Short Communication

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New lipophilic organic nitrates: candidates for chronic skin disease therapy

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Abstract: Organic nitrates are widely used, but their chronic efficacy is blunted due to the development of tolerance. The properties of new tolerance free organic nitrates were studied. Their lipophilicity profile and passive diffusion across polydimethylsiloxane membrane and pig ear-skin, and their efficacy in tissue regeneration using HaCaT keratinocytes were evaluated. The permeation results show that these nitrates have a suitable profile for NO topical administration on the skin. Furthermore, the derivatives with higher NO release exerted a pro-healing effect on HaCaT cells. This new class of organic nitrates might be a promising strategy for the chronic treatment of skin pathologies.

Keywords: lipophilicity; organic nitrates; skin permeation; tolerance; wound healing.

Nitric oxide (NO) is one of the smallest bioactive molecules produced by mammalian cells. NO controls pivotal physiological functions, such as neurotransmission and vascular tone, by activation of its primary physiological effector, which is the soluble guanylate cyclase (Förstermann et al. 1994; Snyder 1992). Evidence of NO synthesis by human skin cells was first reported in 1992 (Heck et al. 1992), and it is now clear that NO plays a key role in maintaining skin homeostasis and orchestrating the skin's response to external stimuli, such as heat, ultraviolet (UV) light, and infection (Cals-Grierson and Ormerod 2004).

Many reports state that exogenous NO donors represent a promising method to promote wound healing (Freedman and Loscalzo 2003) by enhancing cell proliferation, collagen deposition, and angiogenic activities, improving tissue regeneration (Ahmed et al. 2022; Suschek et al. 2022; Zhang et al. 2019; Zhou et al. 2016). Furthermore, topical application of NO donors has been proven to be effective in controlling infection and partially reversing impaired healing (Ghaffari et al. 2006), treating skin microcirculatory dysfunction, producing analgesic action (Ganzarolli de Oliveira 2016), as an antibacterial agent (Urzedo et al. 2020), and as a cytotoxic agent toward cancer cells (Rolim et al. 2019).

Thus, the use of a low-molecular-weight NO donor to provide controlled release of NO in localized areas resulted a good therapeutic strategy. Some organic nitrates are already widely used in the medical field; in particular, GTN has long been used in the treatment of angina pectoris, acute myocardial infarction (Jugdutt 1992), congestive heart failure (Leier et al. 1981), and blood pressure control. In addition, GTN has been successful in the treatment of children with anal fissures when administered as an ointment and as an alternative to sildenalfil (Viagra) when used topically as a spray to the penile shaft (Wang et al. 2002).

A very important limitation in the practical use of GTN and other organic nitrates is the marked attenuation of their effects following continuous exposure to high doses of these drugs. This phenomenon is known as *tolerance* (Daiber et al. 2008, 2012; Gori 2020). Tolerance to the vascular effects of organic nitrates is a well-established phenomenon, probably mostly related to their bioactivation process, thus it has been reported also in other contexts. For instance, a number of studies have shown that topical application of GTN to the anus can heal fissures and cause reversible relaxation of the internal sphincter (Carapeti et al. 1999). Although, an acquired tolerance to GTN after topical use in the treatment

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of chronic anal fissure has been reported in men, and this may account for some treatment failures (Altomare et al. 2000; Watson et al. 1996). Albeit it is well established that most organic nitrates cause nitrate tolerance, the mechanisms of tolerance onset are not yet fully elucidated, which increases the need to develop new NO donor candidates in order to shed new lights on the puzzle of nitrate pharmacology and overcome the development of tolerance (Mizuno et al. 2020; Münzel et al. 2005; Omidkhoda et al. 2020).

Our research group previously described the synthesis of novel organic nitrates (Figure 1) and their properties as vasodilator agents *in vitro* and *ex vivo* (Chegaev et al. 2006, 2009; Marini et al. 2022). Compounds **2a**, **2b** and **3a**, **3b** are the two pairs of *erythro* and *threo* diasteromers which arise from the introduction on the terminal methylene group of the achiral GTN of a phenyl and a benzyl group, respectively, while compounds **4**, **5** (racemic mixtures) and **6**, **7** are the products formally obtained from these models by the deletion of one or two nitrooxy groups, respectively. All of these new compounds showed an interesting vasodilator profile, and newsworthy, the trinitrooxy-substituted compounds did not induce direct tolerance and cross-tolerance with GTN.

Based on this promising behaviour, we decided to further characterize these organic nitrates by focusing on their *in vitro* ADME properties. Specifically, we evaluated the solubility and the lipophilicity profile of all the compounds, as well as their passive diffusion across either polydimethylsiloxane membrane (PDMS), an isotropic polymer widely employed as an alternative model for percutaneous penetration *in vitro* (Neupane et al. 2020), or pig ear-skin, which provides a physiological and functional barrier mimicking human skin (Pulsoni et al. 2022; Sekkat and Guy 2001). In addition, given the central role of nitric oxide as an agent involved in wound healing, we assessed the potential efficacy of these organic nitrates in tissue regeneration using human HaCaT keratinocytes.

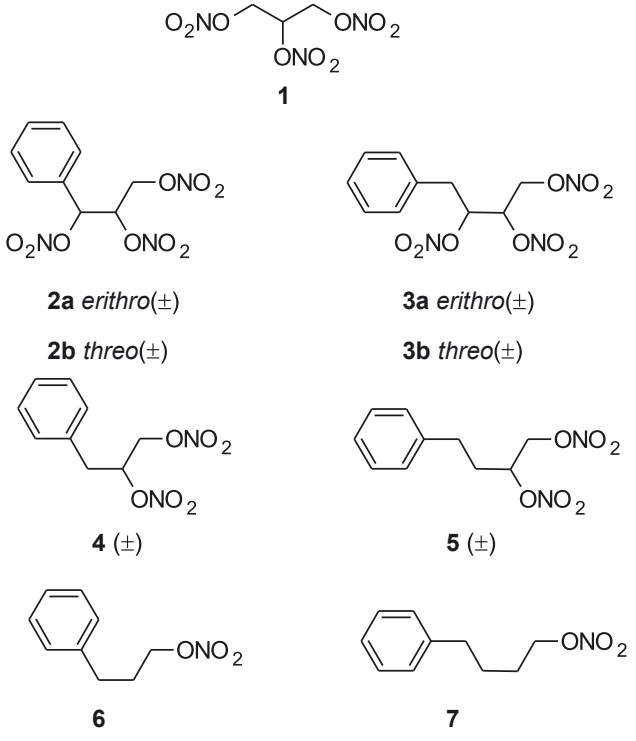

Figure 1: Structures of the compounds under study.

Solubility and lipophilicity are key physicochemical parameter that, along with molecular weight, influence various aspects of the pharmacokinetic profile of drugs (Avdeef 2003). It is well documented that diffusion across membranes and percutaneous absorption are affected by solubility, hydrophobicity (octanol–water partition coefficient, log P_{oct}) and molecular size (molecular mass, MW) of the penetrant (Cronin et al. 1999; Karadzovska et al. 2013; Pecoraro et al. 2019). The relevant physicochemical parameters that we decided to evaluate for our compounds are reported in Table S1 (Supplementary Material). The compounds differ widely in their molecular weights, which range from 195 to 317 daltons, a prerequisite for good penetration. The hydrophilic-lipophilic balance of the compounds under study was evaluated as both calculated partition coefficient (CLOGP) and measured octanol–water partition coefficient (log P_{oct}). The experimental lipophilicity values of the products ranged from 3.33 to 4.03 and were definitively higher than those of GTN. The calculated lipophilicity values of our nitroxy substituted compounds, and of GTN as well, are in good agreement with those measured (see *diff* (log $P^{exp\text{-}calc}$), Table S1).

For the compounds under study, solubility was assessed in water/ethanol/propylene glycol, the vehicle in which GTN, our reference in the permeation experiments, is formulated. As expected, solubility is inversely correlated with lipophilicity.

The cumulative amounts of drugs permeated through dimethylsiloxane and pig ear-skin are shown in Figure S1A and B (see Supplementary Material), respectively.

Studies on quantitative structure–permeability relationships suggest that the mechanism of flux through PDMS membranes is mainly influenced by hydrogen-bonding effects (Cronin et al. 1998; Geinoz et al. 2002). Human skin is a much more complex system than synthetic membranes, and consequently it is not surprising that the mechanisms of permeation through this organ are different (Avdeef 2003). Potts and Guy have shown that the coefficients of permeability through the skin of a large set of compounds can be reasonably described by the linear combination of their log P_{oct} and their molecular weight (MW) (Sekkat and Guy 2001). The greater the lipophilicity of the products,

the higher their ability to partition into the skin, while the larger the size of the products, the less they can diffuse through the skin. PDMS membranes are frequently used as appropriate replacements for excised human skin, despite the different mechanisms of permeation through the two systems. Generally speaking, the flux across PDMS membranes is higher than that through human or porcine skin (Cronin et al. 1998; Geinoz et al. 2004).

Table 1 shows the permeability coefficients (K_p), calculated from Equations (2) and (3) (see Supplementary Material) using the flux values (J).

Analysis of the fluxes measured across the PDMS membrane (K_p*sil*) values collected in Table 1 shows that the ease of permeation of the products under study through the PDMS membrane follows the series **7** > **6** > **5** > **4** > **2b** > **3a** > **2a** > **3b** > **GTN.** This trend is well described by Equation (1), which shows that permeation increases with lipophilicity and decreases slightly with MW.

$$\log K_p = 0.3348 \log P - 0.00547\,\mathrm{MW} - 0.7055 \qquad (1)$$

$$r^2 = 0.98;\ n = 9$$

No relevant amount of each compound was retained by the membrane.

As mentioned above, porcine skin is well known to be a reliable model of the human cutaneous barrier. This is particularly true when studying lipophilic substances (Dick and Scott 1992; Supe and Takudage 2021; Todo 2017). As expected, the fluxes measured across the pig ear-skin (K_p*skin*), found for the products under study are lower than those measured for the artificial membrane. They follow the series **6** > 7 > **GTN** > **4** > **2b** > **2a**. The low solubility of **3a**, **3b**, **5** in vehicle prevented us from studying their permeation through pig skin. In this limited set of compounds, the highest K_p*skin* values are those of compounds **6** and 7, which show the highest lipophilicities and lowest molecular weights. All the products tested were partially retained by the pig skin membrane (Table 1), suggesting that the NO released by compounds might act in the skin, due to the presence of different drug-metabolizing enzymes (Pyo and Maibach 2019; Svensson 2009).

NO in the skin is known to be involved in different processes, so these organic nitrates have a good potential for topical delivery of NO and consequently they could be useful in the treatment of several cutaneous affections (Adler and Friedman 2015; Weller 2003), such as Raynaud's phenomenon (Nahir et al. 1986; Wigley and Flavahan 2016), infectious skin diseases, and acne vulgaris (Baldwin et al. 2016; Ganzarolli de Oliveira 2016; Schairer et al. 2012; Seabra et al. 2005). In particular, NO plays a key role in the regulation of various wound healing processes, including inflammatory response, cell proliferation, collagen formation, antimicrobial action, and angiogenesis (Enoch and Leaper 2005; Malone-Povolny et al. 2019). The important role of NO in wound healing undoubtedly attracts intense research activity on NO-based wound healing therapy. In order to translate these statements and apply our physicochemical findings in an *in vitro* context, we decided to evaluate the pro-healing effect of these NO donors, using human HaCat keratinocytes. First of all, we assessed whether NO donors exerted a cytotoxic effect on the cells (Figure S2, Supplementary Material). HaCaT cells were treated with increasing concentration of NO donors (0.1–1–10–100 μM) for 48 h and cell viability was carried out by MTT assay. We

Table 1: Permeation parameters of the compounds on the dimethylsiloxane membranes and pig-ear skin (see Supplementary Material for detailed experimental conditions).

Compd.	Dimethylsiloxane membrane		Pig ear skin			
	Jsil	K_p*sil* × 10^{3a}	*Jskin*	K_p*skin* × 10^{3b} (cm/h^{-1})	Accumulated compound (μg/cm^2)	Accumulated/permeated compound
1 (GTN)	8.08 ± 0.61	35.59 ± 2.70	16.1 ± 1.2	16.10 ± 4.21	215.6 ± 2.2	0.35
2a	21.18 ± 0.54	69.89 ± 1.81	0.58 ± 0.12	7.38 ± 2.04	36.4 ± 2.5	3.00
2b	22.01 ± 0.74	72.62 ± 2.44	0.36 ± 0.04	5.37 ± 1.88	33.3 ± 1.2	5.5
3a	21.25 ± 0.63	67.00 ± 1.91	–	–	21.2 ± 1.0	–
3b	17.00 ± 1.16	53.57 ± 3.66	–	–	19.3 ± 2.7	–
4	22.80 ± 0.89	94.15 ± 3.68	1.36 ± 0.31	12.38 ± 5.53	77.5 ± 3.2	2.66
5	35.40 ± 1.25	138.17 ± 4.88	–	–	38.4 ± 1.4	–
6	46.95 ± 0.71	259.14 ± 3.94	7.45 ± 1.1	38.96 ± 4.53	117.7 ± 3.3	0.72
7	52.56 ± 1.23	269.27 ± 6.50	3.89 ± 0.86	27.14 ± 5.33	58.6 ± 2.5	0.71

[a]Permeability coefficient at 1 h: K_p = J/C_d (cm h^{-1}); J = flow (μg cm^{-2} h^{-1}); C_d = donor phase concentration (μg cm^{-3}); vehicle, EtOH/H_2O/propylene glycol 50/49/1 v/v. [b]Permeability coefficient at 24 h – lag-time: K_p = J/S (cm h^{-1}); J = flow (μg cm^{-2} h^{-1}); S = solubility (μg cm^{-3}) in vehicle, H_2O/EtOH/propylene glycol 80/19/1 v/v; for GTN, S = 1000 μg cm^{-3} (Perganit® solution).

found that all NO donors did not affect the proliferation of HaCaT cells (Figure S2). Next, the effective production of NO in cells released by these compounds was measured after 48 h treatment. As expected, we found that the trinitrooxy substituted compounds (**2a**, **2b**, **3a**, **3b**) released a higher amount of NO compared to the mononitroxy and dinitroxy substituted compounds **6**, **7** and **4**, **5** and the control (Figure S3, Supplementary Material). In previous *in vitro* and *ex vivo* studies (Chegaev et al. 2009; Marini et al. 2022), the vasodilating profile of the trinitrooxy substituted compounds did not show significant differences between the *erythro* and *threo* diasteromers; however, NO release in HaCat cells showed a trend of slight increase for the *threo* isomers **2b** and **3b** with respect to *erithro* analogues **2a** and **3a**. Therefore, to evaluate the effect of NO donors on cell migration, we performed the wound healing assay on HaCaT cells after incubation with compounds **2b** and **3b**. After 24 h compound **2b** significantly increased the percentage of wound closure whilst an increasing trend was observed for **3b** (Figure 2A and B). This pro-healing effect

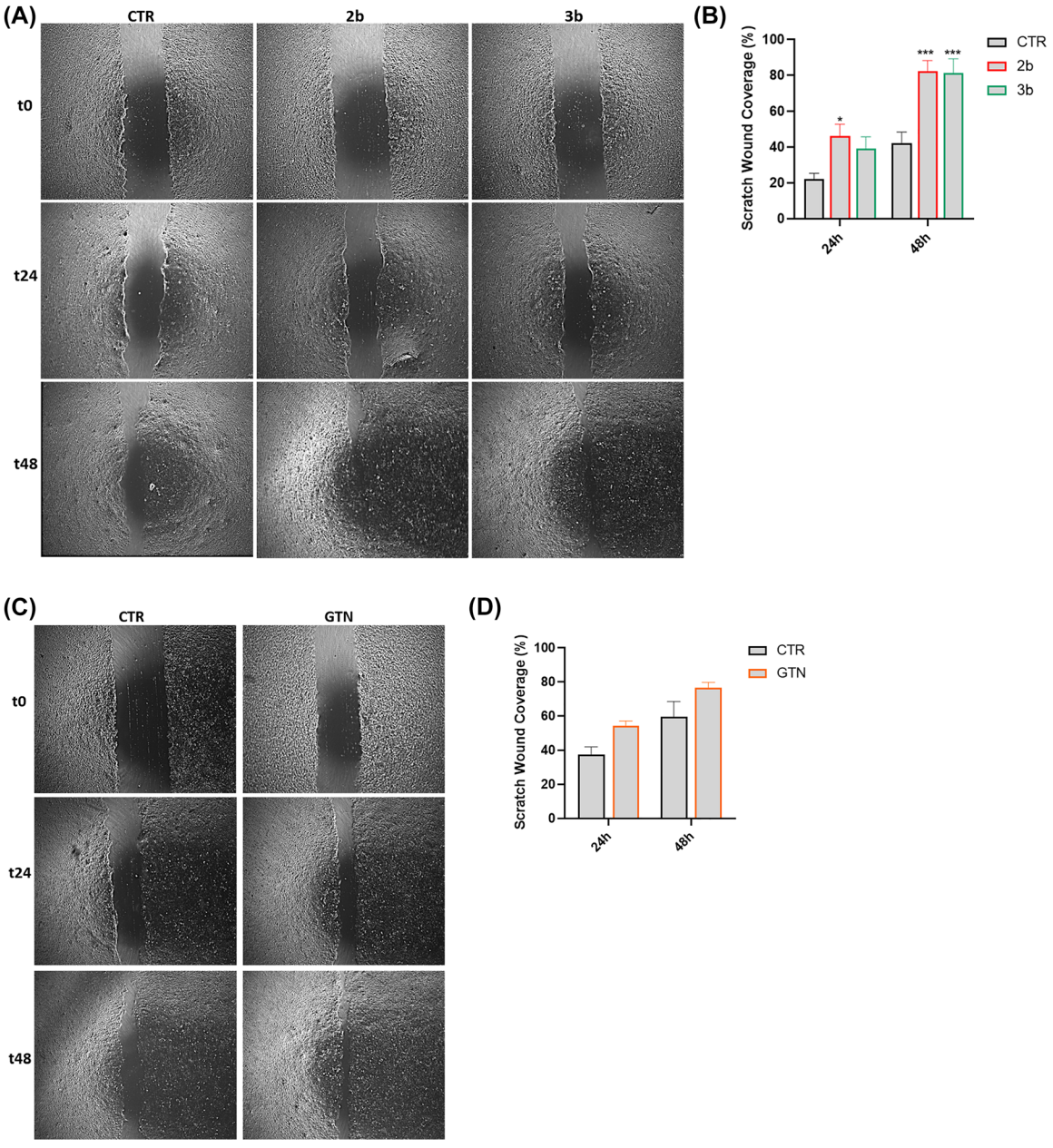

Figure 2: Representative example of a wound healing assay performed using HaCaT cells (A and C) and quantification of the healed wound area (B and D).

was further reinforced at 48 h, where both **2b** and **3b** significantly increased the wound closure rate confirming their efficacy in promoting skin repair (Figure 2A and B). This efficacy was particularly evident when **2b** and **3b** were compared with reference compound **1** (GTN) as shown in Figure 2C and D. In fact, a trend toward increased wound closure rate was also observed for **1**, but this effect was not significant (Figure 2D).

Chronic wounds are wounds that have entered a state of pathological inflammation, and such chronicity can have severe ramifications for diabetic patients, as they are the leading cause of diabetes-associated amputations (Malone-Povolny et al. 2019). Inflamed wounds provide a suitable environment for bacterial colonization; the increasing prevalence of antibiotic-resistant bacteria makes antibiotics treatment less effective, motivating the use of a topical antimicrobial agent that does not foster resistance. Therefore, due to their antimicrobial effects, widely used NO donors such as organic nitrates, could be attractive candidates for chronic wound therapy. On the other hand, chronic use of organic nitrates induces tolerance. In this context, the trinitrooxy substituted compounds **2b** and **3b** represent a real innovation. Indeed, both of these two molecules have showed significant pro-healing effects and promising vasodilating profiles without development of tolerance (Marini et al. 2022).

In conclusion, we have identified novel NO donors with a high lipophilic profile which would facilitate their topic administration. Moreover, their pro-healing effect might be proposed as an add-on treatment for inflammatory skin disease, even in a chronic therapy protocol, thanks to the absence of tolerance development.

Thus, the results obtained undoubtedly support the hypothesis that this new class of NO donor organic nitrates can represent a promising strategy for the treatment of skin diseases, due to their pro-healing effect and suitable physicochemical profile.

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