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Intestinal absorption of *S*-nitrosothiols: Permeability and transport mechanisms

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ABSTRACT

S-Nitrosothiols, a class of NO donors, demonstrate potential benefits for cardiovascular diseases. Drugs for such chronic diseases require long term administration preferentially through the oral route. However, the absorption of *S*-nitrosothiols by the intestine, which is the first limiting barrier for their vascular bioavailability, is rarely evaluated. Using an *in vitro* model of intestinal barrier, based on human cells, the present work aimed at elucidating the mechanisms of intestinal transport (passive or active, paracellular or transcellular pathway) and at predicting the absorption site of three *S*-nitrosothiols: *S*-nitrosoglutathione (GSNO), *S*-nitroso-*N*-acetyl-L-cysteine (NACNO) and *S*-nitroso-*N*-acetyl-D-penicillamine (SNAP). These *S*-nitrosothiols include different skeletons carrying the nitroso group, which confer different physico-chemical characteristics and biological activities (antioxidant and anti-inflammatory). According to the values of apparent permeability coefficient, the three *S*-nitrosothiols belong to the medium class of permeability. The evaluation of the bidirectional apparent permeability demonstrated a passive diffusion of the three *S*-nitrosothiols. GSNO and NACNO preferentially cross the intestinal barrier though the transcellular pathway, while SNAP followed both the trans- and paracellular pathways. Finally, the permeability of NACNO was favoured at pH 6.4, which is close to the pH of the jejunal part of the intestine. Through this study, we determined the absorption mechanisms of *S*-nitrosothiols and postulated that they can be administrated through the oral route.

1. Introduction

Nitric oxide (NO) is a gaseous mediator with a short half-life (less than 5 s [1]). Due to its radical nature and oxidative activity, NO is involved in various signalling pathways among different cellular types and physiological systems. NO is continuously synthesised by oxydoreductases, *i.e.* the three endothelial, inducible or neuronal isoforms of NO synthases. The decrease in NO bioavailability, linked to vascular endothelium dysfunction and oxidative stress, plays a major role in ageing and cardiovascular chronic diseases like atherosclerosis, angina pectoris and stroke. As a result, the restoration of NO bioavailability, using among NO donors the physiologically occurring *S*-nitrosothiols, is a therapeutic key to treat cardiovascular diseases [2–7]. *S*-Nitrosothiols are formed by *S*-nitrosation – *i.e.* formation of a covalent bond between NO and a reduced thiol function of a cysteine residue belonging to high or low molecular weight proteins or peptides. *In vivo*, *S*-nitrosothiols like *S*-nitrosoalbumin, *S*-nitrosohemoglobin and *S*-nitrosoglutathione (GSNO) are the physiological forms of NO storage and transport [8]. Indeed, the formation of the *S*-NO bond extends NO half-life from 45 min up to several hours [9–10] and limits the oxidative/nitrosative stress induced by NO oxidation into peroxynitrite ions ($ONOO^-$) [11]. Despite the therapeutic potential of *S*-nitrosothiols, their half-life linked to their physico-chemical instability (heat, light, metallic cations,…) and/or enzymatic (redoxines or, for GSNO only, γ-glutamyltransferase) degradation, is too short for chronic diseases treatment [12].

Nowadays, many preclinical studies focused on cardiovascular therapeutics using *S*-nitrosothiols [6,13–17]. For example, daily intraperitoneal administration of *S*-nitroso-*N*-acetyl-L-cysteine (NACNO) for two weeks shows anti-atherosclerotic effects in mice [13]. However, compared to the oral route, the intraperitoneal administration is less suitable for chronic treatments. GSNO administration through the oral route in a context of stroke [14] results in neuroprotective effects: GSNO maintains the blood-brain barrier integrity, reduces peroxynitrite formation and stabilises several deleterious factors *via* *S*-nitrosation [13–16]. Despite such beneficial effects following oral administration, to the best of our knowledge, no study evaluated the mechanisms of

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intestinal absorption of GSNO and other *S*-nitrosothiols. Only Pinheiro et al. [17] demonstrated that oral administration of nitrite and nitrate ions (stable NO derived species) to rats increased the concentration of circulating *S*-nitrosothiols, thus produced antihypertensive effects. This study indirectly proves the intestinal absorption of *S*-nitrosothiols without elucidated the underlying mechanisms. However, the understanding of the intestinal absorption mechanisms of *S*-nitrosothiols is a prerequisite to control the dose and the kinetic of NO reaching its action sites.

To predict the intestinal absorption of drugs, the Biopharmaceutical Classification System (BCS) [18] defines four classes based on the physico-chemical properties (solubility) and intestinal permeability of drugs. The intestinal permeability of a drug is characterised, using *in vitro* or *ex vivo* models, by apparent permeability coefficient (Papp) from low permeability ($< 1 \times 10^{-6}$ cm.s^{-1}) to high permeability ($\geq 10 \times 10^{-6}$ cm.s^{-1}) including also a medium permeability class [19]. Thus far, only one of our studies was interested in the improvement and the prolongation of GSNO intestinal absorption by proposing alginate/chitosan nanocomposite formulation [20]. Using an *in vitro* intestinal barrier model of differentiated Caco-2 cells, we showed low intestinal permeability for GSNO with a Papp of 0.83×10^{-7} cm.s^{-1}. The nanocomposite formulation delayed GSNO absorption up to 24 h (1 h for free GSNO) and multiplied by four the Papp value (3.41×10^{-7} cm.s^{-1}) even if GSNO stayed in the low class of permeability [20]. This study showed the ability for GSNO to cross the intestinal barrier model and the possibility to modulate its kinetics of absorption. This opens new therapeutic applications in the treatment of chronic pathologies linked to a decrease of NO bioavailability.

Intestinal absorption of low molecular weight molecules is mainly driven by their physico-chemical properties such as lipophilicity, correlated with the octanol/water partition coefficient, expressed as a logarithmic value (log P), and the ionisation constant (pKa). For *S*-nitrosothiols, the log P value is driven by the skeleton carrying NO. GSNO, NACNO and *S*-nitroso-*N*-acetyl-D-penicillamine (SNAP), the three main *S*-nitrosothiols described in the literature, are characterised by calculated log P value of −2.70, −0.47 and 1.08, respectively [2]. The skeleton carrying NO presents also different therapeutic properties linked with its chemical structure. GSNO is a physiological *S*-nitrosothiol [21], present in the cytosol at a high concentration especially in erythrocytes [22], platelets and cerebral tissue. Its reduced glutathione (GSH) skeleton shows an antioxidant chemical structure thanks to its thiol functional group and forms, with the glutathione disulphide (GSSG), the intracellular redox buffer. NACNO and SNAP are synthetic *S*-nitrosothiols. NACNO with its *N*-acetyl-L-cysteine (NAC) skeleton possesses also an antioxidant activity in accordance with its chemical structure (thiol function). Furthermore, NAC is already used in human medicine as a mucolytic agent (oral administration) or as the antidote in acetaminophen intoxication [23]. SNAP shows in addition to its antioxidant properties (thiol function), an anti-inflammatory skeleton, *N*-acetyl-D-penicillamine (NAP) is used in the treatment of Wilson's disease (Trolovol®) and rheumatoid arthritis.

In this study, using an *in vitro* cell model of intestinal barrier, we propose to elucidate the intestinal transport mechanisms of *S*-nitrosothiols and NO in relation to their physico-chemical properties. Three different conditions were studied, i) permeability from the apical to the basolateral compartment, ii) permeability from the basolateral to the apical compartment to highlight an active transport such as drug influx/efflux, or a passive diffusion, and iii) permeability from an acidified apical compartment, mimicking the luminal intestinal pH of the jejunum, the major site of amino acid absorption [24].

2. Material and methods

2.1. Material and reagents

Eagle's Minimum Essential Medium (EMEM), foetal bovine serum (FBS), sodium pyruvate, penicillin 10 000 U.mL^{-1} and streptomycin 10 mg.mL^{-1} mix, trypsin, non-essential amino acids, glutamine, Hank's Balanced Salt Solution (HBSS Ca^{2+}/Mg^{2+}), sodium nitrate ($NaNO_3$), 2,3-diaminonaphthalene (DAN), 1.0 M hydrochloric acid (HCl) solution, propranolol hydrochloride, furosemide salt, triethylamine, 2-(*N*-morpholino)ethanesulfonic acid (MES), Trisma base (Tris), sodium chloride (NaCl), Igepal CA-630, sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), neocuproine and *N*-ethylmaleimide (NEM) were purchased from Sigma, France. Mercuric chloride ($HgCl_2$), orthophosphoric acid and sodium tetraborate were purchased from Prolabo (VWR). Sodium nitrite ($NaNO_2$) from Merck, sodium hydroxide (NaOH) from VWR Chemicals, methanol from Carlo Erba Reagents and acetonitrile was from Biosolve. Nitrite/nitrate fluorimetric kit was purchased from Cayman Chemical (Ref. 780051).

2.2. S-Nitrosothiols synthesis

GSNO, NACNO and SNAP were synthesised according to a previously described method [25]. Briefly, GSH, NAC or NAP were incubated with one equivalent of sodium nitrite under acidic condition. Then, the pH was shifted to 7.4 using a phosphate buffered saline (PBS 0.148 M) solution. The final concentration was assessed by UV–Vis. spectrophotometry (Shimadzu; UV-spectrophotometer; UV-1800) using the specific molar absorbance of the *S*-NO bond at 334 nm for GSNO and NACNO (ε_{GSNO} = 922 M^{-1} cm^{-1}; ε_{NACNO} = 900 M^{-1} cm^{-1}) and at 340 nm for SNAP (ε_{SNAP} = 1020 M^{-1} cm^{-1}).

2.3. Caco-2 cells culture and cytocompatibility

Intestinal Caco-2 cells (ATCC® HTB-37™) from passage 36 to 45 were grown in complete medium consisting of EMEM supplemented with 10% (v/v) of FBS, 4 mM of glutamine, 100 U/mL of penicillin, 100 U/mL of streptomycin, 1% (v/v) of non-essential amino acids. Cells were cultivated at 37 °C under 5% CO_2 (v/v) in a humidified incubator. Caco-2 cells were seeded in 96-wells plates at 2×10^4 cells/well 24 h before experiment. They were then exposed to each *S*-nitrosothiol (from 10 to 100 μM) for 24 h at 37 °C, complete medium being used as control. Cytocompatibility was assessed through metabolic activity with the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The absorbance of extracted formazan crystals was read at 570 nm with a reference at 630 nm (EL 800 microplate reader, Bio-TEK Instrument, Inc®, France). Metabolic activity in control condition was considered as 100%.

2.4. Intestinal permeability of reference molecules and S-nitrosothiols

Caco-2 cells were seeded at 2×10^6 cell/cm^2 on cell culture inserts (Transwell®, Corning, USA, membrane with 0.4 μm pore size, 1.12 cm^2 area or 4.97 cm^2) disposed in a 12-wells or 6-wells plate, respectively. The complete medium was replaced every two days during the first week of cell proliferation. During the second week, the medium was replaced every day until the differentiated cell monolayer was formed (14–15 days). The formation of the barrier was followed by transepithelial electrical resistance (TEER) measurement using a Millicell®-Electrical Resistance system (Millipore, USA) and validated for TEER values higher than 500 Ω.cm^2.

The bidirectional permeability of each *S*-nitrosothiol across the Caco-2 monolayer was evaluated from the apical to basolateral (A-B) compartment, mimicking physiological permeability conditions (intestinal lumen to blood compartment), and from the basolateral to the apical (B-A) (Fig. 1) compartment in HBSS at pH 7.4 to evaluate possible efflux mechanisms. A third condition evaluates the importance of the influence of luminal pH adjusted to 6.4 with 0.5 M MES solution in the apical compartment to determine the intestinal site of absorption (intestinal segment).

The concentration of *S*-nitrosothiols used to study the permeability