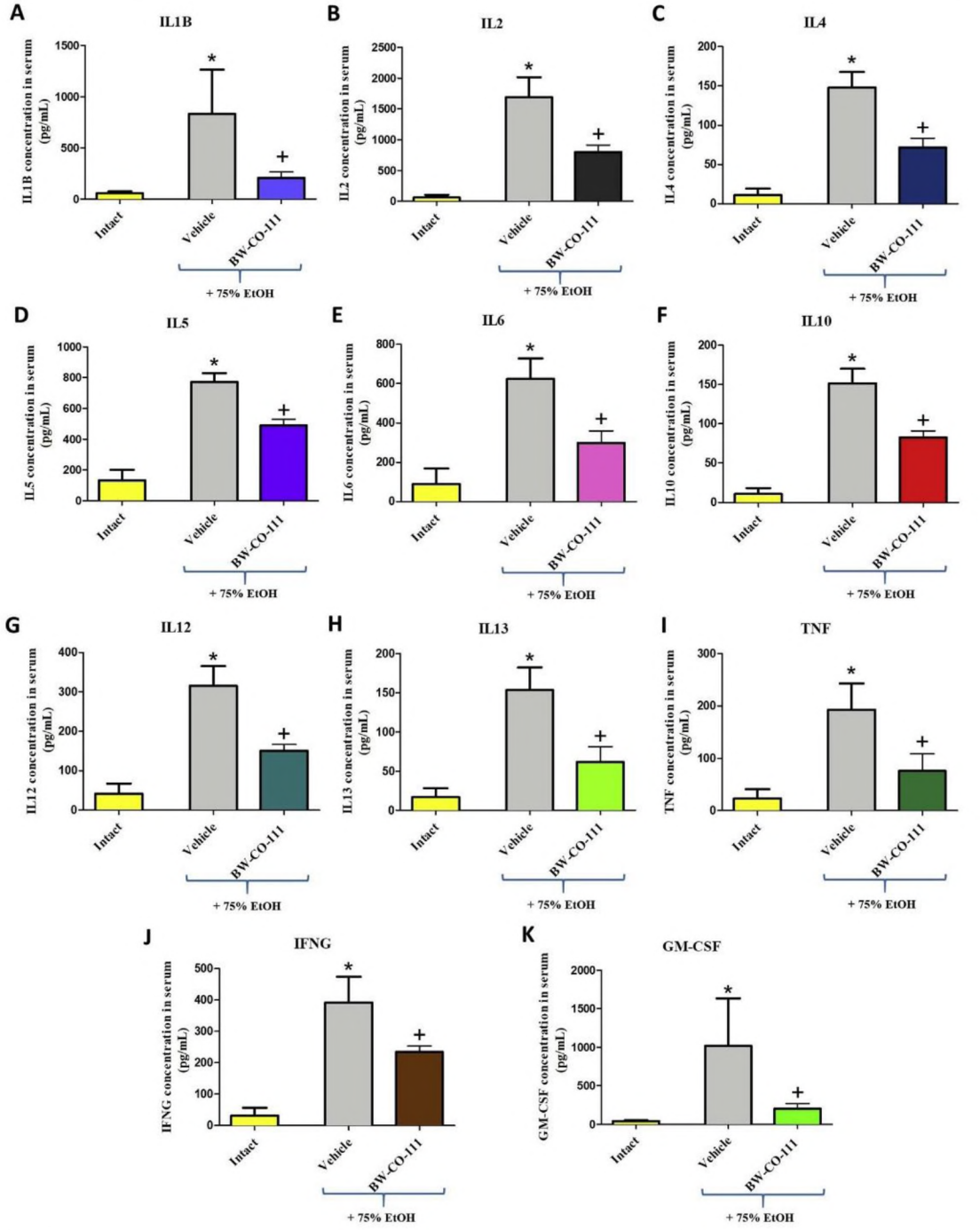


Figure 7 Changes in serum concentration of interleukin IL1B (A), IL2 (B), IL4 (C), IL5 (D), IL6 (E), IL10 (F), IL12 (G), IL13 (H), tumor necrosis factor TNF (I), interferon IFNG (J), and granulocyte-macrophage colony-stimulating factor GM-CSF (K) in rats pretreated i.g. with vehicle or BW-CO-111 (0.1 mg/kg) and administered with 75% ethanol (EtOH). Intact refers to serum concentration of cytokines in rats without any treatments. Results are mean ± SEM of 5 samples per each experimental group. $^{*}P < 0.05$ compared with respective values obtained in intact rats; $^{+}P < 0.05$ compared with respective values obtained in vehicle-control group.

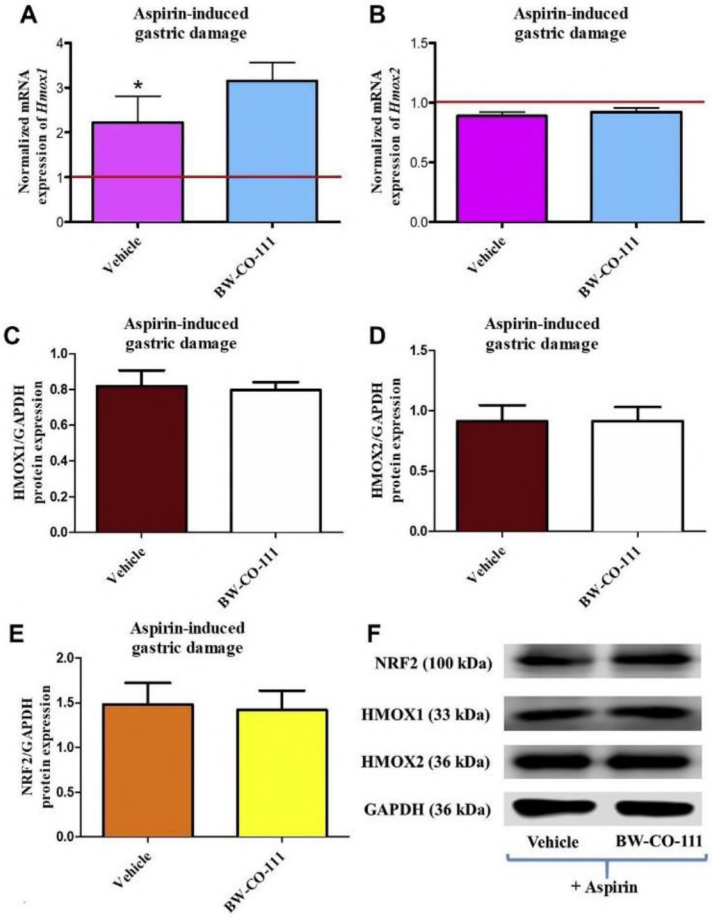


Figure 8 *Hmox1* (A), *Hmox2* (B) mRNA and HMOX1 (C, F), HMOX2 (D, F), NRF2 (E, F) proteins expression in gastric mucosa of rats pretreated i.g. with vehicle or BW-CO-111 (0.1 mg/kg) and administered 30 min later with aspirin (125 mg/kg, i.g.). Results are mean ± SEM of 5 rats per experimental group. Red line indicates baseline value of mRNA expression in healthy gastric mucosa without any treatments. $^{*}P < 0.05$ compared with healthy gastric mucosa.

hydrogen sulfide. In practice, such results also set a window for effective doses for future considerations.

We have observed in our present study that BW-CO-111 maintained upregulated gastric mucosal mRNA expression of endogenous CO producing *Hmox1* in rats administered with aspirin or ethanol. Indeed, it has been previously observed that mRNA and protein expression for HMOX1 was increased and decreased, respectively in gastric mucosa administered with ethanol or aspirin[35,78]. Such results suggest that in contrast to CORM-2, BW-CO-111 can attenuate gastric mucosal necrotic lesions and drug-induced gastrotoxicity without additional synergistic stimulation of endogenous CO production. The observed earlier CORM-2-mediated upregulation of *Hmox1* mRNA in gastric mucosa exposed to ethanol or aspirin could be due to the presence of Ruthenium in the structure of CORM-2, which in fact, did not interfere with the potential gastroprotective activity of this CO donor[35,78]. It needs to be noted that recent studies have found that ruthenium-based CO-RMs such as CORM-2 can participate in many reactions under physiological conditions[68–74]. Thus, it is reasonable to conclude that the interpretation of the protective gastroprotective effects of CORM-2 can be attributed to yet unrecognized factors and mechanisms besides the release of CO.

Interestingly, BW-CO-111 (0.1 mg/kg, i.g.) downregulated gastric mucosal mRNA expression for anti-inflammatory *Anxa1*, upregulated mucosal mRNA expression for *Tgfbr2* and *r3* and maintained elevated TGFB1 and TGFB2 serum concentration in rats administered with ethanol. It has been previously reported that CORM-2 can antagonize TGFB activity through internalization of TGFB receptor 1 (ALK5) inhibiting profibrotic effect of this inflammatory factor[102]. However, we observed that *Anxa1* mRNA expression was decreased in rats administered with high dose of aspirin but pretreatment with BW-CO-111 increased the gastric mucosal mRNA expression for this protein and similarly to rats administered with ethanol, this CO-prodrug maintained elevated TGFB1 and TGFB2 serum contents and maintained upregulated mucosal mRNA expression for *Tgfbr3* by aspirin. It

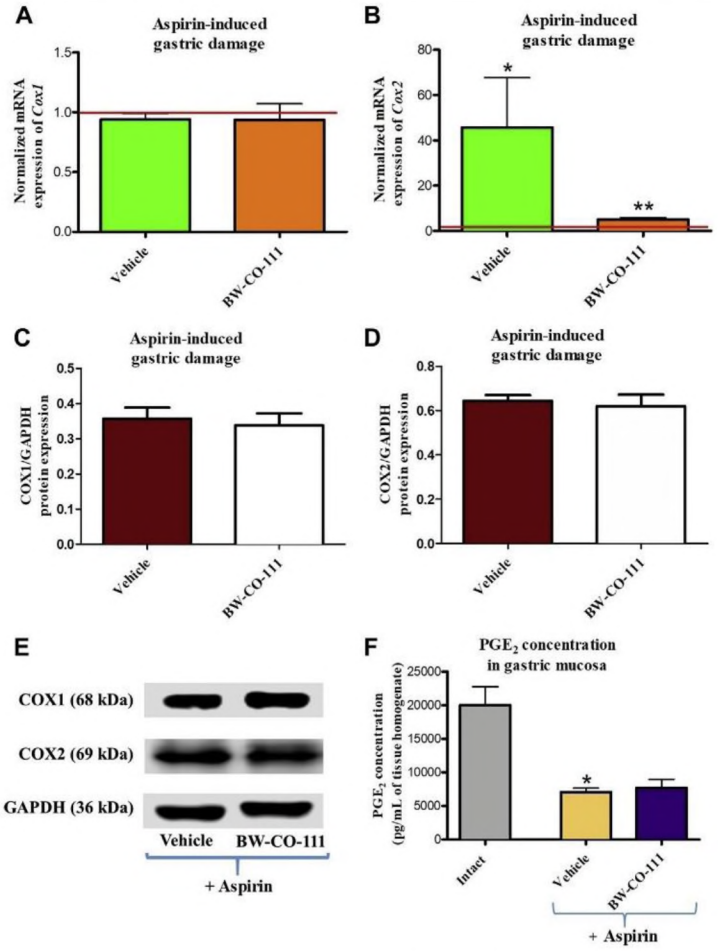


Figure 9 Effect of pretreatment with vehicle or BW-CO-111 (0.1 mg/kg) administered 30 min prior aspirin (125 mg/kg, i.g.) on alterations in *Cox1* (A), *Cox2* (B) mRNA, COX1 (C, E), COX2 (D, E) proteins expression and the mucosal concentrations of prostaglandin E_2 (PGE_2, F). Results are mean ± SEM of 5 rats per experimental group. Red line indicates baseline value of mRNA expression in healthy gastric mucosa without any treatments (Intact). $^*P < 0.05$ compared with healthy gastric mucosa; $^{**}P < 0.05$ compared with respective values obtained in vehicle-pretreated group.

is worth highlighting that ANXA1 is assumed to exert its anti-inflammatory activity due to inhibition of prostaglandins (PGs) biosynthesis[103,104]. However, PGE_2 produced by the activity of COXs is a crucial component of gastric mucosal barrier[105]. Interestingly, it has been reported that ANXA1 is involved in gastroprotection of dexamethasone against indomethacin-induced gastric damage under experimental conditions similar to those with aspirin in our study, in which the PG generation is also suppressed[106]. This could explain the discrepancies in *Anxa1* mRNA expression between gastric mucosa pretreated with BW-CO-111 and compromised by ethanol and aspirin. In chronic ulcer study, CO-releasing CORM-2 accelerated gastric ulcer healing increasing *Anxa1* mRNA expression at ulcer margin after 3 days of treatment and maintaining TGFB2 serum concentration after 6 days of treatments[107]. Additionally, it has been reported that CO-releasing CORM-A1 increased TGFB production by pancreatic lymph node cells resulting in enhancement of beta cells regeneration in experimental mice model of diabetes[108].

CO donors were reported to modulate inflammatory response in various experimental models including digestive system pathologies[6,109–111]. In our study, we have observed that pretreatment with BW-CO-111 (0.1 mg/kg, i.g.) attenuated the systemic inflammatory response induced by 75% ethanol application as reflected in the decreased serum concentrations of eleven inflammatory biomarkers including IL1B, IL2, IL4, IL5, IL6, IL10, IL12, IL13, TNF, IFNG, and GM-CSF. Moreover, BW-CO-111 downregulated gastric mucosal mRNA expression of pro-inflammatory *iNos* and *Cox2* elevated in gastric mucosa exposed to aspirin. Similarly, it has been reported previously that CORM-2 (5 mg/kg, i.g.) prevented ischemia/reperfusion-induced gastric damage in rats by mechanism involving the decrease in the systemic levels of these cytokines[80].

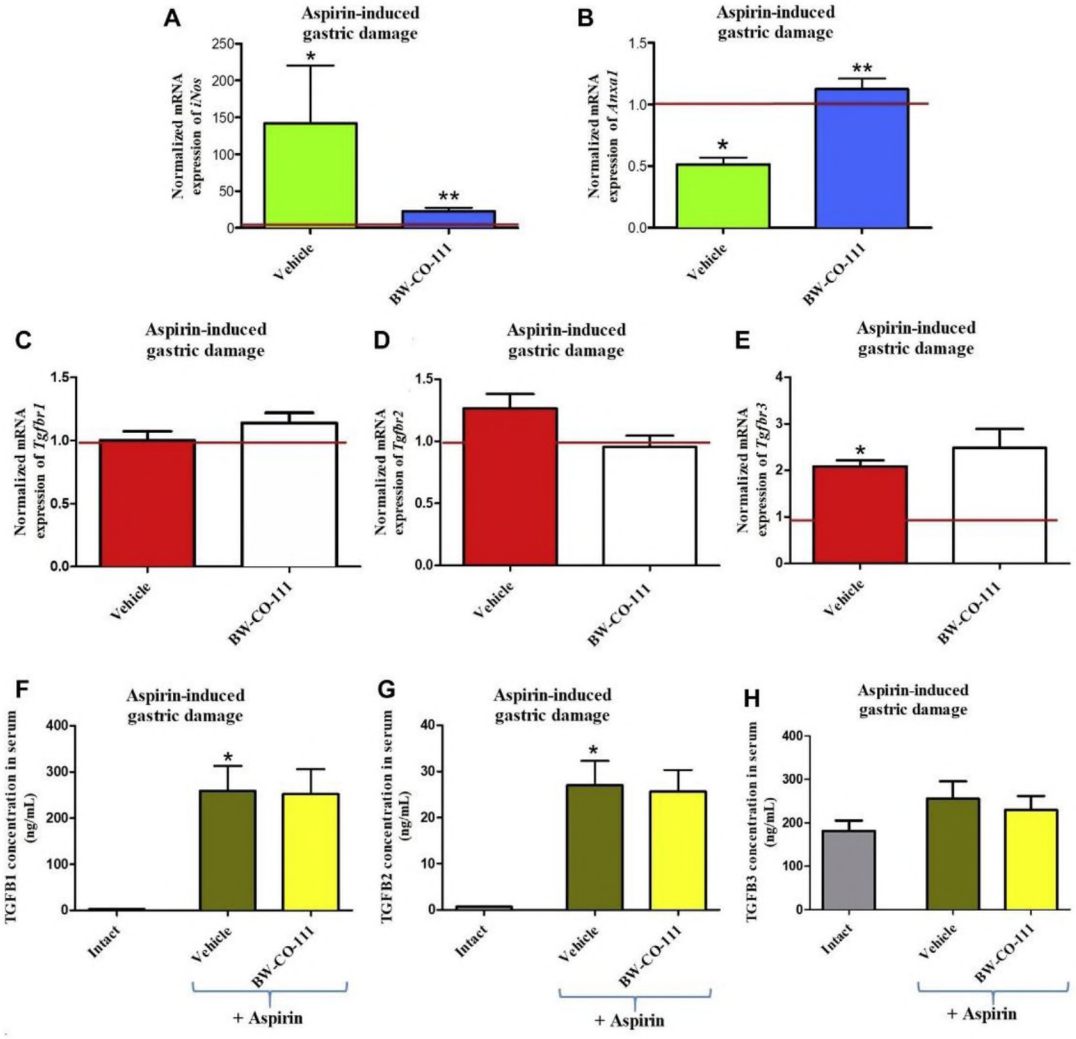


Figure 10 Expression of *iNos* (A), *Anxa1* (B) and *Tgfb* receptor 1 (*Tgfbr1)* (C), *Tgfbr2* (D), *Tgfbr3* (E) mRNA in gastric mucosa and the changes in TGFB1 (F), TGFB2 (G), TGFB3 (H) serum concentrations of rats pretreated i.g. with vehicle or BW-CO-111 (0.1 mg/kg, i.g.) 30 min before administration of aspirin (125 mg/kg, i.g.). Intact refers to healthy rats which were not treated with aspirin. Results are mean ± SEM of 5 samples per each experimental group with statistical significance marked only if 2-fold up- or downregulation was reached. Results are expressed as fold change of normalized gastric mucosal *iNos*, *Anxa1* and *Tgfbr1*, *Tgfbr2*, *Tgfbr3* mRNA expression. Red line indicates baseline value of mRNA expression in healthy gastric mucosa without any treatments (Intact). $*P < 0.05$ compared with intact rats; $**P < 0.05$ compared with respective values obtained in vehicle-pretreated group.

Finally, BW-CO-111 (0.1 mg/kg, i.g.) maintained upregulated gastric mucosal protein expression for COX2 in rats as a result of treatment with aspirin or ethanol. However, BW-CO-111 increased cytoprotective PGE_2 mucosal content in gastric mucosa compromised by ethanol but not by aspirin. In the case of aspirin treatment, BW-CO-111 was able to alleviate the damaging effect without directly altering decreased PGE_2 gastric mucosal production caused by this drug. Since the expression of *Cox2* mRNA but not that of *Cox1* was elevated in gastric mucosa of ethanol treated rats with or without BW-CO-111 pretreatment comparing with healthy gastric mucosa, one would assume that COX2 derived PG are predominantly responsible for this CO donor-induced protection and the increase in GBF. Thus, we assume that this CO donor enhanced the activity of PGE_2 system being involved in the physiological defensive mechanism against necrotic effects of ethanol.

Taken together, we conclude that BW-CO-111 (0.1 mg/kg) applied i.g. protected gastric mucosa against necrotic ethanol-induced and aspirin-induced damage due to its ability to release CO possibly responsible for the increase in gastric

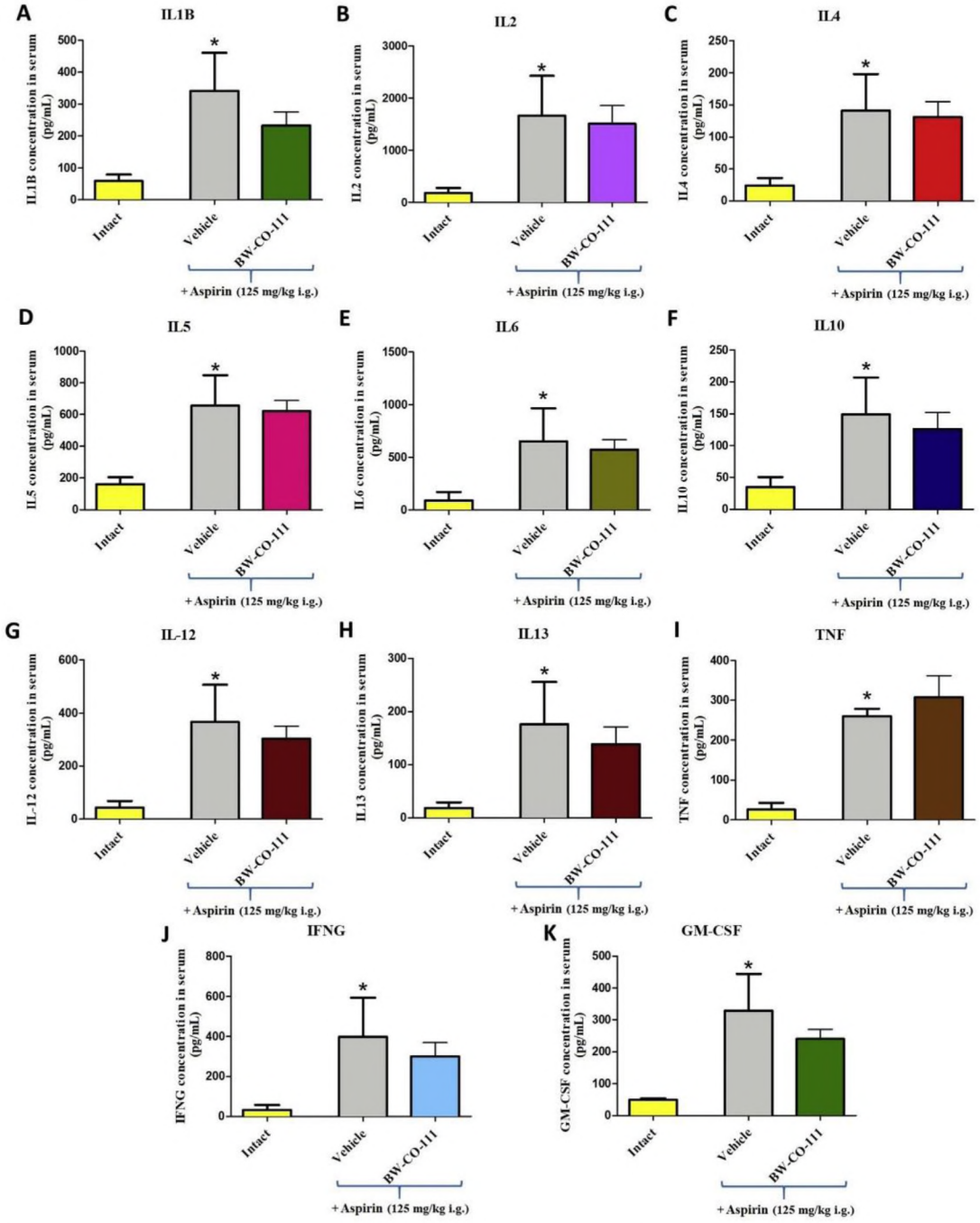


Figure 11 Alterations in serum concentration of interleukin IL1B (A), IL2 (B), IL4 (C), IL5 (D), IL6 (E), IL10 (F), IL12 (G), IL13 (H), tumor necrosis factor TNF (I), interferon IFNG (J), and granulocyte-macrophage colony-stimulating factor GM-CSF (K) in rats pretreated i.g. with vehicle or BW-CO-111 (0.1 mg/kg) and administered with aspirin (125 mg/kg, i.g.). Intact refers to serum of healthy rats without any treatments. Results are mean ± SEM of 5 samples per each experimental group. $^{*}P < 0.05$ compared with respective values obtained in intact rats.

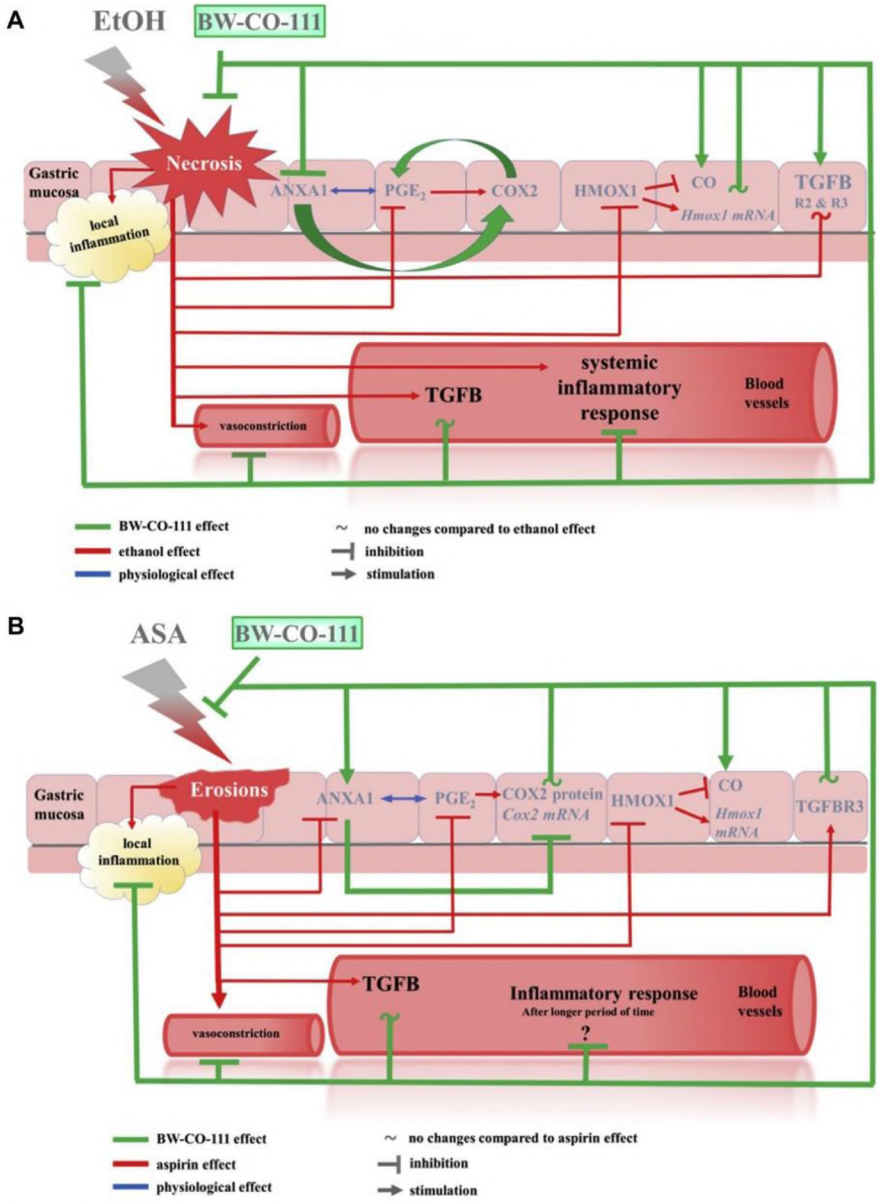


Figure 12 Possible mechanisms of BW-CO-111-mediated gastroprotection against ethanol- (A) and aspirin-induced (B) gastric damage.

microcirculation observed in our present study. However, because the pathophysiology of gastric damage differs between topical administration of ethanol and aspirin, the possible mechanisms of BW-CO-111-mediated gastroprotection seems to be somewhat different in these two models. Its gastroprotective activity was also associated with the similar significant attenuation of systemic and gastric mucosal inflammatory response possibly mediated by the anti-inflammatory ANXA1, TGFB and its receptors activity. Based on our present evidence, the NRF2 triggering effect of HMOX pathway by BW-CO-111 is questionable. Moreover, BW-CO-111 could affect COX2/PGE_2 resulting in gastroprotection against ethanol-induced gastric damage and this CO donor could counteract the alterations in the COX2/PGE_2 pathway in gastric mucosa induced by aspirin (Fig. 12A and B, respectively).

Acknowledgments

This study was supported by statutory grant for Marcin Magierowski received from Jagiellonian University Medical College

(N41/DBS/000106, Poland). Part of molecular and biochemical assessments in Marcin Magierowski's laboratory were supported by a grant from National Science Centre Poland (UMO-2019/33/B/NZ4/00616). Marcin Magierowski received financial support from the Foundation for Polish Science (START 62.2018, Poland). We would like to acknowledge Anna Chmura, MSc for her input related to the technical preparation of histological slides. Binghe Wang acknowledges the financial support from the Georgia Research Alliance Eminent Scholar Fund, and internal resources at Georgia State University (USA) including its Department of Chemistry and the Molecular Basis of Disease program.

Author contributions

Conceptualization: Dominik Bakalarz and Marcin Magierowski. Supervision: Binghe Wang and Marcin Magierowski. Funding acquisition: Binghe Wang and Marcin Magierowski. Investigation: Dominik Bakalarz, Marcin Surmiak, Xiaoxiao Yang, Dagmara Wójcik, Edyta Korbut, Zbigniew Śliwowski, Grzegorz Ginter, Grzegorz Buszewicz, Tomasz Brzozowski, Jakub Cieszkowski, Urszula Głowacka, Katarzyna Magierowska, Zhixiang Pan, and Marcin Magierowski. Methodology: Dominik Bakalarz, Marcin Surmiak, Xiaoxiao Yang, Edyta Korbut, and Grzegorz Buszewicz. Visualization: Dominik Bakalarz, Xiaoxiao Yang, Katarzyna Magierowska, and Marcin Magierowski. Writing-original draft: Dominik Bakalarz and Marcin Magierowski. Writing-review and editing: Dominik Bakalarz, Edyta Korbut, Tomasz Brzozowski, Xiaoxiao Yang, Binghe Wang and Marcin Magierowski.

Conflicts of interest

The authors have nothing to declare.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2020.08.005.

References

1. Wu L, Wang R. Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. *Pharmacol Rev* 2005;**57**:585–630.
2. Magierowska K, Brzozowski T, Magierowski M. Emerging role of carbon monoxide in regulation of cellular pathways and in the maintenance of gastric mucosal integrity. *Pharmacol Res* 2018;**129**:56–64.
3. Takeuchi K, Aihara E, Kimura M, Dogishi K, Hara T, Hayashi S. Gas mediators involved in modulating duodenal $HCO3^-$ secretion. *Curr Med Chem* 2012;**19**:43–54.
4. Shefa U, Kim D, Kim MS, Jeong NY, Jung J. Roles of gasotransmitters in synaptic plasticity and neuropsychiatric conditions. *Neural Plast* 2018;**2018**:1824713.
5. Lee H, Choi YK. Regenerative effects of heme oxygenase metabolites on neuroinflammatory diseases. *Int J Mol Sci* 2018;**20**:78.
6. Ryter SW. Heme oxygenase-1/carbon monoxide as modulators of autophagy and inflammation. *Arch Biochem Biophys* 2019;**678**:108186.
7. Figueiredo-Pereira C, Dias-Pedroso D, Soares NL, Vieira HLA. CO-mediated cytoprotection is dependent on cell metabolism modulation. *Redox Biol* 2020;**32**:101470.
8. Lin X, Lv J, Ge D, Bai H, Yang Y, Wu J. Heme oxygenase-1 alleviates eosinophilic inflammation by inhibiting STAT3-SOCS3 signaling. *Pediatr Pulmonol* 2020;**55**:1440–7.
9. Faizan M, Muhammad N, Niazi KUK, Hu Y, Wang Y, Wu Y, et al. CO-releasing materials: an emphasis on therapeutic implications, as release and subsequent cytotoxicity are the part of therapy. *Materials* 2019;**12**:1643.
10. Ji X, Damera K, Zheng Y, Yu B, Otterbein LE, Wang B. Toward carbon monoxide-based therapeutics: critical drug delivery and developability issues. *J Pharmaceut Sci* 2016;**105**:406–16.
11. Motterlini R, Otterbein LE. The therapeutic potential of carbon monoxide. *Nat Rev Drug Discov* 2010;**9**:728–43.
12. Adach W, Olas B. Carbon monoxide and its donors—heir implications for medicine. *Future Med Chem* 2019;**11**:61–73.
13. Stojak M, Kaczara P, Motterlini R, Chlopicki S. Modulation of cellular bioenergetics by CO-releasing molecules and NO-donors inhibits the interaction of cancer cells with human lung microvascular endothelial cells. *Pharmacol Res* 2018;**136**:160–71.
14. Uddin MJ, Pak ES, Ha H. Carbon monoxide releasing molecule-2 protects mice against acute kidney injury through inhibition of ER stress. *Korean J Physiol Pharmacol* 2018;**22**:567–75.
15. Cheng Y, Rong J. Therapeutic potential of heme oxygenase-1/carbon monoxide system against ischemia–reperfusion injury. *Curr Pharmaceut Des* 2017;**23**:3884–98.
16. Abe T, Yazawa K, Fujino M, Imamura R, Hatayama N, Kakuta Y, et al. High-pressure carbon monoxide preserves rat kidney grafts from apoptosis and inflammation. *Lab Invest* 2017;**97**:468–77.
17. Correa-Costa M, Gallo D, Csizmadia E, Gomperts E, Lieberum JL, Hauser CJ, et al. Carbon monoxide protects the kidney through the central circadian clock and CD39. *Proc Natl Acad Sci U S A* 2018;**115**:E2302–10.
18. Vera T, Henegar JR, Drummond HA, Rimoldi JM, Stec DE. Protective effect of carbon monoxide-releasing compounds in ischemia-induced acute renal failure. *J Am Soc Nephrol* 2005;**16**:950–8.
19. Suliman HB, Carraway MS, Ali AS, Reynolds CM, Welty-Wolf KE, Piantadosi CA. The CO/HO system reverses inhibition of mitochondrial biogenesis and prevents murine doxorubicin cardiomyopathy. *J Clin Invest* 2007;**117**:3730–41.
20. Kim HH, Choi S. Therapeutic aspects of carbon monoxide in cardiovascular disease. *Int J Mol Sci* 2018;**19**:2381.
21. Andreadou I, Iliodromitis EK, Rassaf T, Schulz R, Papapetropoulos A, Ferdinandy P. The role of gasotransmitters NO, H_2S and CO in myocardial ischaemia/reperfusion injury and cardioprotection by preconditioning, postconditioning and remote conditioning. *Br J Pharmacol* 2015;**172**:1587–606.
22. Zheng YQ, Ji XY, Yu BC, Ji KL, Gallo D, Csizmadia E, et al. Enrichment-triggered prodrug activation demonstrated through mitochondria-targeted delivery of doxorubicin and carbon monoxide. *Nat Chem* 2018;**10**:787–94.
23. Sun J, Guo E, Yang J, Yang Y, Liu S, Hu J, et al. Carbon monoxide ameliorates hepatic ischemia/reperfusion injury *via* sirtuin 1-mediated deacetylation of high-mobility group box 1 in rats. *Liver Transplant* 2017;**23**:510–26.
24. Li S, Fujino M, Takahara T, Li XK. Protective role of heme oxygenase-1 in fatty liver ischemia–reperfusion injury. *Med Mol Morphol* 2019;**52**:61–72.
25. Che X, Fang Y, Si X, Wang J, Hu X, Reis C, et al. The role of gaseous molecules in traumatic brain injury: an updated review. *Front Neurosci* 2018;**12**:392.
26. Choi YK, Maki T, Mandeville ET, Koh SH, Hayakawa K, Arai K, et al. Dual effects of carbon monoxide on pericytes and neurogenesis in traumatic brain injury. *Nat Med* 2016;**22**:1335–41.
27. Parfenova H, Pourcyrous M, Fedinec AL, Liu J, Basuroy S, Leffler CW. Astrocyte-produced carbon monoxide and the carbon monoxide donor CORM-A1 protect against cerebrovascular dysfunction caused by prolonged neonatal asphyxia. *Am J Physiol Heart Circ Physiol* 2018;**315**:H978–88.

28. Wang P, Yao L, Zhou LL, Liu YS, Chen MD, Wu HD, et al. Carbon monoxide improves neurologic outcomes by mitochondrial biogenesis after global cerebral ischemia induced by cardiac arrest in rats. *Int J Biol Sci* 2016;**12**:1000–9.
29. Hegazi RAF, Rao KN, Mayle A, Sepulveda AR, Otterbein LE, Plevy SE. Carbon monoxide ameliorates chronic murine colitis through a heme oxygenase 1-dependent pathway. *J Exp Med* 2005; **202**:1703–13.
30. Sheikh SZ, Hegazi RA, Kobayashi T, Onyiah JC, Russo SM, Matsuoka KM, et al. An anti-inflammatory role for carbon monoxide and heme oxygenase-1 in chronic Th2-mediated murine colitis. *J Immunol* 2011;**186**:5506–13.
31. Steiger C, Uchiyama K, Takagi T, Mizushima K, Higashimura Y, Gutmann M, et al. Prevention of colitis by controlled oral drug delivery of carbon monoxide. *J Control Release* 2016;**239**:128–36.
32. Takagi T, Naito Y, Mizushima K, Akagiri S, Suzuki, Hirata I, et al. Inhalation of carbon monoxide ameliorates TNBS-induced colitis in mice through the inhibition of TNF-α expression. *Dig Dis Sci* 2010; **55**:2797–804.
33. Takagi T, Naito Y, Uchiyama K, Suzuki T, Hirata I, Mizushima K, et al. Carbon monoxide liberated from carbon monoxide-releasing molecule exerts an anti-inflammatory effect on dextran sulfate sodium-induced colitis in mice. *Dig Dis Sci* 2011;**56**:1663–71.
34. Uddin MJ, Jeong SO, Zheng M, Chen Y, Cho GJ, Chung HT, et al. Carbon monoxide attenuates dextran sulfate sodium-induced colitis *via* inhibition of GSK-3beta signaling. *Oxid Med Cell Longev* 2013; **2013**:210563.
35. Magierowski M, Magierowska K, Hubalewska-Mazgaj M, Adamski J, Bakalarz D, Sliwowski Z, et al. Interaction between endogenous carbon monoxide and hydrogen sulfide in the mechanism of gastroprotection against acute aspirin-induced gastric damage. *Pharmacol Res* 2016;**114**:235–50.
36. Magierowski M, Magierowska K, Szmyd J, Surmiak M, Sliwowski Z, Kwiecien S, et al. Hydrogen sulfide and carbon monoxide protect gastric mucosa compromised by mild stress against alendronate injury. *Dig Dis Sci* 2016;**61**:3176–89.
37. Jasnos K, Magierowski M, Kwiecien S, Brzozowski T. Carbon monoxide in human physiology—its role in the gastrointestinal tract. *Postepy Hig Med Dosw* 2014;**68**:101–9.
38. Magierowski M, Magierowska K, Hubalewska-Mazgaj M, Sliwowski Z, Ginter G, Pajdo R, et al. Carbon monoxide released from its pharmacological donor, tricarbonyldichlororuthenium (II) dimer, accelerates the healing of pre-existing gastric ulcers. *Br J Pharmacol* 2017;**174**:3654–68.
39. McKendrick JG, Snodgrass W. On the physiological action of carbon monoxide of nickel. *Br Med J* 1891;**1**:1215.
40. Motterlini R, Foresti R. Biological signaling by carbon monoxide and carbon monoxide-releasing molecules. *Am J Physiol Cell Physiol* 2017;**312**:C302–13.
41. Vummaleti SV, Branduardi D, Masetti M, de Vivo M, Motterlini R, Cavalli A. Theoretical insights into the mechanism of carbon monoxide (CO) release from CO-releasing molecules. *Chemistry* 2012; **18**:9267–75.
42. Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE, Green CJ. Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. *Circ Res* 2002;**90**:E17–24.
43. Jeremias HF, Lousa D, Hollmann A, Coelho AC, Baltazar CS, Seixas JD, et al. Study of the interactions of bovine serum albumin with a molybdenum(II) carbonyl complex by spectroscopic and molecular simulation methods. *PLoS One* 2018;**13**:e0204624.
44. Romanski S, Stamellou E, Jaraba JT, Storz D, Kramer BK, Hafner M, et al. Enzyme-triggered CO-releasing molecules (ET-CORMs): evaluation of biological activity in relation to their structure. *Free Radic Biol Med* 2013;**65**:78–88.
45. Stamellou E, Storz D, Botov S, Ntasis E, Wedel J, Sollazzo S, et al. Different design of enzyme-triggered CO-releasing molecules (ET-CORMs) reveals quantitative differences in biological activities in terms of toxicity and inflammation. *Redox Biol* 2014;**2**:739–48.
46. Yin H, Fang J, Liao L, Nakamura H, Maeda H. Styrene-maleic acid copolymer-encapsulated CORM2, a water-soluble carbon monoxide (CO) donor with a constant CO-releasing property, exhibits therapeutic potential for inflammatory bowel disease. *J Control Release* 2014;**187**:14–21.
47. Hasegawa U, van der Vlies AJ, Simeoni E, Wandrey C, Hubbell JA. Carbon monoxide-releasing micelles for immunotherapy. *J Am Chem Soc* 2010;**132**:18273–80.
48. Pierri AE, Huang PJ, Garcia JV, Stanfill JG, Chui M, Wu G, et al. A photoCORM nanocarrier for CO release using NIR light. *Chem Commun* 2015;**51**:2072–5.
49. Dordelmann G, Pfeiffer H, Birkner A, Schatzschneider U. Silicium dioxide nanoparticles as carriers for photoactivatable CO-releasing molecules (PhotoCORMs). *Inorg Chem* 2011;**50**:4362–7.
50. Matson JB, Webber MJ, Tamboli VK, Weber B, Stupp SI. A peptide-based material for therapeutic carbon monoxide delivery. *Soft Matter* 2012;**8**:2689–92.
51. Antony LA, Slanina T, Sebej P, Solomek T, Klan P. Fluorescein analogue xanthene-9-carboxylic acid: a transition-metal-free CO releasing molecule activated by green light. *Org Lett* 2013;**15**: 4552–5.
52. Palao E, Slanina T, Muchova L, Solomek T, Vitek L, Klan P. Transition-metal-free CO-releasing BODIPY derivatives activatable by visible to NIR light as promising bioactive molecules. *J Am Chem Soc* 2016;**138**:126–33.
53. Peng P, Wang C, Shi Z, Johns VK, Ma L, Oyer J, et al. Visible-light activatable organic CO-releasing molecules (PhotoCORMs) that simultaneously generate fluorophores. *Org Biomol Chem* 2013;**11**: 6671–4.
54. Anderson SN, Richards JM, Esquer HJ, Benninghoff AD, Arif AM, Berreau LM. A structurally-tunable 3-hydroxyflavone motif for visible light-induced carbon monoxide-releasing molecules (CORMs). *ChemistryOpen* 2015;**4**:590–4.
55. Popova M, Soboleva T, Ayad S, Benninghoff AD, Berreau LM. Visible-light-activated quinolone carbon-monoxide-releasing molecule: prodrug and albumin-assisted delivery enables anticancer and potent anti-inflammatory effects. *J Am Chem Soc* 2018; **140**:9721–9.
56. Schatzschneider U. Novel lead structures and activation mechanisms for CO-releasing molecules (CORMs). *Br J Pharmacol* 2015;**172**: 1638–50.
57. Belcher JD, Gomperts E, Nguyen J, Chen C, Abdulla F, Kiser ZM, et al. Oral carbon monoxide therapy in murine sickle cell disease: beneficial effects on vaso-occlusion, inflammation and anemia. *PLoS One* 2018;**13**:e0205194.
58. Ji X, Wang B. Strategies toward organic carbon monoxide prodrugs. *Acc Chem Res* 2018;**51**:1377–85.
59. Ji X, Wang B. Toward carbon monoxide based therapeutics: carbon monoxide in a pill. *Pharm Pat Anal* 2017;**6**:171–7.
60. Ji X, Aghoghovbia RE, De La Cruz LKC, Pan Z, Yang X, Yu B, et al. Click and release: a high-content bioorthogonal prodrug with multiple outputs. *Org Lett* 2019;**21**:3649–52.
61. Ji X, Ji K, Chittavong V, Aghoghovbia RE, Zhu M, Wang B. Click and fluoresce: a bioorthogonally activated smart probe for wash free fluorescent labeling of biomolecules. *J Org Chem* 2017;**82**: 1471–6.
62. Pan Z, Ji X, Chittavong V, Li W, Ji K, Zhu M, et al. Organic CO-prodrugs: structure CO-release rate relationship studies. *Chem Eur J* 2017;**23**:9838–45.
63. Wang D, Viennois E, Ji K, Damera K, Draganov A, Zheng Y, et al. A click-and-release approach to CO prodrugs. *Chem Commun* 2014;**50**: 15890–3.
64. Ji X, Pan Z, Li C, Kang T, De La Cruz LKC, Yang L, et al. Esterase-sensitive and pH-controlled carbon monoxide prodrugs for treating systemic inflammation. *J Med Chem* 2019;**62**:3163–8.
65. Ji X, Ji K, Chittavong V, Yu B, Pan Z, Wang B. An esterase-activated click and release approach to metal-free CO-prodrugs. *Chem Commun* 2017;**53**:8296–9.

66. Pan Z, Zhang J, Chittavong V, Ji X, Wang B. Organic CO prodrugs activated by endogenous ROS. *Org Lett* 2018;**20**:8−11.
67. De La Cruz K, Benoit SL, Pan Z, Maier R, Ji X, Wang B. Click, release, and fluoresce: a chemical strategy for a cascade prodrug system for codelivery of carbon monoxide, a drug payload, and a fluorescent reporter. *Org Lett* 2018;**20**:897−900.
68. Southam HM, Smith TW, Lyon RL, Liao C, Trevitt CR, Middlemiss LA, et al. A thiol-reactive Ru(II) ion, not CO release, underlies the potent antimicrobial and cytotoxic properties of CO-releasing molecule-3. *Redox Biol* 2018;**18**:114−23.
69. Seixas JD, Chaves-Ferreira M, Montes-Grajales D, Goncalves AM, Marques AR, Saraiva LM, et al. An *N*-acetyl cysteine ruthenium tricarbonyl conjugate enables simultaneous release of CO and ablation of reactive oxygen species. *Chemistry* 2015;**21**:14708−12.
70. Santos-Silva T, Mukhopadhyay A, Seixas JD, Bernardes GJ, Romao CC, Romao MJ. CORM-3 reactivity toward proteins: the crystal structure of a Ru(II) dicarbonyl-lysozyme complex. *J Am Chem Soc* 2011;**133**:1192−5.
71. McLean S, Mann BE, Poole RK. Sulfite species enhance carbon monoxide release from CO-releasing molecules: implications for the deoxymyoglobin assay of activity. *Anal Biochem* 2012;**427**:36−40.
72. Nobre LS, Jeremias H, Romao CC, Saraiva LM. Examining the antimicrobial activity and toxicity to animal cells of different types of CO-releasing molecules. *Dalton Trans* 2016;**45**:1455−66.
73. Santos-Silva T, Mukhopadhyay A, Seixas JD, Bernardes GJ, Romao CC, Romao MJ. Towards improved therapeutic CORMs: understanding the reactivity of CORM-3 with proteins. *Curr Med Chem* 2011;**18**:3361−6.
74. Yuan Z, Yang X, De La Cruz LK, Wang B. Nitro reduction-based fluorescent probes for carbon monoxide require reactivity involving a ruthenium carbonyl moiety. *Chem Commun* 2020;**56**:2190−3.
75. Ji X, Zhou C, Ji K, Aghoghovbia R, Pan Z, Chittavong V, et al. Click and release: a chemical strategy toward developing gasotransmitter prodrugs by using an intramolecular Diels−Alder reaction. *Angew Chem Int Ed Engl* 2016;**55**:15846−51.
76. Lee JS, An JM, Kang EA, Han YM, Kim YS, Lee HJ, et al. Host nuclear factor erythroid 2-related factor-2 defense system determines the outcome of dextran sulfate sodium-induced colitis in mice. *J Physiol Pharmacol* 2018;**69**:755−67.
77. Kim KJ, Park JM, Lee JS, Kim YS, Kangwan N, Han YM, et al. Oligonol prevented the relapse of dextran sulfate sodium-ulcerative colitis through enhancing NRF2-mediated antioxidative defense mechanism. *J Physiol Pharmacol* 2018;**69**:359−71.
78. Magierowska K, Magierowski M, Hubalewska-Mazgaj M, Adamski J, Surmiak M, Sliwowski Z, et al. Carbon monoxide (CO) released from tricarbonyldichlororuthenium (II) dimer (CORM-2) in gastroprotection against experimental ethanol-induced gastric damage. *PLoS One* 2015;**10**:e0140493.
79. Magierowski M, Magierowska K, Hubalewska-Mazgaj M, Surmiak M, Sliwowski Z, Wierdak M, et al. Cross-talk between hydrogen sulfide and carbon monoxide in the mechanism of experimental gastric ulcers healing, regulation of gastric blood flow and accompanying inflammation. *Biochem Pharmacol* 2018;**149**:131−42.
80. Magierowska K, Korbut E, Hubalewska-Mazgaj M, Surmiak M, Chmura A, Bakalarz D, et al. Oxidative gastric mucosal damage induced by ischemia/reperfusion and the mechanisms of its prevention by carbon monoxide-releasing tricarbonyldichlororuthenium (II) dimer. *Free Radic Biol Med* 2019;**145**:198−208.
81. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008;**3**:1101−8.
82. Wallace JL. Recent advances in gastric ulcer therapeutics. *Curr Opin Pharmacol* 2005;**5**:573−7.
83. Ham M, Kaunitz JD. Gastroduodenal mucosal defense. *Curr Opin Gastroenterol* 2008;**24**:665−73.
84. Tarnawski AS, Ahluwalia A. Increased susceptibility of aging gastric mucosa to injury and delayed healing: clinical implications. *World J Gastroenterol* 2018;**24**:4721−7.
85. Krzysiek-Maczka G, Wrobel T, Targosz A, Szczyrk U, Strzalka M, Ptak-Belowska A, et al. *Helicobacter pylori*-activated gastric fibroblasts induce epithelial-mesenchymal transition of gastric epithelial cells *in vitro* in a TGF-beta-dependent manner. *Helicobacter* 2019;**24**:e12653.
86. Kauffman G. Aspirin-induced gastric mucosal injury: lessons learned from animal models. *Gastroenterol* 1989;**96**:606−14.
87. Bjarnason I, Scarpignato C, Takeuchi K, Rainsford KD. Determinants of the short-term gastric damage caused by NSAIDs in man. *Aliment Pharmacol Ther* 2007;**26**:95−106.
88. Compare D, Pica L, Rocco A, De Giorgi F, Cuomo R, Sarnelli G, et al. Effects of long-term PPI treatment on producing bowel symptoms and SIBO. *Eur J Clin Invest* 2011;**41**:380−6.
89. Wallace JL, Ianaro A, de Nucci G. Gaseous mediators in gastrointestinal mucosal defense and injury. *Dig Dis Sci* 2017;**62**:2223−30.
90. Wallace JL, Dicay M, McKnight W, Martin GR. Hydrogen sulfide enhances ulcer healing in rats. *FASEB J* 2007;**21**:4070−6.
91. Nicolau LA, Silva RO, Damasceno SR, Carvalho NS, Costa NR, Aragao KS, et al. The hydrogen sulfide donor, Lawesson's reagent, prevents alendronate-induced gastric damage in rats. *Braz J Med Biol Res* 2013;**46**:708−14.
92. Magierowski M, Magierowska K, Surmiak M, Hubalewska-Mazgaj M, Kwiecien S, Wallace JL, et al. The effect of hydrogen sulfide-releasing naproxen (ATB-346) *versus* naproxen on formation of stress-induced gastric lesions, the regulation of systemic inflammation, hypoxia and alterations in gastric microcirculation. *J Physiol Pharmacol* 2017;**68**:749−56.
93. Wallace JL, Nagy P, Feener TD, Allain T, Ditroi T, Vaughan DJ, et al. A proof-of-concept, phase 2 clinical trial of the gastrointestinal safety of a hydrogen sulfide-releasing anti-inflammatory drug. *Br J Pharmacol* 2020;**177**:769−77.
94. Stucki D, Krahl H, Walter M, Steinhausen J, Hommel K, Brenneisen P, et al. Effects of frequently applied carbon monoxide releasing molecules (CORMs) in typical CO-sensitive model systems—a comparative *in vitro* study. *Arch Biochem Biophys* 2020;**687**:108383.
95. Fiorucci S, Antonelli E, Distrutti E, Rizzo G, Mencarelli A, Orlandi S, et al. Inhibition of hydrogen sulfide generation contributes to gastric injury caused by anti-inflammatory nonsteroidal drugs. *Gastroenterology* 2005;**129**:1210−24.
96. Konturek SJ, Brzozowski T, Piastucki I, Radecki T. Prevention of ethanol and aspirin-induced gastric mucosal lesions by paracetamol and salicylate in rats: role of endogenous prostaglandins. *Gut* 1982;**23**:536−40.
97. Kocer G, Nasircilar Ulker S, Senturk UK. The contribution of carbon monoxide to vascular tonus. *Microcirculation* 2018;**25**:e12495.
98. de Backer O, Elinck E, Sips P, Buys E, Brouckaert P, Lefebvre RA. Role of the soluble guanylyl cyclase alpha1/alpha2 subunits in the relaxant effect of CO and CORM-2 in murine gastric fundus. *Naunyn-Schmiedeberg's Arch Pharmacol* 2008;**378**:493−502.
99. Moon H, Jang JH, Jang TC, Park GH. Carbon monoxide ameliorates 6-hydroxydopamine-induced cell death in C6 glioma cells. *Biomol Ther (Seoul)* 2018;**26**:175−81.
100. Magierowska K, Magierowski M, Surmiak M, Adamski J, Mazur-Bialy AI, Pajdo R, et al. The protective role of carbon monoxide (CO) produced by heme oxygenases and derived from the CO-releasing molecule CORM-2 in the pathogenesis of stress-induced gastric lesions: evidence for non-involvement of nitric oxide (NO). *Int J Mol Sci* 2016;**17**:442.
101. Magierowska K, Bakalarz D, Wójcik D, Korbut E, Danielak A, Głowacka U, et al. Evidence for cytoprotective effect of carbon monoxide donor in the development of acute esophagitis leading to acute esophageal epithelium lesions. *Cells* 2020;**9**:1203.
102. Hovater MB, Ying WZ, Agarwal A, Sanders PW. Nitric oxide and carbon monoxide antagonize TGF-beta through ligand-independent internalization of TbetaR1/ALK5. *Am J Physiol Ren Physiol* 2014;**307**:F727−35.

103. Perretti M, D'Acquisto F. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nat Rev Immunol* 2009;**9**:62–70.
104. Goppelt-Struebe M, Wolter D, Resch K. Glucocorticoids inhibit prostaglandin synthesis not only at the level of phospholipase A2 but also at the level of cyclo-oxygenase/PGE isomerase. *Br J Pharmacol* 1989;**98**:1287–95.
105. Tarnawski A, Ahluwalia A, Jones MK. Gastric cytoprotection beyond prostaglandins: cellular and molecular mechanisms of gastroprotective and ulcer healing actions of antacids. *Curr Pharmaceut Des* 2013;**19**:126–32.
106. Zanardo RC, Perretti M, Wallace JL. Annexin-1 is an endogenous gastroprotective factor against indomethacin-induced damage. *Am J Physiol Gastrointest Liver Physiol* 2005;**288**:G481–6.
107. Magierowska K, Bakalarz D, Wojcik D, Chmura A, Hubalewska-Mazgaj M, Licholai S, et al. Time-dependent course of gastric ulcer healing and molecular markers profile modulated by increased gastric mucosal content of carbon monoxide released from its pharmacological donor. *Biochem Pharmacol* 2019;**163**:71–83.
108. Nikolic I, Saksida T, Vujicic M, Stojanovic I, Stosic-Grujicic S. Anti-diabetic actions of carbon monoxide-releasing molecule (CORM)-A1: immunomodulation and regeneration of islet beta cells. *Immunol Lett* 2015;**165**:39–46.
109. Lin CC, Hsiao LD, Cho RL, Yang CM. CO-releasing molecule-2 induces Nrf2/ARE-dependent heme oxygenase-1 expression suppressing TNF-alpha-induced pulmonary inflammation. *J Clin Med* 2019;**8**:436.
110. Takagi T, Naito Y, Tanaka M, Mizushima K, Ushiroda C, Toyokawa Y, et al. Carbon monoxide ameliorates murine T-cell-dependent colitis through the inhibition of Th17 differentiation. *Free Radic Res* 2018;**52**:1328–35.
111. Babu D, Motterlini R, Lefebvre RA. CO and CO-releasing molecules (CO-RMs) in acute gastrointestinal inflammation. *Br J Pharmacol* 2015;**172**:1557–73.

International Journal of
Molecular Sciences

MDPI

Article

Novel Hydrogen Sulfide (H_2S)-Releasing BW-HS-101 and Its Non-H_2S Releasing Derivative in Modulation of Microscopic and Molecular Parameters of Gastric Mucosal Barrier

Dominik Bakalarz [1,2], Edyta Korbut [1], Zhengnan Yuan [3], Bingchen Yu [3], Dagmara Wójcik [1], Aleksandra Danielak [1], Katarzyna Magierowska [1], Slawomir Kwiecień [1], Tomasz Brzozowski [1], Monika Marcinkowska [4], Binghe Wang [3,*] and Marcin Magierowski [1,*]

1 Department of Physiology, Jagiellonian University Medical College, 31531 Cracow, Poland; dominik.bakalarz@uj.edu.pl (D.B.); edyta.korbut@uj.edu.pl (E.K.); dagmara1.wojcik@uj.edu.pl (D.W.); aleksandra26.danielak@student.uj.edu.pl (A.D.); katarzyna.magierowska@uj.edu.pl (K.M.); skwiecien@cm-uj.krakow.pl (S.K.); mpbrzozo@cyf-kr.edu.pl (T.B.)
2 Department of Forensic Toxicology, Institute of Forensic Research, 31033 Cracow, Poland
3 Department of Chemistry and Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA 30302, USA; zyuan3@gsu.edu (Z.Y.); yubingchen2013@gmail.com (B.Y.)
4 Faculty of Pharmacy, Jagiellonian University Medical College, 30688 Cracow, Poland; monika.marcinkowska@uj.edu.pl
* Correspondence: bwang31@gsu.edu (B.W.); m.magierowski@uj.edu.pl (M.M.)

Citation: Bakalarz, D.; Korbut, E.; Yuan, Z.; Yu, B.; Wójcik, D.; Danielak, A.; Magierowska, K.; Kwiecień, S.; Brzozowski, T.; Marcinkowska, M.; et al. Novel Hydrogen Sulfide (H_2S)-Releasing BW-HS-101 and Its Non-H_2S Releasing Derivative in Modulation of Microscopic and Molecular Parameters of Gastric Mucosal Barrier. *Int. J. Mol. Sci.* **2021**, *22*, 5211. https://doi.org/10.3390/ijms22105211

Academic Editor: Khosrow Kashfi

Received: 19 March 2021
Accepted: 11 May 2021
Published: 14 May 2021

Abstract: Hydrogen sulfide (H_2S) is an endogenously produced molecule with anti-inflammatory and cytoprotective properties. We aimed to investigate for the first time if a novel, esterase-sensitive H_2S-prodrug, BW-HS-101 with the ability to release H_2S in a controllable manner, prevents gastric mucosa against acetylsalicylic acid-induced gastropathy on microscopic and molecular levels. Wistar rats were pretreated intragastrically with vehicle, BW-HS-101 (0.5–50 μmol/kg) or its analogue without the ability to release H_2S, BW-iHS-101 prior to ASA administration (125 mg/kg, intragastrically). BW-HS-101 was administered alone or in combination with nitroarginine (L-NNA, 20 mg/kg, intraperitoneally) or zinc protoporphyrin IX (10 mg/kg, intraperitoneally). Gastroprotective effects of BW-HS-101 were additionally evaluated against necrotic damage induced by intragastrical administration of 75% ethanol. Gastric mucosal damage was assessed microscopically, and gastric blood flow was determined by laser flowmetry. Gastric mucosal DNA oxidation and PGE_2 concentration were assessed by ELISA. Serum and/or gastric protein concentrations of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, VEGF, GM-CSF, IFN-γ, TNF-α, and EGF were determined by a microbeads/fluorescent-based multiplex assay. Changes in gastric mucosal iNOS, HMOX-1, SOCS3, IL1-R1, IL1-R2, TNF-R2, COX-1, and COX-2 mRNA were assessed by real-time PCR. BW-HS-101 or BW-iHS-101 applied at a dose of 50 μmol/kg protected gastric mucosa against ASA-induced gastric damage and prevented a decrease in the gastric blood flow level. H_2S prodrug decreased DNA oxidation, systemic and gastric mucosal inflammation with accompanied upregulation of SOCS3, and EGF and HMOX-1 expression. Pharmacological inhibition of nitric oxide (NO) synthase but not carbon monoxide (CO)/heme oxygenase (HMOX) activity by L-NNA or ZnPP, respectively, reversed the gastroprotective effect of BW-HS-101. BW-HS-101 also protected against ethanol-induced gastric injury formation. We conclude that BW-HS-101, due to its ability to release H_2S in a controllable manner, prevents gastric mucosa against drugs-induced gastropathy, inflammation and DNA oxidation, and upregulate gastric microcirculation. Gastroprotective effects of this H_2S prodrug involves endogenous NO but not CO activity and could be mediated by cytoprotective and anti-inflammatory SOCS3 and EGF pathways.

Keywords: hydrogen sulfide prodrugs; BW-HS-101; non-steroidal anti-inflammatory drugs; gastrotoxicity; molecular gastroenterology; gastrointestinal pharmacology

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs), including acetylsalicylic acid (ASA, the active ingredient of aspirin), exert analgesic, anti-pyretic, and anti-inflammatory effects. The mechanism of action of ASA is through the inhibition of cyclooxygenases (COX-1, COX-2), enzymes involved in the synthesis of pro-inflammatory but also cytoprotective prostaglandins (PGs) such as PGE_2. However, inhibition of COXs also reduces thromboxane synthesis, leading to decreased platelet aggregation and an antithrombotic effect. Therefore, ASA is widely used for prevention of ischemic stroke and myocardial infarction [1]. Despite the beneficial effects of ASA on the cardiovascular system, the adverse effects of this drug within the gastrointestinal (GI) tract, such as gastric mucosal injuries, remain a major limitation of its therapeutic effectiveness in humans [2]. The pathogenesis of this ASA-induced gastrotoxicity is attributed to its topical and systemic effects [3,4] since this drug irreversibly inhibits COX-1 and COX-2. Deficiency of PGs in the gastric mucosa is involved in the impairment of gastric mucosal integrity. Moreover, ASA itself is chemically irritating and cytotoxic for gastric epithelial cells [5].

Hydrogen sulfide (H_2S) is an intracellular gaseous signaling molecule and participates in many physiological and pathological processes within the GI tract and in other parts of the body [6,7]. Numerous studies have shown that H_2S serves as an anti-oxidative, anti-inflammatory, and cytoprotective agent [8,9]. For instance, H_2S has been reported to inhibit the adherence of leukocytes to blood vessel walls [10]. This simple inorganic molecule has also been shown to have a vasodilatory effect, similar to its sister endogenous gaseous mediators, carbon monoxide (CO) and nitric oxide (NO) [11–15]. H_2S is produced endogenously by the metabolism of L-cysteine, mainly with the involvement of two pyridoxal-5-phosphate (P5P, vitamin B6)-dependent enzymes: cystathionine-γ-lyase (CTH) and cystathionine-β-synthase (CBS) [16,17]. Alternatively, H_2S is biosynthesized with the participation of mercaptopyruvate-3-sulfurtransferase (MPST) with the coactivity of cysteine aminotransferase [18]. H_2S can also be produced from sulfates without enzymatic activity by colonic bacteria [19].

Previous studies have shown that diallyl disulfide (DADS), a H_2S donor derived from garlic, inhibited proliferation of human colon cancer HT29 cells in a dose-dependent manner [20]. Moreover, pretreatment with sodium hydrosulfide (NaHS) prevented NSAIDs-induced gastrotoxicity [21]. It has been shown that H_2S inhibited lipid peroxidation and formation of acute stress-induced or ischemia/reperfusion-gastric damage [22,23]. Wallace et al. observed that daily intragastric treatment with L-cysteine or Lawesson's reagent, a H_2S donor, accelerated the healing of experimental chronic gastric ulcers [24]. In addition to its gastroprotective effects, NaHS was also observed to increase dose-dependently the secretion of HCO_3^- ions in the duodenum of rats, which resulted in the maintenance of intestinal mucosal integrity [25]. Interestingly, pretreatment with a synthetic slowly-release H_2S donor, GYY4137, reduced ischemia/reperfusion-induced gastric injury in rats [22]. Importantly, GI-safe H_2S-releasing derivatives of conventional NSAIDs have been developed recently. ATB-346, a naproxen derivative conjugated with 4-hydroxythiobenzamide, has been shown to have significantly reduced gastrotoxicity in pre-clinical experiments and in phase-2 clinical trials [26]. Therefore, the implementation of novel H_2S-releasing molecules seems to be a rational approach for the development of treatment options for pathologies in the GI. BW-HS-101 is a novel organic and esterase sensitive H_2S-releasing compound, which in contrast to sulfide salts like NaHS exerts controlled release rates of this gaseous mediator with a half-life about 13 min [27]. This H_2S prodrug has been reported to decrease lipopolysaccharide (LPS)-induced inflammatory responses in vitro [27].

In this study, we aimed to investigate for the first time if pretreatment with a novel H_2S prodrug, BW-HS-101 applied intragastrically (i.g.), protects gastric mucosa against ASA-induced injury and if this compound affects gastric blood flow (GBF). We have included the analogue of the H_2S-prodrug, BW-iHS-101, without the ability to release this gaseous mediator in this study (Figure 1). We aimed to additionally confirm the possible gastroprotective effect of BW-HS-101 against necrotic injuries induced by i.g.

administration of 75% ethanol. We focused on possible modulation of systemic and GI inflammatory responses and alterations in molecular patterns. We also examined the possible involvement of endogenous CO and NO produced by heme oxygenase (HMOX) or NO synthase (NOS) activities, respectively, in gastric mucosal integrity maintenance by BW-HS-101.

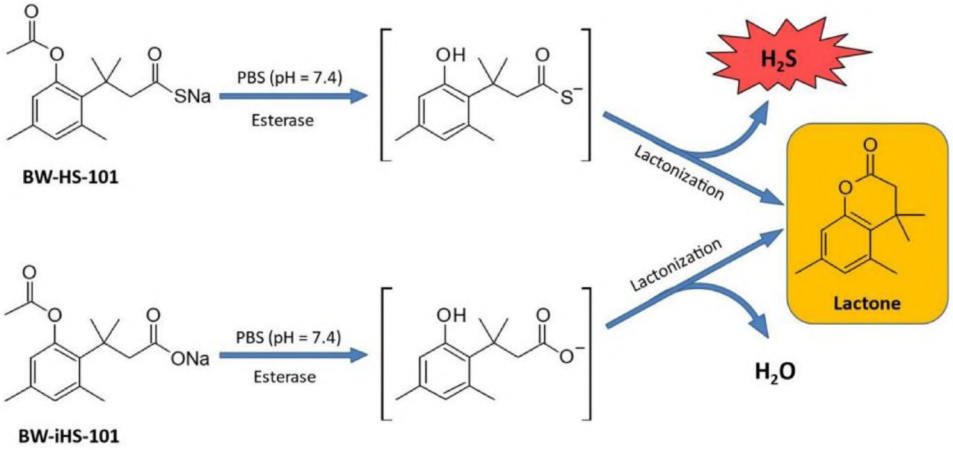


Figure 1. Hydrolysis of BW-HS-101 and BW-iHS-101 leading to the generation of lactone and hydrogen sulfide (H_2S) or water (H_2O), respectively.

2. Results

2.1. Chemical Conversion of BW-HS-101 and BW-iHS-101

Figure 1 demonstrates the biochemical conversions of BW-HS-101 and BW-iHS-101. Due to the activity of esterase, both compounds are hydrolyzed leading to the generation of the lactone product as an inactive metabolite, and H_2S or H_2O for BW-HS-101 and BW-iHS-101, respectively (Figure 1).

2.2. Possible Effects on the Gastric Damage Score, Gastric Blood Flow Alterations, and Systemic and Gastric Mucosal Inflammation. Pharmacological Evaluation of the Involvement of Endogenous NO and CO Biosynthesis Pathways

Figure 2(A1) shows that pretreatment with BW-HS-101 applied i.g. at a dose of 50 but not 0.5 or 5 μmol/kg significantly decreased ASA-induced gastric damage score by more than 50% as compared with vehicle-treated rats ($p < 0.05$). BW-iHS-101 (50 μmol/kg i.g.) but not lactone (50 μmol/kg i.g.) significantly decreased the ASA-induced gastric damage score as compared with vehicle (Figure 2(A1); $p < 0.05$). Additionally, Figure 2(A1) shows that i.g. pretreatment with nitroarginine (L-NNA, 20 mg/kg applied intraperitoneally (i.p.)) but not zinc protoporphyrin IX (ZnPP, 10 mg/kg i.p.) in combination with BW-HS-101 (50 μmol/kg i.g.) significantly decreased ASA-induced gastric damage score as compared with rats administered with BW-HS-101 (50 μmol/kg i.g.) alone ($p < 0.05$).

Figure 2(A2) shows representative microscopic photomicrographs of gastric mucosa pretreated with vehicle, BW-HS-101, BW-iHS-101 or lactone applied i.g. at a dose of 50 μmol/kg and administered with ASA (125 mg/kg i.g.). Topical application of ASA induced hemorrhagic erosions of the epithelial surface penetrating more than 250 μm into gastric mucosa, even reaching lamina propria with the width being more than 500 μm and with submucosal leukocytes infiltration (Figure 2(A2)). These alterations were not observed in intact gastric mucosa (Figure 2(A2)). Pretreatment with BW-HS-101 or BW-iHS-101 but not with lactone limited bleeding and the injury range to the superficial epithelium (Figure 2(A2)). Pretreatment with BW-HS-101 applied in combination with ZnPP but not with L-NNA limited gastric damage to the superficial epithelium (Figure 2(A2)).

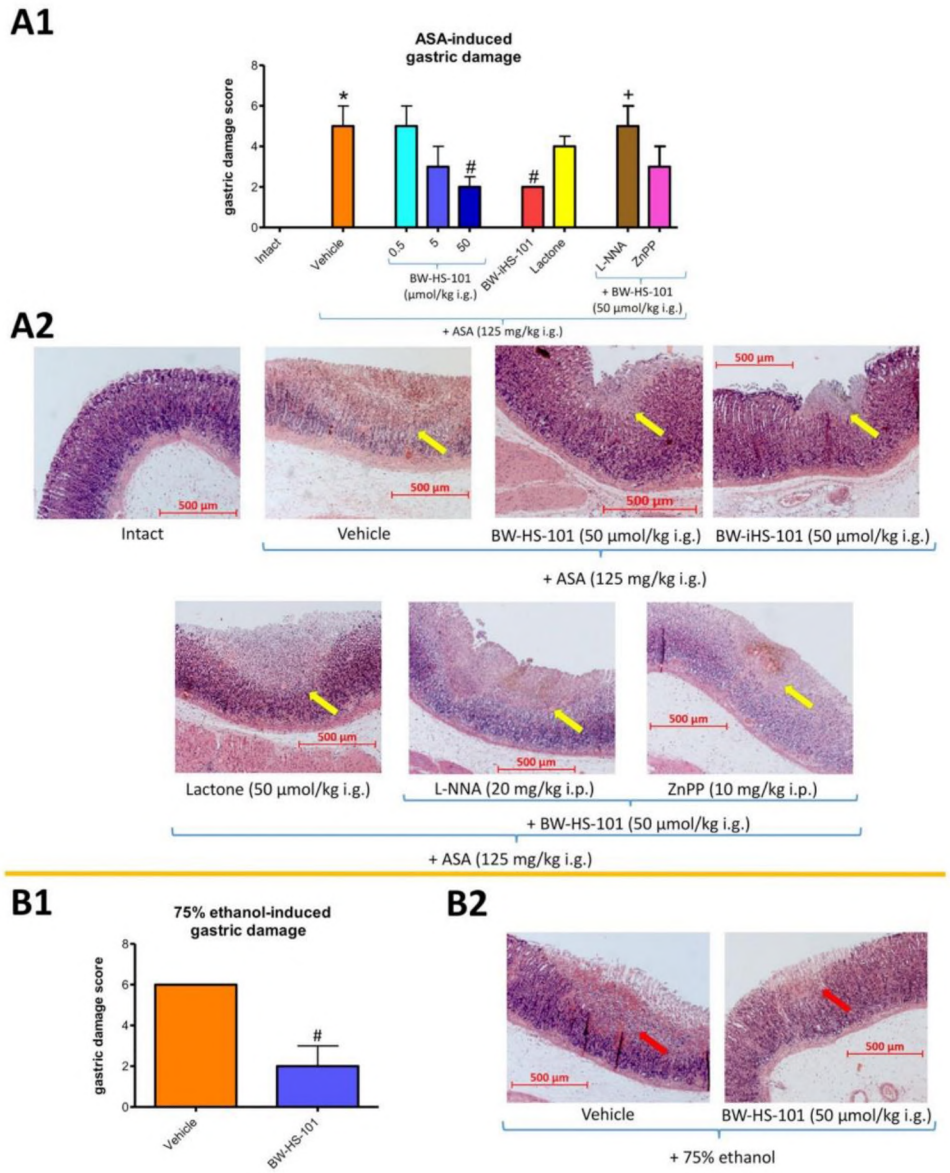


Figure 2. Gastric damage score (**A1**,**B1**) and representative histological slides (**A2**,**B2**) of gastric mucosa of rats administered i.g. with acetylsalicylic acid (ASA, 125 mg/kg) or 1 mL of 75% ethanol. Results are median ± interquartile range of five rats per each experimental group. Asterisk (*) indicates significant change as compared with intact ($p < 0.05$). Hash (#) indicates significant change as compared with vehicle ($p < 0.05$). Cross (+) indicates significant change as compared with BW-HS-101 (50 μmol/kg) ($p < 0.05$). (**A1**): Gastric damage score in rats administered with ASA and pretreated i.g. 30 min earlier with vehicle, BW-HS-101 (0.5–50 μmol/kg), BW-iHS-101 (50 μmol/kg), lactone (50 μmol/kg) or BW-HS-101 (50 μmol/kg) combined with nitroarginine (L-NNA, 20 mg/kg i.p.) or zinc protoporphyrin IX (ZnPP, 10 mg/kg i.p.). (**A2**): Representative histological slides of gastric mucosal damage induced by ASA (yellow arrows) in rats pretreated i.g. with vehicle, BW-HS-101 (50 μmol/kg), BW-iHS-101 (50 μmol/kg), lactone (50 μmol/kg), or BW-HS-101 (50 μmol/kg) combined with L-NNA or ZnPP. (**B1**): Gastric damage score in rats administered with 75% ethanol and pretreated i.g. 30 min earlier with vehicle or BW-HS-101 (50 μmol/kg). (**B2**): Representative histological slides of gastric mucosal damage induced by 75% ethanol (red arrows) in rats pretreated i.g. with vehicle or BW-HS-101 (50 μmol/kg).

Figure 2(B1) shows that pretreatment with BW-HS-101 (50 μmol/kg i.g.) significantly decreased ethanol-induced gastric damage score as compared with vehicle-treated rats ($p < 0.05$). Figure 2(B2) shows that topical application of 75% ethanol induced necrotic and hemorrhagic erosions of the epithelial surface penetrating more than 250 μm into the lamina propria of gastric mucosa with the width being more than 500 μm and with submucosal leukocytes infiltration. Pretreatment with BW-HS-101 reduced bleeding and limited the gastric erosion development to the superficial epithelium (Figure 2(B2)).

Administration of ASA significantly decreased the GBF as compared with intact gastric mucosa ($p < 0.05$) (Table 1). Pretreatment with BW-HS-101 (50 μmol/kg i.g.) or BW-iHS-101 (50 μmol/kg i.g.) but not lactone (50 μmol/kg i.g.) significantly increased GBF in rats with ASA-induced gastric damage as compared to the group pretreated with vehicle ($p < 0.05$, Table 1). Pretreatment with BW-HS-101 (50 μmol/kg i.g.) significantly increased GBF as compared with BW-iHS-101 (50 μmol/kg i.g.) ($p < 0.05$, Table 1). Pretreatment with L-NNA (20 mg/kg i.p.) but not with ZnPP (10 mg/kg i.p.) in combination with BW-HS-101 (50 μmol/kg i.g.) significantly decreased GBF in rats with ASA-induced gastric damage as compared to the group pretreated with BW-HS-101 applied alone ($p < 0.05$, Table 1). Pretreatment with ZnPP (10 mg/kg i.p.) but not with L-NNA (20 mg/kg i.p.) in combination with BW-HS-101 (50 μmol/kg i.g.) significantly increased GBF in rats with ASA-induced gastric damage as compared to the group pretreated with vehicle ($p < 0.05$, Table 1).

Table 1. Gastric blood flow (GBF) in gastric mucosa of rats pretreated i.g. with vehicle, BW-HS-101 (50 μmol/kg), BW-iHS-101 (50 μmol/kg), lactone (50 μmol/kg), or BW-HS-101 applied in combination with nitroarginine (L-NNA, 20 mg/kg i.p.) or zinc protoporphyrin IX (ZnPP, 10 mg/kg i.p.), 30 min before i.g. administration of acetylsalicylic acid (ASA, 125 mg/kg). Intact refers to the values obtained in healthy gastric mucosa without ASA-induced gastric damage. Results are mean ± SEM of five rats per group. Significant changes as compared with the respective values in intact gastric mucosa are indicated by asterisk (*) ($p < 0.05$). Hash (#) indicates significant changes as compared with vehicle administered with ASA ($p < 0.05$). Hat (^) indicates significant difference as compared with BW-HS-101 ($p < 0.05$).

Experimental Group	GBF [% of Control]
Intact	99.99 ± 2.371
Vehicle + ASA	58.45 ± 2.795 *
BW-HS-101 + ASA	81.16 ± 1.232 #
BW-iHS-101 + ASA	70.05 ± 2.646 #^
Lactone + ASA	64.25 ± 1.963
BW-HS-101 + L-NNA + ASA	66.18 ± 3.206 ^
BW-HS-101 + ZnPP + ASA	84.54 ± 4.113

Figure 3 shows that in rats administered with ASA, serum concentrations of IL-1β (A), TNF-α (B), IL-10 (C), and VEGFA (D) were significantly increased as compared with intact animals ($p < 0.05$). Pretreatment with BW-HS-101 (50 μmol/kg) and BW-iHS-101 (50 μmol/kg) but not with lactone (50 μmol/kg) significantly decreased serum concentrations of IL-1β (A), TNF-α (B), IL-10 (C) and VEGFA (D) as compared with vehicle ($p < 0.05$).

Figure 4A–F shows that in rats administered with ASA, gastric mucosal mRNA expression of inducible NOS (iNOS) (A), HMOX-1 (B), suppressor of cytokine signaling 3 (SOCS3) (C), interleukin (IL)1-receptor 1 (R1) (D), IL-1R2 (E), tumor necrosis factor (TNF)-receptor 2 (R2) (F) was significantly increased as compared with intact animals ($p < 0.05$). Pretreatment with BW-HS-101 (50 μmol/kg) or BW-iHS-101 (50 μmol/kg) significantly decreased IL-1R2 mRNA expression (E) but did not significantly affected mRNA expression of iNOS (A), HMOX-1 (B), IL1-R1 (D), and TNF-R2 (F) as compared with vehicle (Figure 4).

BW-HS-101 but not BW-iHS-101 significantly upregulated SOCS3 mRNA expression as compared with vehicle ($p < 0.05$, Figure 4C). Figure 4G–L shows that in rats pretreated with lactone (50 µmol/kg), gastric mucosal mRNA expression of iNOS (G), HMOX-1 (H), SOCS3 (I), IL1-R1 (J), IL-1R2 (K), TNF-R2 (L) was not significantly altered as compared with vehicle.

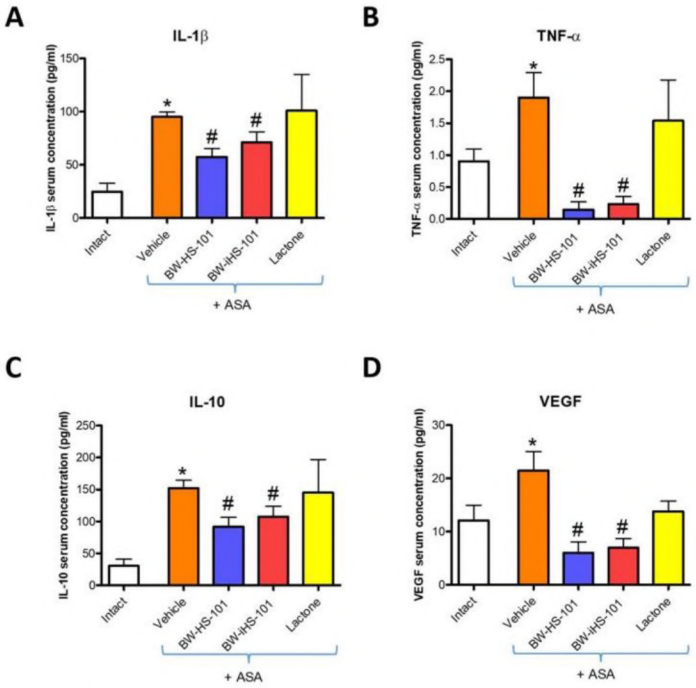


Figure 3. Concentration of interleukin (IL)-1β (**A**), tumor necrosis factor (TNF)-α (**B**), IL-10 (**C**), or vascular endothelial growth factor A (VEGF) (**D**) in serum of rats administered i.g. with acetylsalicylic acid (ASA, 125 mg/kg) and pretreated 30 min earlier with vehicle, BW-HS-101 (50 µmol/kg), BW-iHS-101 (50 µmol/kg), or lactone (50 µmol/kg). Results are mean ± SEM of five rats per each experimental group. Significant changes as compared with the respective values in intact rats are indicated by asterisk (*) ($p < 0.05$). Hash (#) indicates significant changes as compared with vehicle ($p < 0.05$).

2.3. Molecular Pattern of Gastric Mucosal Proteins

Figure 5 shows that pretreatment with BW-HS-101 (50 µmol/kg i.g.) but not with BW-iHS-101 (50 µmol/kg i.g.) significantly decreased gastric mucosal concentration of IL-1α (A), IL-1β (B), IL-2 (C), IL-4 (D), IL-6 (E), IL-10 (F), granulocyte-macrophage colony-stimulating factor (GM-CSF) (H), interferon (IFN)γ (I), TNF-α (J) as compared with vehicle in rats administered with ASA ($p < 0.05$). Figure 5 shows that BW-HS-101 and BW-iHS-101 significantly increased gastric mucosal protein concentration of epidermal growth factor (EGF) (K) but not vascular endothelial growth factor A (VEGF) (L) or IL-13 (G) as compared with vehicle in rats administered with ASA ($p < 0.05$). Based on the above-reported data, the lactone-pretreated group was not included in this experiment. Alterations in inflammatory and oxidative response markers within gastric mucosa exposed to ASA vs. intact were also in part confirmed as shown on Figures 3 and 4. The intact group was also not analyzed within this experimental series, since the alterations in the abovementioned targets concentration on a systemic level after administration of ASA vs. intact were previously reported [28].

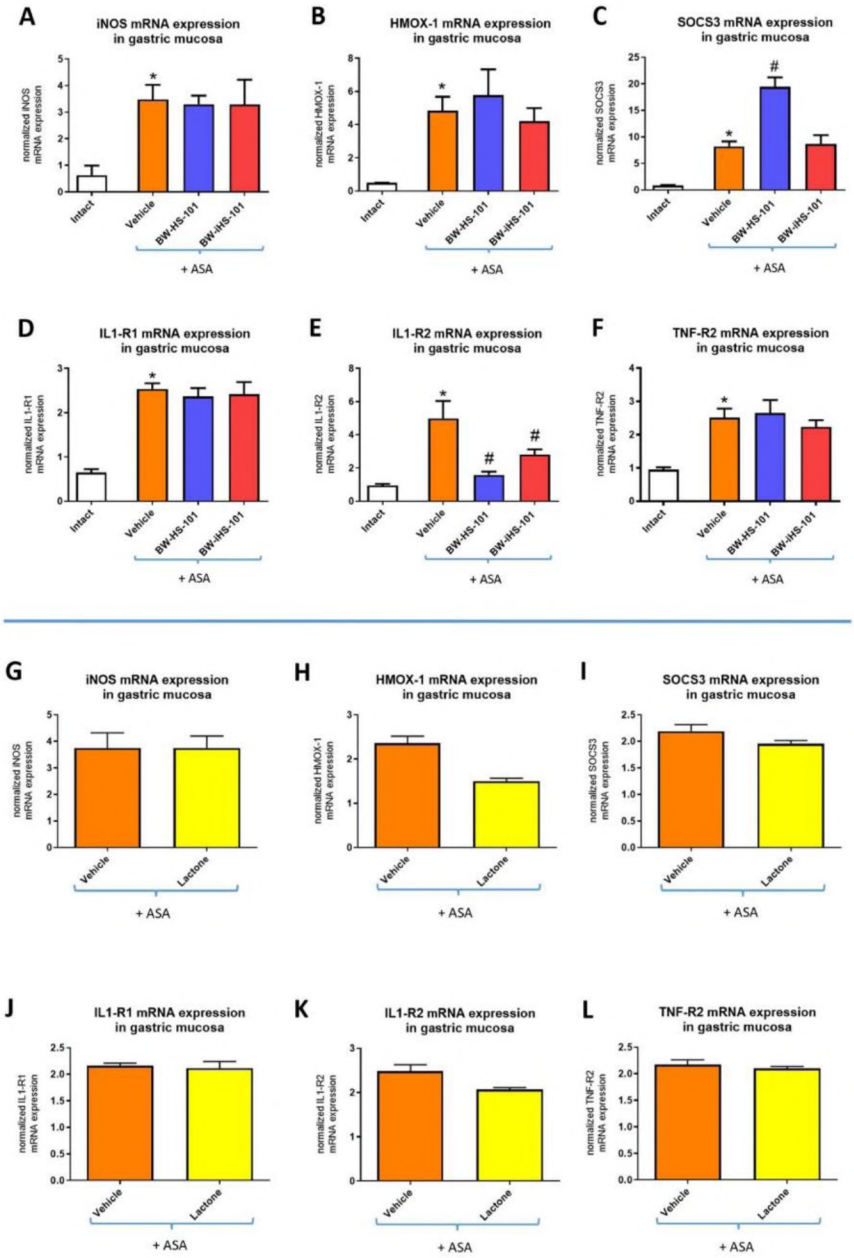


Figure 4. Gastric mucosal mRNA expression of inducible nitric oxide synthase (iNOS) (**A**,**G**), heme oxygenase (HMOX)-1 (**B**,**H**), suppressor of cytokine signaling 3 (SOCS3) (**C**,**I**), interleukin (IL) receptor (R)1 (**D**,**J**), IL-R2 (**E**,**K**), and tumor necrosis factor (TNF)-R2 (**F**,**L**) in rats administered i.g. with acetylsalicylic acid (ASA, 125 mg/kg) and pretreated 30 min earlier with vehicle, BW-HS-101 (50 μmol/kg), BW-iHS-101 (50 μmol/kg) or lactone (50 μmol/kg). Results are mean ± SEM of five rats per each experimental group. Significant changes as compared with the respective values in intact rats are indicated by asterisk (*) ($p < 0.05$). Hash (#) indicates significant changes as compared with vehicle ($p < 0.05$). (**G**–**L**): Results were reported on separate figures because technically the data were calculated based on the different reference sample and the fold-change values for the same vehicle group are different than on panels (**A**–**F**).

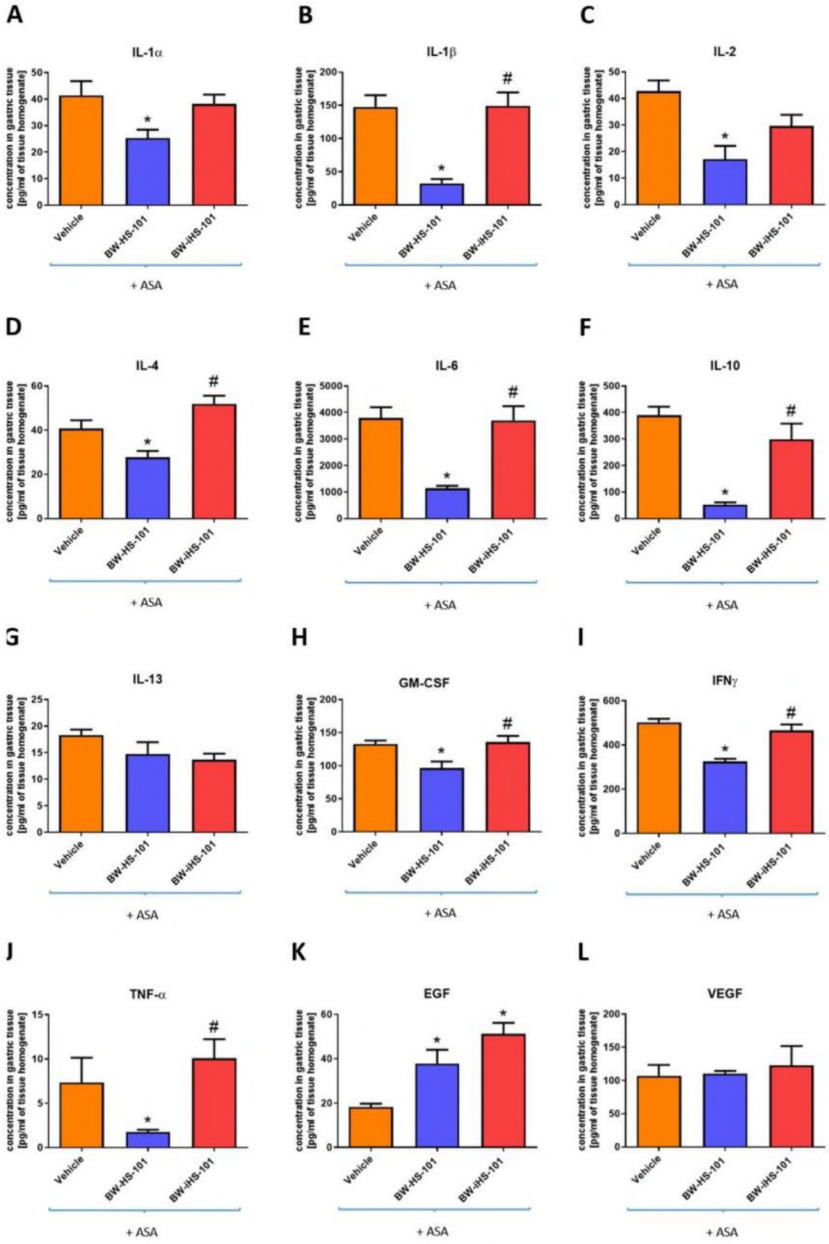


Figure 5. Concentration of interleukin (IL)-1α (**A**), IL-1β (**B**), IL-2 (**C**), IL-4 (**D**), IL-6 (**E**), IL-10 (**F**), IL-13 (**G**), granulocyte-macrophage colony-stimulating factor (GM-CSF) (**H**), interferon (IFN) γ (**I**), tumor necrosis factor (TNF)-α (**J**), epidermal growth factor (EGF) (**K**), or vascular endothelial growth factor (VEGF) (**L**) in gastric mucosa of rats administered i.g. with acetylsalicylic acid (ASA, 125 mg/kg) and pretreated 30 min earlier with vehicle, BW-HS-101 (50 μmol/kg), BW-iHS-101 (50 μmol/kg) or lactone (50 μmol/kg). Results are mean ± SEM of five rats per each experimental group. Significant changes as compared with the respective values in vehicle-treated rats are indicated by asterisk (*) ($p < 0.05$). Hash (#) indicates significant changes as compared with BW-HS-101 ($p < 0.05$).

2.4. Possible Alterations in the Gastric Mucosal Prostaglandins/Cyclooxygenase Pathway Activity

Figure 6A shows that in rats administered with ASA gastric mucosal concentration of PGE_2 was significantly decreased as compared with intact animals ($p < 0.05$). Pretreatment with BW-HS-101 (50 µmol/kg) or BW-iHS-101 (50 µmol/kg) did not significantly affected PGE_2 concentration as compared with vehicle (Figure 6A). Figure 6B shows that in rats administered with ASA gastric mucosal mRNA expression of COX-1 was not significantly altered as compared with intact animals. Pretreatment with BW-HS-101 (50 µmol/kg) or BW-iHS-101 (50 µmol/kg) did not significantly affected COX-1 mRNA expression as compared with vehicle (Figure 6B). Figure 6C shows that in rats administered with ASA gastric mucosal mRNA expression of COX-2 was significantly upregulated as compared with intact animals ($p < 0.05$). Pretreatment with BW-HS-101 (50 µmol/kg) or BW-iHS-101 (50 µmol/kg) significantly decreased COX-2 mRNA expression as compared with vehicle ($p < 0.05$, Figure 6C). Based on the above-reported data, the lactone-pretreated group was not included in this experiment.

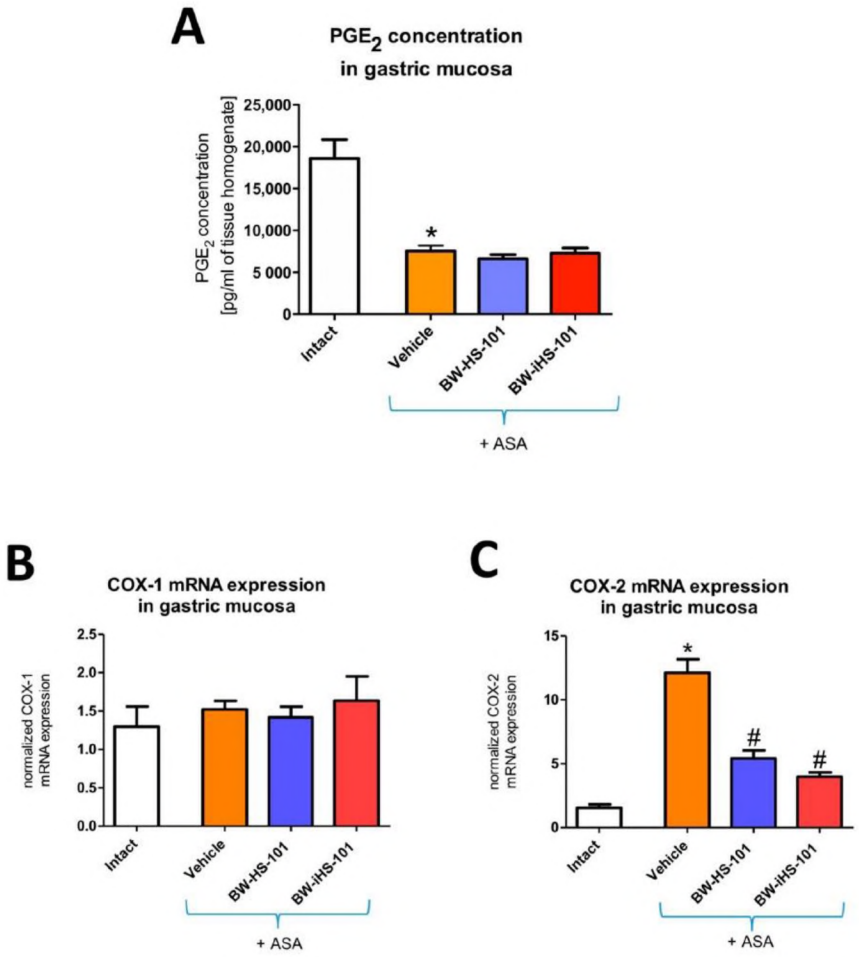


Figure 6. Gastric mucosal concentration of prostaglandin E_2 (**A**) and mRNA expression of cyclooxygenase (COX)-1 (**B**) and COX-2 (**C**) in rats administered i.g. with acetylsalicylic acid (ASA, 125 mg/kg) and pretreated 30 min earlier with vehicle, BW-HS-101 (50 µmol/kg) or BW-iHS-101 (50 µmol/kg). Results are mean ± SEM of five rats per each experimental group. Significant changes as compared with the respective values in intact rats are indicated by asterisk (*) ($p < 0.05$). Hash (#) indicates significant changes as compared with vehicle ($p < 0.05$).

2.5. Oxidation of DNA in Gastric Mucosa

Figure 7 shows that in rats administered with ASA gastric mucosal concentration of 8-hydroxy-deoxyguanosine (8-OHdG) was significantly increased as compared with intact animals ($p < 0.05$). Pretreatment with BW-HS-101 (50 µmol/kg) or BW-iHS-101 (50 µmol/kg) significantly decreased gastric mucosal 8-OHdG concentration as compared with vehicle ($p < 0.05$, Figure 7). Based on the above-reported data, the lactone-pretreated group was not included in this experiment.

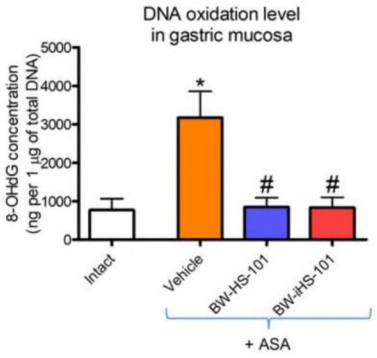


Figure 7. Gastric mucosal concentration of 8-hydroxy-deoxyguanosine (8-OHdG) in rats administered i.g. with acetylsalicylic acid (ASA, 125 mg/kg) and pretreated 30 min earlier with vehicle, BW-HS-101 (50 µmol/kg) or BW-iHS-101 (50 µmol/kg). Results are mean ± SEM of five rats per each experimental group. Significant changes as compared with the respective values in intact rats are indicated by asterisk (*) ($p < 0.05$). Hash (#) indicates significant changes as compared with vehicle ($p < 0.05$).

2.6. Bioinformatic Evaluation of Possible Molecular Targets

Supplementary Tables S1 and S2 show that based on bioinformatic analysis there is no specific molecular target for BW-HS-101 and BW-iHS-101, respectively, with probability of interaction higher than 0.12 (calculated scores 0–0.11) [29].

3. Discussion

H_2S as an endogenous gaseous mediator has been shown to be involved in regulation of many physiological functions within the cardiovascular and digestive systems [30–32]. H_2S-releasing compounds were reported to exert anti-inflammatory and anti-oxidative properties under experimental conditions [33–36]. NaHS was shown to protect gastric mucosa against damage induced by ASA, alendronates, ethanol or by the exposure to stress or ischemia/reperfusion [37–40]. This chemical, together with Lawesson's reagent, has been also reported to accelerate gastric ulcer healing [24]. Nevertheless, the balance between beneficial/toxic effects of H_2S was strictly dose-dependent [22,41]. Therefore, over the last few years, several H_2S-prodrugs were developed, capable of releasing this molecule in a somewhat controllable manner [27,42–44]. GYY4137, a pharmacological H_2S donor, was observed to protect GI tract against acute oxidative injury and to exert chemoprevention effects [22,45]. A novel mitochondria-targeted H_2S donor, AP-39, was shown to be protective against myocardial reperfusion injury [46]. Interestingly, new derivatives of NSAIDs were developed, such as H_2S-releasing naproxen (ATB-346) or ketoprofen (ATB-352) [26,47]. These compounds were shown to have reduced GI toxicity with comparable or even more effective anti-inflammatory activity [26,47].

In our study, we have investigated for the first time the gastroprotective effect of novel organic H_2S-prodrug, BW-HS-101 against the damage induced by the exposure to ASA or ethanol. BW-HS-101 is an esterase-sensitive compound releasing H_2S in a controllable manner with a half-life of 13 min [27]. Importantly, esterases were reported to be active

within gastric mucosa [48,49]. We observed that i.g. topical pretreatment with BW-HS-101 or BW-iHS-101 as its analogue without the ability to release H_2S before exposure to a high dose of ASA (125 mg/kg i.g.) protected gastric mucosa against the damage induced by this NSAID. Moreover, gastroprotection of BW-HS-101 was further confirmed since this H_2S-prodrug effectively prevented necrotic gastric mucosal damage induced by i.g. application of 75% ethanol. This effect of H_2S-prodrug was accompanied by elevated GBF. Interestingly, BW-HS-101 did not reverse gastric mucosal PGE_2 production, which is inhibited by well-known ASA activity. Pretreatment with BW-HS-101 decreased ASA-induced DNA oxidation in gastric mucosa. This is consistent with previously published data showing that NaHS decreased lipid peroxidation in the stomach induced by topical administration of ASA [50]. Our bioinformatic analysis of possible molecular targets of BW-HS-101 did not reveal any protein target that BW-HS-101 could interact with (calculated scores 0–0.11). Thus, we focused on oxidative- and inflammatory-response markers specific for gastric mucosal barrier maintenance, selected based on our previously published data [51]. BW-HS-101 exerted anti-inflammatory effects on the systemic level observed as decreased serum contents of IL-1β, TNF-α, IL-10, or VEGFA and locally as expressed by decreased gastric mucosal mRNA fold changes for IL-1R2 and COX-2. Importantly, BW-HS-101 maintained elevated anti-oxidative HMOX-1 and further enhanced anti-inflammatory SOCS3 mRNA expression increased in gastric mucosa exposed to ASA. Additionally, pretreatment with BW-HS-101 maintained upregulated mRNA expression of iNOS as a result of ASA administration. HMOX is an enzyme involved in the endogenous production of another gaseous mediator, CO [52]. While iNOS activity results in generation of endogenous NO [53]. Moreover, CO and NO, together with H_2S were reported to contribute to the maintenance of gastric mucosal integrity and in modulation of gastric microcirculation [13]. Interestingly, our study revealed that pharmacological inhibition of NOS but not HMOX activity reversed the gastroprotective and vasodilatory effects of BW-HS-101. Thus, we assume that BW-HS-101 exerts gastroprotective activity via NO/NOS and SOCS3 pathways. However, CO/HMOX-1 contribution in BW-HS-101-mediated gastroprotection may exist, but not to a significant degree. Additionally, it has been reported previously that the H_2S donor, NaHS, reduced chronic heart failure, possibly due to upregulation of HMOX-1 mRNA expression [54]. Moreover, NaHS-mediated gastroprotection against ASA-induced gastric damage has been shown to be NO biosynthesis-dependent [50].

Interestingly, we observed in our study that BW-iHS-101, chemically not able to release H_2S, exerted similar gastroprotection against ASA-induced erosions on the microscopic level as compared with BW-HS-101. BW-iHS-101 also elevated gastric microcirculation but to a lower extent than BW-HS-101. Nevertheless, both compounds did not affect gastric mucosal PGE_2 production and both decreased mRNA expression and/or serum concentration of inflammatory response markers such as IL-1R2, IL-1β, TNF-α, IL-10, and VEGFA, in parallel with reduced DNA oxidation. The bioinformatic analysis performed for BW-iHS-101 did not indicate any possible target protein (calculated scores 0–0.11). As it has been reported previously, BW-HS-101 or BW-iHS-101 is converted in the presence of esterase to a lactone derivative and H_2S or H_2O, respectively (Figure 1) [27]. Therefore, to evaluate the potential activity related with the biological effects of the tested compounds, we also evaluated the lactone for its possible molecular effectiveness within the gastric mucosa. We have found that the lactone derivative was biologically inactive. Our data has shown that pretreatment with lactone itself did not reduce the gastric damage score and did not affect GBF. Moreover, this compound was not effective in terms of inflammatory response inhibition since IL-1β, TNF-α, IL-10, VEGFA serum concentration and IL-R1, TNF-R2, IL-1R1, IL-1R2, and iNOS gastric mucosal mRNA expression were not decreased after subsequent administration of ASA. Importantly, in parallel with decreased NSAID-induced gastrotoxicity, BW-HS-101 and BW-iHS-101 maintained upregulated gastric mucosal mRNA expression for anti-oxidative HMOX-1 as well as increased concentration of EGF protein. However, H_2S-releasing BW-HS-101 but not BW-iHS-101 upregulated gastric mucosal mRNA expression of anti-inflammatory SOCS-3 accompanied by decreased gastric mucosal

concentration of inflammatory markers such as IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFNγ, TNF-α. This suggests that due to its ability to release H_2S, BW-HS-101 is an effective gastroprotective compound with a different anti-inflammatory molecular pattern compared to BW-iHS-101.

According to experimental protocol, there was about 1.5–2 h between the pretreatments and the termination of experiments. Over this relatively short period of time, we were unable to observe any possible side effects of BW-HS-101 or BW-iHS-101 administration and toxicological analysis was not within the scope of our study.

4. Conclusions

We conclude that possibly because of the similar chemical structure both, BW-HS-101 and BW-iHS-101 exert gastroprotective effect against NSAID-induced gastric damage. Nevertheless, due to the ability of BW-HS-101 to release H_2S, this compound, in contrast to BW-iHS-101, more effectively upregulated gastric microcirculation and induced antioxidative and anti-inflammatory pathways as reflected by increased gastric mucosal mRNA expression for HMOX-1 and SOCS3, respectively, possibly leading to inhibition of local inflammatory response within gastric mucosa exposed to ASA (Figure 8).

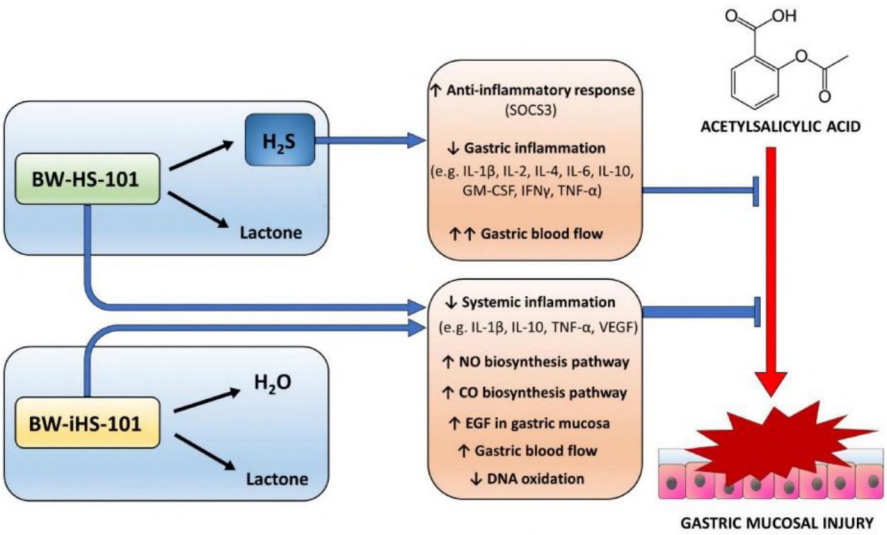


Figure 8. Overview on the possible mechanisms of BW-HS-101 and BW-iHS-101 gastroprotection against acetylsalicylic acid-induced injury. Abbreviations: H_2S—hydrogen sulfide, NO—nitric oxide, CO—carbon monoxide, SOCS3—suppressor of cytokine signaling 3, IL—interleukin, TNF—tumor necrosis factor, VEGF—vascular endothelial growth factor A, EGF—epidermal growth factor, GM-CSF—granulocyte-macrophage colony-stimulating factor.

5. Materials and Methods

5.1. Experimental Design

Experimental design involved fifty-five male Wistar rats with the age of 8–10 weeks and with average weight of 250–300 g. According to experimental protocol there was approximately 1.5–2 h between the first treatment and the termination of the experiment. Thus, weight changes were not assumed to be significantly altered after such a short period of time. Animals were fasted for 24 h with free access to tap water before each experiment. All procedures were approved by the 1st Local Ethical Committee for Care and Use of Experimental Animals, held by Faculty of Pharmacy, Jagiellonian University Medical College in Cracow (Decision No.: 311/2019; Date: 17 July 2019). Experiments were run with implications for replacement, refinement, or reduction (the 3Rs) principle and in compliance with the ARRIVE guidelines. Rats were randomly assigned to the

appropriate experimental groups (five rats each) and were pretreated i.g. by orogastric tube with (1) 1mL of dimethyl sulfoxide (DMSO)/H_2O (1:9) as vehicle, (2–4) H_2S prodrug BW-HS-101 (0.5–50 μmol/kg), (5) its analogue, BW-iHS-101, without the ability to release H_2S or (6) inactive metabolite of these compounds (lactone). BW-iHS-101 and lactone were applied at a dose of 50 μmol/kg, which is the equivalent of the effective dose of BW-HS-101 capable of reducing aspirin-induced gastric injury area by more than 50%. BW-HS-101 (50 μmol/kg) was also administered i.g. in combination with HMOX inhibitor, (7) ZnPP (10 mg/kg i.p.), or NOS inhibitor, (8) L-NNA (20 mg/kg i.p.), applied with the dose regimen based on previously described experiments [50]. After 30 min, animals were administered i.g. with 1.5 mL of ASA (125 mg/kg dissolved in 0.2 M HCl), based on previously implemented and described experimental model of ASA-induced gastric mucosal damage [50]. In separate experimental series, rats were pretreated i.g. with (9) vehicle or (10) BW-HS-101 (50 μmol/kg) 30 min before i.g. administration of 1 mL of 75% ethanol [28]. Because the molecular effects of BW-HS-101 and BW-iHS-101 on HMOX-1 and iNOS mRNA expression were similar, additional experimental groups with BW-iHS-101 + L-NNA or BW-iHS-101 + ZnPP were not employed to also comply with the 3R principle (by reduced number of animals involved in this protocol). Additionally, rats administered i.g. with vehicle without any additional treatments or gastric injury were considered as (11) the intact group.

All compounds and chemicals were purchased from Sigma Aldrich (Schnelldorf, Germany) unless otherwise stated. BW-HS-101 and BW-iHS-101 were synthesized by the Wang group (Georgia State University, Atlanta, GA, USA) following procedures described previously [27].

5.2. BW-HS-101 and BW-iHS-101 Synthesis and Chemical Conversion to Lactone

BW-HS-101 and BW-iHS-101 were synthesized following procedures described previously [27].

Conversion of BW-iHS-101 into the lactone was performed in vitro. Briefly, into the solution of a BW-iHS-101 (0.26 mmol) in MeOH (1.3 mL), 2 N NaOH (1.3 mL) was added, and the reaction was stirred for 2 h. Next, 10% HCl was added to acidify the reaction solution to the pH < 2. This acidified solution with precipitated crystals was extracted with ethyl acetate (13 mL × 3). The combined organic layers were washed with water (13 mL), dried (Na_2SO_4), filtered, and concentrated to yield a white solid product, which was characterized by LC-QTOF-MS analysis, using a 1200 series chromatograph and 6520 accurate-Mass QTOFMS (Agilent Technologies, Santa Clara, CA, USA), with the identification of the [M + H^+] peak (205.1028), corresponding to the calculated mass of protonated lactone. In the LC chromatogram, the lactone was the only product with a retention time of 6.2 min.

5.3. GBF Determination, Microscopic Gastric Damage Assessment and Biological Samples Collection

One hour after administration of ASA or ethanol, under isoflurane anesthesia, the abdomen was opened for the GBF measurement by laser flowmetry, as described previously [55]. Briefly, the GBF was determined in the oxyntic part of the gastric mucosa using laser flowmeter (Laserflo, model BPM2, Blood Perfusion Monitor, Vasamedics, Saint Paul, MN, USA). Average values of three measurements were expressed as % of the average value determined in healthy gastric mucosa (% of control). Serum samples were collected from the vena cava and was stored at −80 °C until further analysis [55]. Stomach was excised, opened along the greater curvature and gastric mucosal samples were scraped off on ice, snap-frozen in liquid nitrogen and stored at −80 °C until further analysis [50]. For microscopic analysis, the gastric tissue sections were excised and fixed in 10% buffered formalin, pH 7.4. Samples were dehydrated by passing them through a series of alcohols with incremental concentrations, equilibrated in xylene for 10–15 min and embedded in paraffin; paraffin blocks were cut into about 4 μm sections using a microtome. The prepared specimens were stained with haematoxylin/eosin (H&E). Tissue slides were evaluated

using a light microscope (AxioVert A1, Carl Zeiss, Oberkochen, Germany) [56]. Digital documentation of histological slides was obtained using ZEN Pro 2.3 software (Carl Zeiss, Oberkochen, Germany) [56].

All erosions/necrotic or inflammatory spots were evaluated based on following scoring criteria:

0 no erosion/necrosis/inflammation
1 length of injury <250 µm
2 length of injury 251–500 µm
3 length of injury 501–2000 µm

and

0 no erosion/necrosis/inflammation
1 depth of injury <500 µm per tissue section
2 depth of injury >500 µm per slide
3 depth of injury—erosion reaching submucosal layer

Median for the sum of abovementioned scoring criteria for all injuries in each slide separately was taken for further data analysis.

5.4. Determination of Gastric Mucosal mRNA Fold Changes by Real-Time PCR

Gastric mucosal mRNA expression fold changes for iNOS, HMOX-1, SOCS3, IL1-R1, IL1-R2, TNF-R2, COX-1, and COX-2 were assessed by real time PCR, as described previously [56]. Briefly, total RNA was isolated using commercially available kit with spin-columns (GeneMATRIX Universal RNA Purification Kit, EURx, Gdansk, Poland) according to manufacturer's protocols. Reversed transcription (RT) was performed using PrimeScript™ RTMasterMix (Perfect Real Time Takara Bio Inc., Kyoto, Japan). RNA concentration was measured using Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). For each RT reaction, total RNA concentration was adjusted to (1 µg) per sample. Samples from healthy (intact) gastric mucosa were further used as reference control during calculations. Expression of mRNA for iNOS, HMOX-1, SOCS3, IL1-R1, IL1-R2, TNF-R2, COX-1, COX-2 and succinate dehydrogenase complex, subunit A (SDHA) and β-actin (ACTB) as reference genes was determined using specific primers as described previously [50,51,57]. PCR reaction was run using thermal cycler Quant Studio 3 (Thermo Fisher Scientific, Waltham, MA, USA) and SYBR Green I dye including kit (SG qPCR Master Mix (2×), EURx, Gdansk, Poland). To maintain the same PCR reaction efficiency in all analyzed samples, the same amount of cDNA per each well was used. After reaction, melting curve for each sample, its technical replicates and for appropriate negative control were analyzed to exclude the data derived from potentially unintended products. Results were analyzed using the $-\Delta\Delta$Ct method [58].

5.5. Luminex Microbeads Fluorescent Assays

Determination of serum concentrations of interleukin (IL)-1β, IL10, tumor necrosis factor (TNF)-α and vascular endothelial growth factor A (VEGF) was performed using Luminex microbeads fluorescent assay (Bio-Rad, Hercules, CA, USA) and Luminex MAG-PIX system (Luminex Corp., Austin, TX, USA). Results were calculated from calibration curves and expressed in pg/mL [28]. Gastric mucosal concentrations of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, GM-CSF, IFN, TNF-α, EGF, and VEGF were determined with the implementation of calibration curve. All samples were standardized in terms of total protein concentration before the assay and results were expressed as pg/mL of the tissue homogenate [28].

5.6. Determination of PGE_2 and 8-OHdG Concentration in Gastric Mucosa

PGE_2 and 8-OdHG concentrations in gastric mucosa were determined as described in detail previously [51]. Briefly, PGE_2 concentrations in gastric mucosal samples obtained from the ulcer margin were determined using PGE_2 ELISA kit (ab133021, Abcam, Cam-

bridge, UK) according to the manufacturer's protocol. The homogenization process of each sample was standardized regarding sample weight and buffer volume and results were expressed in pg/mL of gastric tissue homogenate. 8-OHdG content as DNA oxidation marker was assessed in DNA isolated from gastric mucosa using ELISA kit (589320, Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's protocol.

5.7. Bioinformatic Analysis of Possible Molecular Targets of BW-HS-101

Possible molecular targets of BW-HS-101 were evaluated using SwissTargetPrediction, which estimates the probability for a tested compound to have indicated protein as a molecular target. Calculated scores higher than 0.5 indicate that the investigated compounds are likely to interact with given protein [59].

5.8. Statistical Analysis

Experiments and data collection were done by operators blinded to the sample identity. Results were analyzed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). Results are presented as mean ± SEM and as median ± range (Figure 2). Statistical analysis was conducted using Student's *t*-test or ANOVA with Dunnett's multiple comparison or Bonferroni post hoc tests if more than two experimental groups were compared. Kruskal–Wallis test was used for the data shown on Figure 2. The size for each experimental group was of $n = 5$. $p < 0.05$ was considered as statistically significant.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22105211/s1, Table S1: Target prediction data for BW-HS-101, Table S2: Target prediction data for BW-iHS-101.

Author Contributions: Conceptualization: D.B., B.W., M.M. (Marcin Magierowski); Investigation: D.B., E.K., D.W., A.D., K.M., S.K., T.B., M.M. (Marcin Magierowski); Investigation (chemical synthesis): Z.Y., B.Y.; Methodology: D.B., Z.Y., B.Y., E.K., M.M. (Monika Marcinkowska), M.M. (Marcin Magierowski); Resources: D.B., Z.Y., M.M. (Monika Marcinkowska), B.W., M.M. (Marcin Magierowski); Supervision and Project Administration: M.M. (Marcin Magierowski); Visualization: D.B., M.M. (Marcin Magierowski); Funding acquisition: M.M. (Marcin Magierowski); Writing—original draft: D.B., M.M. (Marcin Magierowski); Writing—review & editing: B.W., M.M. (Marcin Magierowski). All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by statutory grant for Marcin Magierowski received from Jagiellonian University Medical College (N41/DBS/000106, Poland). Zhengnan Yuan acknowledge the financial support of the Brains and Behaviors pre-doctoral fellowship program. Binghe Wang acknowledge the general financial support of the Georgia Research Alliance Eminent Scholar endowment and other internal resources at Georgia State University.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the 1st Local Ethical Committee for Care and Use of Experimental Animals held by Faculty of Pharmacy, Jagiellonian University Medical College in Cracow (Decision No.: 311/2019; Date: 17 July 2019).

Informed Consent Statement: Not applicable.

Data Availability Statement: All the data supporting the conclusions is included within the manuscript and is available on request from the corresponding author.

Acknowledgments: Authors would like to acknowledge Anna Chmura, for technical preparation of histological slides.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Fang, J.; George, M.G.; Gindi, R.M.; Hong, Y.; Yang, Q.; Ayala, C.; Ward, B.W.; Loustalot, F. Use of low-dose aspirin as secondary prevention of atherosclerotic cardiovascular disease in US adults (from the National Health Interview Survey, 2012). *Am. J. Cardiol.* **2015**, *115*, 895–900. [CrossRef] [PubMed]
2. Sostres, C.; Lanas, A. Gastrointestinal effects of aspirin. *Nat. Rev. Gastroenterol. Hepatol.* **2011**, *8*, 385–394. [CrossRef] [PubMed]

3. Laine, L. Nonsteroidal anti-inflammatory drug gastropathy. *Gastrointest. Endosc. Clin. N. Am.* **1996**, *6*, 489–504. [CrossRef]
4. Matsui, H.; Shimokawa, O.; Kaneko, T.; Nagano, Y.; Rai, K.; Hyodo, I. The pathophysiology of non-steroidal anti-inflammatory drug (NSAID)-induced mucosal injuries in stomach and small intestine. *J. Clin. Biochem. Nutr.* **2011**, *48*, 107–111. [CrossRef] [PubMed]
5. Cryer, B.; Mahaffey, K.W. Gastrointestinal ulcers, role of aspirin, and clinical outcomes: Pathobiology, diagnosis, and treatment. *J. Multidiscip. Health* **2014**, *7*, 137–146. [CrossRef]
6. Wallace, J.L.; Ferraz, J.G.; Muscara, M.N. Hydrogen Sulfide: An Endogenous Mediator of Resolution of Inflammation and Injury. *Antioxid. Redox Signal.* **2012**, *17*, 58–67. [CrossRef] [PubMed]
7. Kajimura, M.; Fukuda, R.; Bateman, R.M.; Yamamoto, T.; Suematsu, M. Interactions of Multiple Gas-Transducing Systems: Hallmarks and Uncertainties of CO, NO, and H_2S Gas Biology. *Antioxid. Redox Signal.* **2010**, *13*, 157–192. [CrossRef]
8. Shen, F.; Zhao, C.-S.; Shen, M.-F.; Wang, Z.; Chen, G. The role of hydrogen sulfide in gastric mucosal damage. *Med. Gas. Res.* **2019**, *9*, 88–92. [CrossRef]
9. Flannigan, K.; Wallace, K.L.F.A.J.L. Hydrogen Sulfide-Based Anti-Inflammatory and Chemopreventive Therapies: An Experimental Approach. *Curr. Pharm. Des.* **2015**, *21*, 3012–3022. [CrossRef]
10. Zanardo, R.C.O.; Brancaleone, V.; Distrutti, E.; Fiorucci, S.; Cirino, G.; Wallace, J.L. Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. *FASEB J.* **2006**, *20*, 2118–2120. [CrossRef]
11. Wang, R. The Gasotransmitter Role of Hydrogen Sulfide. *Antioxid. Redox Signal.* **2003**, *5*, 493–501. [CrossRef] [PubMed]
12. Wang, R. Two's company, three's a crowd: Can H_2S be the third endogenous gaseous transmitter? *FASEB J.* **2002**, *16*, 1792–1798. [CrossRef]
13. Magierowska, K.; Brzozowski, T.; Magierowski, M. Emerging role of carbon monoxide in regulation of cellular pathways and in the maintenance of gastric mucosal integrity. *Pharmacol. Res.* **2018**, *129*, 56–64. [CrossRef]
14. Dilek, N.; Papapetropoulos, A.; Toliver-Kinsky, T.; Szabo, C. Hydrogen sulfide: An endogenous regulator of the immune system. *Pharmacol. Res.* **2020**, *161*, 105119. [CrossRef]
15. Jasnos, K.; Magierowski, M.; Kwiecień, S.; Brzozowski, T. Carbon monoxide in human physiology—Its role in the gastrointestinal tract. *Postępy Higieny i Medycyny Doświadczalnej* **2014**, *68*, 101–109. [CrossRef]
16. Stipanuk, M.H.; Beck, P.W. Characterization of the enzymic capacity for cysteine desulphhydration in liver and kidney of the rat. *Biochem. J.* **1982**, *206*, 267–277. [CrossRef]
17. Swaroop, M.; Bradley, K.; Ohura, T.; Tahara, T.; Roper, M.D.; Rosenberg, L.E.; Kraus, J.P. Rat cystathionine beta-synthase. Gene organization and alternative splicing. *J. Biol. Chem.* **1992**, *267*, 11455–11461. [CrossRef]
18. Shibuya, N.; Mikami, Y.; Kimura, Y.; Nagahara, N.; Kimura, H. Vascular Endothelium Expresses 3-Mercaptopyruvate Sulfurtransferase and Produces Hydrogen Sulfide. *J. Biochem.* **2009**, *146*, 623–626. [CrossRef]
19. Singh, S.B.; Lin, H.C. Hydrogen Sulfide in Physiology and Diseases of the Digestive Tract. *Microorganisms* **2015**, *3*, 866–889. [CrossRef]
20. Su, Q.; Huang, Y.-S.; Xie, N.; Su, J.; Huang, C.; Liao, Q.-J. Diallyl disulfide inhibits the proliferation of HT-29 human colon cancer cells by inducing differentially expressed genes. *Mol. Med. Rep.* **2011**, *4*, 553–559. [CrossRef]
21. Fiorucci, S.; Antonelli, E.; Distrutti, E.; Rizzo, G.; Mencarelli, A.; Orlandi, S.; Zanardo, R.; Renga, B.; Di Sante, M.; Morelli, A.; et al. Inhibition of Hydrogen Sulfide Generation Contributes to Gastric Injury Caused by Anti-Inflammatory Nonsteroidal Drugs. *Gastroenterology* **2005**, *129*, 1210–1224. [CrossRef] [PubMed]
22. Magierowski, M.; Magierowska, K.; Hubalewska-Mazgaj, M.; Sliwowski, Z.; Pajdo, R.; Ginter, G.; Kwiecien, S.; Brzozowski, T. Exogenous and Endogenous Hydrogen Sulfide Protects Gastric Mucosa against the Formation and Time-Dependent Development of Ischemia/Reperfusion-Induced Acute Lesions Progressing into Deeper Ulcerations. *Molecules* **2017**, *22*, 295. [CrossRef] [PubMed]
23. Lou, L.-X.; Geng, B.; Du, J.-B.; Tang, C.-S. Hydrogen Sulphide-Induced Hypothermia Attenuates Stress-Related Ulceration in Rats. *Clin. Exp. Pharmacol. Physiol.* **2007**, *35*, 223–228. [CrossRef] [PubMed]
24. Wallace, J.L.; Dicay, M.; McKnight, W.; Martin, G.R. Hydrogen sulfide enhances ulcer healing in rats. *FASEB J.* **2007**, *21*, 4070–4076. [CrossRef]
25. Ise, F.; Takasuka, H.; Hayashi, S.; Takahashi, K.; Koyama, M.; Aihara, E.; Takeuchi, K. Stimulation of duodenal HCO3− secretion by hydrogen sulphide in rats: Relation to prostaglandins, nitric oxide and sensory neurones. *Acta Physiol.* **2010**, *201*, 117–126. [CrossRef]
26. Wallace, J.L.; Nagy, P.; Feener, T.D.; Allain, T.; Ditrói, T.; Vaughan, D.J.; Muscara, M.N.; de Nucci, G.; Buret, A.G. A proof-of-concept, Phase 2 clinical trial of the gastrointestinal safety of a hydrogen sulfide-releasing anti-inflammatory drug. *Br. J. Pharmacol.* **2020**, *177*, 769–777. [CrossRef]
27. Zheng, Y.; Yueqin, Z.; Ji, K.; Pan, Z.; Chittavong, V.; Wang, B. Esterase-Sensitive Prodrugs with Tunable Release Rates and Direct Generation of Hydrogen Sulfide. *Angew. Chem. Int. Ed.* **2016**, *55*, 4514–4518. [CrossRef]
28. Bakalarz, D.; Surmiak, M.; Yang, X.; Wójcik, D.; Korbut, E.; Śliwowski, Z.; Ginter, G.; Buszewicz, G.; Brzozowski, T.; Cieszkowski, J.; et al. Organic carbon monoxide prodrug, BW-CO-111, in protection against chemically-induced gastric mucosal damage. *Acta Pharm. Sin. B* **2021**, *11*, 456–475. [CrossRef]

29. Da Silva Hage-Melim, L.I.; Federico, L.B.; de Oliveira, N.K.S.; Francisco, V.C.C.; Correia, L.C.; de Lima, H.B.; Gomes, S.Q.; Barcelos, M.P.; Francischini, I.A.G.; de Paula de Silva, C.H.T. Virtual screening, ADME/Tox predictions and the drug repurposing concept for future use of old drugs against the COVID-19. *Life Sci.* **2020**, *256*, 117963. [CrossRef]
30. Sun, H.; Yang, J.; Shi, Y.; Wang, Y.; Li, C.; Zhu, M. Hydrogen sulfide in the nucleus tractus solitarii regulates gastric acid secretion in rats. *J. Physiol. Pharmacol.* **2020**, *71*, 1–5.
31. Cacanyiova, S.; Majzunova, M.; Golas, S.; Berenyiova, A. The role of perivascular adipose tissue and endogenous hydrogen sulfide in vasoactive responses of isolated mesenteric arteries in normotensive and spontaneously hypertensive rats. *J. Physiol. Pharmacol.* **2019**, *70*, 295–306.
32. Lasheen, N.N.; Elayat, W.M.; Elrefai, M.F.M.; Zaki, W.S.; Ahmed, E.H.; El Sheikh, R.M.N.; Rayas, D.S.A.A.; Gad, F.R.S. Possible role of garlic oil in ameliorating renal injury after liver ischemia/reperfusion in rats. *J. Physiol. Pharmacol.* **2020**, *70*, 765–778.
33. Gemici, B.; Wallace, J.L. Anti-inflammatory and Cytoprotective Properties of Hydrogen Sulfide. In *Regulated Cell Death Part A: Apoptotic Mechanisms*; Elsevier BV: Amsterdam, The Netherlands, 2015; Volume 555, pp. 169–193.
34. Lazarević, M.; Mazzon, E.; Momčilović, M.; Basile, M.S.; Colletti, G.; Petralia, M.C.; Bramanti, P.; Nicoletti, F.; Miljković, Đ. The H_2S Donor GYY4137 Stimulates Reactive Oxygen Species Generation in BV2 Cells While Suppressing the Secretion of TNF and Nitric Oxide. *Molecules* **2018**, *23*, 2966. [CrossRef]
35. Magierowski, M.; Jasnos, K.; Kwiecień, S.; Brzozowski, T. Role of hydrogen sulfide in the physiology of gastrointestinal tract and in the mechanism of gastroprotection. *Postępy Higieny i Medycyny Doświadczalnej* **2013**, *67*, 150–156. [CrossRef]
36. Yu, L.; Park, B.M.; Ahn, Y.J.; Lee, G.-J.; Kim, S.H. Hydrogen sulfide donor, NaHS, stimulates ANP secretion via the KATP channel and the NOS/sGC pathway in rat atria. *Peptides* **2019**, *111*, 89–97. [CrossRef]
37. De Araújo, S.; Oliveira, A.P.; Sousa, F.B.; Souza, L.K.; Pacheco, G.; Filgueiras, M.C.; Nicolau, L.A.; Brito, G.A.C.; Cerqueira, G.S.; Silva, R.O.; et al. AMPK activation promotes gastroprotection through mutual interaction with the gaseous mediators H_2S, NO, and CO. *Nitric Oxide* **2018**, *78*, 60–71. [CrossRef]
38. Mard, S.A.; Neisi, N.; Solgi, G.; Hassanpour, M.; Darbor, M.; Maleki, M. Gastroprotective Effect of NaHS Against Mucosal Lesions Induced by Ischemia–Reperfusion Injury in Rat. *Dig. Dis. Sci.* **2012**, *57*, 1496–1503. [CrossRef]
39. Medeiros, J.V.R.; Bezerra, V.H.; Gomes, A.S.; Barbosa, A.L.R.; Lima-Júnior, R.C.P.; Soares, P.M.G.; Brito, G.A.C.; Ribeiro, R.A.; Cunha, F.Q.; Souza, M.H.L.P. Hydrogen Sulfide Prevents Ethanol-Induced Gastric Damage in Mice: Role of ATP-Sensitive Potassium Channels and Capsaicin-Sensitive Primary Afferent Neurons. *J. Pharmacol. Exp. Ther.* **2009**, *330*, 764–770. [CrossRef]
40. Sun, H.-Z.; Zheng, S.; Lu, K.; Hou, F.-T.; Bi, J.-X.; Liu, X.-L.; Wang, S.-S. Hydrogen sulfide attenuates gastric mucosal injury induced by restraint water-immersion stressviaactivation of KATPchannel and NF-κB dependent pathway. *World J. Gastroenterol.* **2017**, *23*, 87–92. [CrossRef]
41. Jeddi, S.; Gheibi, S.; Kashfi, K.; Carlström, M.; Ghasemi, A. Dose-Dependent Effects of Long-Term Administration of Hydrogen Sulfide on Myocardial Ischemia–Reperfusion Injury in Male Wistar Rats: Modulation of RKIP, NF-κB, and Oxidative Stress. *Int. J. Mol. Sci.* **2020**, *21*, 1415. [CrossRef]
42. Zheng, Y.; Ji, X.; Ji, K.; Wang, B. Hydrogen sulfide prodrugs—A review. *Acta Pharm. Sin. B* **2015**, *5*, 367–377. [CrossRef] [PubMed]
43. Li, L.; Whiteman, M.; Guan, Y.Y.; Neo, K.L.; Cheng, Y.; Lee, S.W.; Zhao, Y.; Baskar, R.; Tan, C.-H.; Moore, P.K. Characterization of a Novel, Water-Soluble Hydrogen Sulfide–Releasing Molecule (GYY4137). *Circulation* **2008**, *117*, 2351–2360. [CrossRef]
44. Gerő, D.; Torregrossa, R.; Perry, A.; Waters, A.; Le-Trionnaire, S.; Whatmore, J.L.; Wood, M.; Whiteman, M. The novel mitochondria-targeted hydrogen sulfide (H_2S) donors AP123 and AP39 protect against hyperglycemic injury in microvascular endothelial cells in vitro. *Pharmacol. Res.* **2016**, *113*, 186–198. [CrossRef]
45. Sakuma, S.; Minamino, S.; Takase, M.; Ishiyama, Y.; Hosokura, H.; Kohda, T.; Ikeda, Y.; Fujimoto, Y. Hydrogen sulfide donor GYY4137 suppresses proliferation of human colorectal cancer Caco-2 cells by inducing both cell cycle arrest and cell death. *Heliyon* **2019**, *5*, e02244. [CrossRef]
46. Karwi, Q.G.; Bornbaum, J.; Boengler, K.; Torregrossa, R.; Whiteman, M.; Wood, M.E.; Schulz, R.; Baxter, G.F. AP39, a mitochondria-targeting hydrogen sulfide (H_2S) donor, protects against myocardial reperfusion injury independently of salvage kinase signalling. *Br. J. Pharmacol.* **2017**, *174*, 287–301. [CrossRef] [PubMed]
47. Costa, S.K.; Muscara, M.N.; Allain, T.; Dallazen, J.; Gonzaga, L.; Buret, A.G.; Vaughan, D.J.; Fowler, C.J.; De Nucci, G.; Wallace, J.L. Enhanced Analgesic Effects and Gastrointestinal Safety of a Novel, Hydrogen Sulfide-Releasing Anti-Inflammatory Drug (ATB-352): A Role for Endogenous Cannabinoids. *Antioxid. Redox Signal.* **2020**, *33*, 1003–1009. [CrossRef] [PubMed]
48. Doyle, W.L. Distribution of Esterase in Gastric Mucosa. *J. Gen. Physiol.* **1954**, *38*, 141–144. [CrossRef] [PubMed]
49. Spenney, J.G. Acetylsalicylic acid hydrolase of gastric mucosa. *Am. J. Physiol. Metab.* **1978**, *234*, E606-10. [CrossRef]
50. Magierowski, M.; Hubalewska-Mazgaj, M.; Magierowska, K.; Wojcik, D.; Sliwowski, Z.; Kwiecien, S.; Brzozowski, T. Nitric oxide, afferent sensory nerves, and antioxidative enzymes in the mechanism of protection mediated by tricarbonyldichlororuthenium(II) dimer and sodium hydrosulfide against aspirin-induced gastric damage. *J. Gastroenterol.* **2018**, *53*, 52–63. [CrossRef]
51. Magierowska, K.; Bakalarz, D.; Wójcik, D.; Korbut, E.; Danielak, A.; Głowacka, U.; Pajdo, R.; Buszewicz, G.; Ginter, G.; Surmiak, M.; et al. Evidence for Cytoprotective Effect of Carbon Monoxide Donor in the Development of Acute Esophagitis Leading to Acute Esophageal Epithelium Lesions. *Cells* **2020**, *9*, 1203. [CrossRef]
52. Vrankova, S.; Zemancikova, A.; Torok, J.; Pechanova, O. Effect of low dose L-NAME pretreatment on nitric oxide/reactive oxygen species balance and vasoactivity in L-NAME/salt-induced hypertensive rats. *J. Physiol. Pharmacol.* **2019**, *70*, 535–544.

53. An, J.M.; Kang, E.A.; Han, Y.M.; Kim, Y.S.; Hong, Y.G.; Hah, B.S.; Hong, S.P.; Hahm, K.B. Dietary threonine prevented stress-related mucosal diseases in rats. *J. Physiol. Pharmacol.* **2019**, *70*, 467–478.
54. Zhang, C.-Y.; Li, X.-H.; Zhang, T.; Fu, J.; Cui, X.-D. Hydrogen sulfide upregulates heme oxygenase-1 expression in rats with volume overload-induced heart failure. *Biomed. Rep.* **2013**, *1*, 454–458. [CrossRef]
55. Magierowski, M.; Magierowska, K.; Hubalewska-Mazgaj, M.; Sliwowski, Z.; Ginter, G.; Pajdo, R.; Chmura, A.; Kwiecien, S.; Brzozowski, T. Carbon monoxide released from its pharmacological donor, tricarbonyldichlororuthenium (II) dimer, accelerates the healing of pre-existing gastric ulcers. *Br. J. Pharmacol.* **2017**, *174*, 3654–3668. [CrossRef] [PubMed]
56. Magierowska, K.; Korbut, E.; Hubalewska-Mazgaj, M.; Surmiak, M.; Chmura, A.; Bakalarz, D.; Buszewicz, G.; Wójcik, D.; Śliwowski, Z.; Ginter, G.; et al. Oxidative gastric mucosal damage induced by ischemia/reperfusion and the mechanisms of its prevention by carbon monoxide-releasing tricarbonyldichlororuthenium (II) dimer. *Free Radic. Biol. Med.* **2019**, *145*, 198–208. [CrossRef]
57. Peinnequin, A.; Mouret, C.; Birot, O.; Alonso, A.; Mathieu, J.; Clarençon, D.; Agay, D.; Chancerelle, Y.; Multon, E. Rat pro-inflammatory cytokine and cytokine related mRNA quantification by real-time polymerase chain reaction using SYBR green. *BMC Immunol.* **2004**, *5*, 3. [CrossRef]
58. Schmittgen, T.D.; Livak, K.J. Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* **2008**, *3*, 1101–1108. [CrossRef]
59. Daina, A.; Michielin, O.; Zoete, V. SwissTargetPrediction: Updated data and new features for efficient prediction of protein targets of small molecules. *Nucleic Acids Res.* **2019**, *47*, W357–W364. [CrossRef]

5. OŚWIADCZENIA WSPÓŁAUTORÓW

(Wymagane co najmniej cztery dla każdej publikacji)

Kraków, dnia 09.06.2021 r.

mgr Dominik Bakalarz
Katedra Fizjologii, Uniwersytet Jagielloński - Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy: Organic carbon monoxide prodrug, BW-CO-111, in protection against chemically-induced gastric mucosal damage. Acta Pharmaceutica Sinica B, 2021; 11(2):456-475, doi: 10.1016/j.apsb.2020.08.005, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 40% i polegał na:

- współudziale w projektowaniu całościowej koncepcji badawczej,
- przygotowaniu testowanych substancji do badań eksperymentalnych *in vivo*,
- procesowaniu pobranego materiału biologicznego do badań biochemicznych i molekularnych (izolacja kwasów nukleinowych, białek, prostaglandyn),
- wykonaniu oznaczenia ekspresji genów w błonie śluzowej żołądka metodą real-time PCR,
- wykonaniu oznaczenia białek w błonie śluzowej żołądka metodą Western Blot,
- współudziale w oznaczeniu poziomu prostaglandyn metodą ELISA,
- współudziale w analizie, opracowaniu i interpretacji uzyskanych wyników,
- przygotowaniu rycin do publikacji,
- przygotowaniu manuskryptu w zakresie opisu części rezultatów oraz dyskusji i finalnych wniosków, a także edycji tekstu.

Dominik Bakalarz

(podpis współautora)

Kraków, dnia 09.06.2021 r.

Dr n.med. Edyta Korbut
Katedra Fizjologii, Uniwersytet Jagielloński - Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy: Organic carbon monoxide prodrug, BW-CO-111, in protection against chemically-induced gastric mucosal damage. Acta Pharmaceutica Sinica B, 2021; 11(2):456-475, doi: 10.1016/j.apsb.2020.08.005, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 3% i polegał na:

- współudziale w wykonaniu oznaczenia poziomu prostaglandyn metodą ELISA

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Dominika Bakalarza jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Dominika Bakalarza polegający na:

- współudziale w projektowaniu całościowej koncepcji badawczej,
- przygotowanie testowanych substancji do badań eksperymentalnych *in vivo*,
- procesowanie pobranego materiału biologicznego do badań biochemicznych i molekularnych (izolacja kwasów nukleinowych, białek, prostaglandyn),
- wykonaniu oznaczenia ekspresji genów w błonie śluzowej żołądka metodą real-time PCR,
- wykonaniu oznaczenia białek w błonie śluzowej żołądka metodą Western Blot,
- współudziale w oznaczeniu poziomu prostaglandyn metodą ELISA,
- współudziale w analizie, opracowaniu i interpretacji uzyskanych wyników,
- przygotowaniu rycin do publikacji,
- przygotowanie manuskryptu w zakresie opisu części rezultatów oraz dyskusji i finalnych wniosków, a także edycja tekstu

Edyta Korbut
(podpis współautora)

Kraków, dnia 09.06.2021 r.

Dr n.med. Katarzyna Magierowska
Katedra Fizjologii, Uniwersytet Jagielloński - Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy: Organic carbon monoxide prodrug, BW-CO-111, in protection against chemically-induced gastric mucosal damage. Acta Pharmaceutica Sinica B, 2021; 11(2):456-475, doi: 10.1016/j.apsb.2020.08.005, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 3% i polegał na:

- współudziale w wykonaniu części badań na zwierzętach oraz w przygotowaniu ryciny koncepcyjnej (Rycina 12)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Dominika Bakalarza jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Dominika Bakalarza polegający na:

- współudziale w projektowaniu całościowej koncepcji badawczej,
- przygotowanie testowanych substancji do badań eksperymentalnych *in vivo*,
- procesowanie pobranego materiału biologicznego do badań biochemicznych i molekularnych (izolacja kwasów nukleinowych, białek, prostaglandyn),
- wykonaniu oznaczenia ekspresji genów w błonie śluzowej żołądka metodą real-time PCR,
- wykonaniu oznaczenia białek w błonie śluzowej żołądka metodą Western Blot,
- współudziale w oznaczeniu poziomu prostaglandyn metodą ELISA,
- współudziale w analizie, opracowaniu i interpretacji uzyskanych wyników,
- przygotowaniu rycin do publikacji,
- przygotowanie manuskryptu w zakresie opisu części rezultatów oraz dyskusji i finalnych wniosków, a także edycja tekstu

Katarzyna Magierowska
(podpis współautora)

Kraków, dnia 08.06.2021 r.

Dr n. med. Zbigniew Śliwowski
Katedra Fizjologii, Uniwersytet Jagielloński - Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy: Organic carbon monoxide prodrug, BW-CO-111, in protection against chemically-induced gastric mucosal damage. Acta Pharmaceutica Sinica B, 2021; 11(2):456-475, doi: 10.1016/j.apsb.2020.08.005, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 3% i polegał na:

- współuczestnictwie w wykonaniu badań eksperymentalnych na zwierzętach

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Dominika Bakalarza jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Dominika Bakalarza polegający na:

- współudziale w projektowaniu całościowej koncepcji badawczej,
- przygotowanie testowanych substancji do badań eksperymentalnych *in vivo*,
- procesowanie pobranego materiału biologicznego do badań biochemicznych i molekularnych (izolacja kwasów nukleinowych, białek, prostaglandyn),
- wykonaniu oznaczenia ekspresji genów w błonie śluzowej żołądka metodą real-time PCR,
- wykonaniu oznaczenia białek w błonie śluzowej żołądka metodą Western Blot,
- współudziale w oznaczeniu poziomu prostaglandyn metodą ELISA,
- współudziale w analizie, opracowaniu i interpretacji uzyskanych wyników,
- przygotowaniu rycin do publikacji,
- przygotowanie manuskryptu w zakresie opisu części rezultatów oraz dyskusji i finalnych wniosków, a także edycja tekstu

Zbigniew Śliwowski
.....
(podpis współautora)

Kraków, dnia 09.06.2021 r.

Dr n. med. Jakub Cieszkowski
Katedra Fizjologii, Uniwersytet Jagielloński - Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy: Organic carbon monoxide prodrug, BW-CO-111, in protection against chemically-induced gastric mucosal damage. Acta Pharmaceutica Sinica B, 2021; 11(2):456-475, doi: 10.1016/j.apsb.2020.08.005, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 3% i polegał na:

- współuczestnictwie w wykonaniu badań eksperymentalnych na zwierzętach

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Dominika Bakalarza jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Dominika Bakalarza polegający na:

- współudziale w projektowaniu całościowej koncepcji badawczej,
- przygotowanie testowanych substancji do badań eksperymentalnych *in vivo*,
- procesowanie pobranego materiału biologicznego do badań biochemicznych i molekularnych (izolacja kwasów nukleinowych, białek, prostaglandyn),
- wykonaniu oznaczenia ekspresji genów w błonie śluzowej żołądka metodą real-time PCR,
- wykonaniu oznaczenia białek w błonie śluzowej żołądka metodą Western Blot,
- współudziale w oznaczeniu poziomu prostaglandyn metodą ELISA,
- współudziale w analizie, opracowaniu i interpretacji uzyskanych wyników,
- przygotowaniu rycin do publikacji,
- przygotowanie manuskryptu w zakresie opisu części rezultatów oraz dyskusji i finalnych wniosków, a także edycja tekstu

.....

(podpis współautora)

Kraków, dnia 09.06.2021 r.

mgr Dominik Bakalarz
Katedra Fizjologii, Uniwersytet Jagielloński - Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy: Novel Hydrogen Sulfide (H2S)-Releasing BW-HS-101 and Its Non-H2S Releasing Derivative in Modulation of Microscopic and Molecular Parameters of Gastric Mucosal Barrier. Int. J. Mol. Sci. 2021, 22(10):5211, doi: 10.3390/ijms22105211, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 50% i polegał na:

- współudziale w projektowaniu całościowej koncepcji badawczej,
- przygotowaniu testowanych substancji do badań eksperymentalnych *in vivo*, w tym konwersji chemicznej badanych związków *in vitro,*
- procesowaniu pobranego materiału biologicznego do badań biochemicznych i molekularnych (izolacja kwasów nukleinowych, białek, prostaglandyn),
- wykonaniu oznaczenia ekspresji genów w błonie śluzowej żołądka metodą real-time PCR,
- wykonaniu oznaczenia białek w błonie śluzowej żołądka metodą Western Blot,
- współudziale w oznaczeniu poziomu prostaglandyn metodą ELISA,
- współudziale w analizie, opracowaniu i interpretacji uzyskanych wyników,
- przygotowaniu rycin do publikacji,
- przygotowaniu manuskryptu w zakresie opisu części rezultatów oraz dyskusji i finalnych wniosków, a także edycji tekstu.

Dominik Bakalarz

(podpis współautora)

Kraków, dnia 09.06.2021 r.

Dr n. med. Katarzyna Magierowska
Katedra Fizjologii, Uniwersytet Jagielloński - Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy: Novel Hydrogen Sulfide (H2S)-Releasing BW-HS-101 and Its Non-H2S Releasing Derivative in Modulation of Microscopic and Molecular Parameters of Gastric Mucosal Barrier. Int. J. Mol. Sci. 2021, 22(10):5211, doi: 10.3390/ijms22105211, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 3% i polegał na:

- współuczestnictwie w wykonaniu badań na zwierzętach

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Dominika Bakalarza jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Dominika Bakalarza polegający na:

- współudziale w projektowaniu całościowej koncepcji badawczej,
- przygotowanie testowanych substancji do badań eksperymentalnych *in vivo*, w tym konwersja chemiczna badanych związków *in vitro*
- procesowanie pobranego materiału biologicznego do badań biochemicznych i molekularnych (izolacja kwasów nukleinowych, białek, prostaglandyn),
- wykonaniu oznaczenia ekspresji genów w błonie śluzowej żołądka metodą real-time PCR,
- wykonaniu oznaczenia białek w błonie śluzowej żołądka metodą Western Blot,
- współudziale w oznaczeniu poziomu prostaglandyn metodą ELISA,
- współudziale w analizie, opracowaniu i interpretacji uzyskanych wyników,
- przygotowaniu rycin do publikacji,
- przygotowanie manuskryptu w zakresie opisu części rezultatów oraz dyskusji i finalnych wniosków, a także edycja tekstu

Katarzyna Magierowska

(podpis współautora)

Kraków, dnia 09.06.2021 r.

Aleksandra Danielak
Katedra Fizjologii, Uniwersytet Jagielloński - Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy: Novel Hydrogen Sulfide (H2S)-Releasing BW-HS-101 and Its Non-H2S Releasing Derivative in Modulation of Microscopic and Molecular Parameters of Gastric Mucosal Barrier. Int. J. Mol. Sci. 2021, 22(10):5211, doi: 10.3390/ijms22105211, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 3% i polegał na:

- współuczestnictwie w przygotowaniu i archiwizacji materiałów w ramach eksperymentów *in vivo*
- współuczestnictwie w procesowaniu materiału biologicznego do oznaczeń biochemicznych i molekularnych oraz zebraniu danych do przygotowania ryciny 6

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Dominika Bakalarza jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Dominika Bakalarza polegający na:

- współudziale w projektowaniu całościowej koncepcji badawczej,
- przygotowanie testowanych substancji do badań eksperymentalnych *in vivo*, w tym konwersja chemiczna badanych związków *in vitro*
- procesowanie pobranego materiału biologicznego do badań biochemicznych i molekularnych (izolacja kwasów nukleinowych, białek, prostaglandyn),
- wykonaniu oznaczenia ekspresji genów w błonie śluzowej żołądka metodą real-time PCR,
- wykonaniu oznaczenia białek w błonie śluzowej żołądka metodą Western Blot,
- współudziale w oznaczeniu poziomu prostaglandyn metodą ELISA,
- współudziale w analizie, opracowaniu i interpretacji uzyskanych wyników,
- przygotowaniu rycin do publikacji,
- przygotowanie manuskryptu w zakresie opisu części rezultatów oraz dyskusji i finalnych wniosków, a także edycja tekstu

Aleksandra Danielak

(podpis współautora)

Kraków, dnia 09.06.2021 r.

Prof. dr hab. Tomasz Brzozowski
Katedra Fizjologii, Uniwersytet Jagielloński - Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy: Novel Hydrogen Sulfide (H2S)-Releasing BW-HS-101 and Its Non-H2S Releasing Derivative in Modulation of Microscopic and Molecular Parameters of Gastric Mucosal Barrier. Int. J. Mol. Sci. 2021, 22(10):5211, doi: 10.3390/ijms22105211, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 3% i polegał na:

- współuczestnictwie w wykonaniu badań na zwierzętach

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Dominika Bakalarza jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Dominika Bakalarza polegający na:

- współudziale w projektowaniu całościowej koncepcji badawczej,
- przygotowanie testowanych substancji do badań eksperymentalnych *in vivo*, w tym konwersja chemiczna badanych związków *in vitro*
- procesowanie pobranego materiału biologicznego do badań biochemicznych i molekularnych (izolacja kwasów nukleinowych, białek, prostaglandyn),
- wykonaniu oznaczenia ekspresji genów w błonie śluzowej żołądka metodą real-time PCR,
- wykonaniu oznaczenia białek w błonie śluzowej żołądka metodą Western Blot,
- współudziale w oznaczeniu poziomu prostaglandyn metodą ELISA,
- współudziale w analizie, opracowaniu i interpretacji uzyskanych wyników,
- przygotowaniu rycin do publikacji,
- przygotowanie manuskryptu w zakresie opisu części rezultatów oraz dyskusji i finalnych wniosków, a także edycja tekstu

.....

(podpis współautora)

Kraków, dnia 08.06.2021 r.

Dr inż. Dagmara Wójcik
Katedra Fizjologii, Uniwersytet Jagielloński - Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy: Novel Hydrogen Sulfide (H2S)-Releasing BW-HS-101 and Its Non-H2S Releasing Derivative in Modulation of Microscopic and Molecular Parameters of Gastric Mucosal Barrier. Int. J. Mol. Sci. 2021, 22(10):5211, doi: 10.3390/ijms22105211, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 3% i polegał na:

- współuczestnictwie w wykonaniu badań na zwierzętach oraz wykonaniu oznaczeń poziomu oksydacji kwasów nukleinowych w materiale biologicznym

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Dominika Bakalarza jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Dominika Bakalarza polegający na:

- współudziale w projektowaniu całościowej koncepcji badawczej,
- przygotowanie testowanych substancji do badań eksperymentalnych *in vivo*, w tym konwersja chemiczna badanych związków *in vitro*
- procesowanie pobranego materiału biologicznego do badań biochemicznych i molekularnych (izolacja kwasów nukleinowych, białek, prostaglandyn),
- wykonaniu oznaczenia ekspresji genów w błonie śluzowej żołądka metodą real-time PCR,
- wykonaniu oznaczenia białek w błonie śluzowej żołądka metodą Western Blot,
- współudziale w oznaczeniu poziomu prostaglandyn metodą ELISA,
- współudziale w analizie, opracowaniu i interpretacji uzyskanych wyników,
- przygotowaniu rycin do publikacji,
- przygotowanie manuskryptu w zakresie opisu części rezultatów oraz dyskusji i finalnych wniosków, a także edycja tekstu

Dagmara Wójcik
.....
(podpis współautora)