Determination of plasma and tissue distribution of 27-hydroxycholesterol after a single oral administration in a mouse model

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Abstract

Background: The side chain 27-hydroxycholesterol has been reported to inhibit the replication of several pathogen viruses, including herpes simplex virus, rhinovirus, rotavirus and SARS-CoV-2, in in vitro and ex vivo models.

Objective: In view of a future potential therapeutic use of 27-hydroxycholesterol, a pilot pharmacokinetic study was set up.

Methods: This active substance was complexed with 2-hydroxypropyl-β-cyclodextrin and orally administered in a single dose to CD1 male mice; its recovery in plasma and a few tissues up to 24 h post-treatment was evaluated.

Results: The absorption of the oxysterol by the small intestine was moderate, due to its physicochemical properties, but still relevant and rapid, showing a peak at 1 h after supplementation and being almost completed 24 h after treatment. 27-Hydroxycholesterol appeared to be a high hepatic extraction drug, possibly with an extrahepatic component contributing to the total clearance.

Conclusions: Following the oral 25 mg/kg dosing, plasma levels of 27-hydroxycholesterol showed an average steady-state concentration similar to that shown to be able to inhibit the replication of all viruses tested so far in in vitro models.

Significance statement

The first pharmacokinetic data relative to a natural oxysterol administered p.o. are reported. Data should contribute to further elucidate oxysterol pathophysiology and guide non-clinical studies aiming at investigating possible therapeutic use of 27-hydroxycholesterol or its analogs.

Keywords

- 27-hydroxycholesterol
- oxysterols
- pharmacokinetics
- antiviral
Introduction

The possible involvement of cholesterol oxidation products, named oxysterols in human pathophysiology, is becoming of multidisciplinary interest. These cholesterol derivatives are in part of non-enzymatic origin, taken up with food or generated endogenously, especially in inflammatory conditions, and in part the result of enzyme-driven metabolic reactions in our body (Schroepfer 2020, Sottero et al. 2019a). Among the oxysterols of enzymatic origin, 27-hydroxycholesterol (27OHC) is definitely the most prevalent, under physiological conditions, in human peripheral blood (Dzeletovic et al. 1995, Leoni & Caccia 2011). It derives from the oxidation of cholesterol at the level of the side chain by cholesterol 27-hydroxylase (CYP27A1) (Björkhem et al. 1994), an ubiquitous mitochondrial enzyme, particularly represented in hepatocytes, monocytes and neutrophils (Björkhem et al. 1994, The Human Protein Atlas a).

27OHC has long been recognized as contributing to cholesterol homeostasis since it regulates one of the two pathways of bile acid synthesis (Javitt 1994), and more recently it has been demonstrated to be a good ligand of quite a number of receptors, like liver X receptors (Janowski et al. 1996), estrogen receptors (DuSell et al. 2008) and toll-like receptors (Gargiulo et al. 2015), in this way intervening in the modulation of a wide array of signaling pathways. On the other hand, as usually happens with multifunctional molecules, relatively high concentrations of 27OHC have been associated with a variety of disease conditions, in particular those conditions whose progression is enhanced by chronic inflammatory reactions, including atherosclerosis, inflammatory bowel disease, Alzheimer’s disease and cancer (Poli et al. 2013, Gargiulo et al. 2015, Poli & Biasi 2016, Testa et al. 2016, Sottero et al. 2019b).

Of note, the large majority of findings provided up to now in support of a given physiological or pathological effect of 27OHC have been obtained in cell cultures and in few in vivo experimental studies, and very little information is available about the kinetic behavior of cells and organisms vs an exogenously supplemented amount of this or any other oxysterol. Only the metabolism of oxysterols, including that of 27OHC, has been investigated in depth (Mutemberezi et al. 2016), while scarce is the knowledge about the actual absorption and distribution of these compounds. This consideration prompted us to set up a pilot pharmacokinetic study of 27OHC orally administered in a single dose to the mouse, evaluating the oxysterol’s recovery in plasma and a few tissues up to 24 h. Oral administration was chosen as the most suitable method considering a future potential therapeutic use of this oxysterol, for instance to reintegrate its physiological blood concentration in cerebrotendinous xanthomatosis, characterized by a deficit of 27-cholesterol hydroxylase (Mignarri et al. 2016) or to inhibit the replication of several pathogen viruses, both enveloped and naked virus, including hepatitis B and C viruses, rotavirus (Lembo et al. 2016) and, last but not the least SARS-CoV-2 (Marcello et al. 2020). The broad-spectrum antiviral potential of 27OHC appears to be due to its ability to impair the intracellular cholesterol homeostasis, necessary for many viruses to replicate (Lembo et al. 2016), with a very high selectivity index, i.e. with no evident cytotoxicity at all (Civra et al. 2014, Marcello et al. 2020).

Materials and methods

Reagents and animals

27-hydroxycholesterol-d₆ and 27OHC were purchased from Avanti Polar Lipids Inc. (USA). Solid phase extraction (SPE) cartridge, SI 100 mg columns, Isolute, were purchased from Biotage (Uppsala, Sweden). The 27OHC administrated to the mice by gavage was synthetized by Aesica Pharmaceuticals (Northumberland, UK) and kindly provided by Panoxyvir Srl (Turin, Italy). All other reagents, including 2-hydroxypropyl-β-cyclodextrin (2HPβCD), were of analytical grade and purchased from Sigma Aldrich. 27OHC was complexed with 2HPβCD in our laboratories (batch 11-2020).

CD1 male mice were provided by Envigo RMS Srl (San Pietro al Natisone, Italy). The animals, identified by the number on the tail and kept three per cage, were acclimatized for about 5 days before treatment, with free access to food and water. Environmental conditions were as follows: temperature 22 ± 2°C, humidity 55 ± 10%, air changes 15/h, light/darkness cycle 12 h, food: pellets 12 mm, Global Diet 2018 certificate (Mucedola, srl, Settimo Milanese, Italy).

In life treatment

For the oral gavage, 27OHC was dissolved in a glycerol solution (2% w/v) containing 2HPβCD (35% w/v), final pH=4. The concentration of 27OHC was 5 mg/mL, 2HPβCD concentration was 350 mg/mL and that of glycerol was 20 mg/mL. The placebo given at time point 0 (pre-treatment) was 2HPβCD (35% w/v) in glycerol solution (2% w/v); 2HPβCD concentration 350 mg/mL and glycerol
concentration 20 mg/ml, pH = 4. Stability of the solutions (1 month) was adequate to the timelines required for the study. Formulation storage condition: room temperature, in the dark.

The time points of the kinetics were 0, 1, 4, 8 and 24 h with four mice for each time point. The amount of 27OHC administered by gavage to the experimental animals was 25 mg/kg body weight. Thus, 5 mL of the 27OHC solution (5 mg/mL) were administered per kilogram body weight. An identical amount of 2HPβCD (35% w/v) in glycerol solution (2% w/v) was administered p.o. to the time 0 group of animals.

### Tissue sampling

At the different time points, the corresponding group of mice was sacrificed by exsanguination from cheek and cava vein under anesthesia, followed by neck dislocation, and blood, lung, brain, liver and intestine were collected. The single blood samples were collected in tubes containing Li heparin, stored in ice and centrifuged at +4°C, 3000 g for 10 min. Plasma samples so obtained were kept at −20°C until bioanalysis. Each organ was rapidly washed in saline, dried and placed in tubes and kept at −20°C until bioanalysis.

### Preparation of tissue homogenates

The organs collected for bioanalysis were homogenised with PBS pH 7 at the dilution of 1 g tissue/10 mL buffer, using Precellys Evolution homogenizer with Lysing kit (CK28 beads), three cycles of 25 s (15 s pause), speed 7500 rpm.

### Sterol quantification by gas chromatography-mass spectrometry

To a screw-capped vial sealed with a teflon septum, 0.15 mL of plasma or 250 mg of tissue homogenate were added together with 50 ng of 27OHC -25, 26, 26, 27, 27-d₆ as internal standards and 50 μL of butyldimethylhydroxytoluene (5g/L) and 50 μL of K3-EDTA (10 g/L) to prevent auto-oxidation. Each vial was flushed with argon for 10 min to remove air. Alkaline hydrolysis was allowed to proceed at room temperature (22°C) with magnetic stirring for 60 min in the presence of ethanolic 1 M potassium hydroxide solution. After hydrolysis, the sterols were extracted twice with 5 mL of cyclohexane. After evaporation, 27OHC was separated from cholesterol and other sterols by elution of the remaining 7 mL on SPE cartridge (SI 100 mg columns, Isolute) with isopropanol:hexane 30:70 v/v. The organic solvents were evaporated under a gentle stream of argon and converted into trimethylsilyl ethers with N,O-bis(trimethylsilyl)trifluoroacetamide (60°C for 60 min). Analysis was performed by GC-MS on a Clarus 600 gas chromatograph (Perkin Elmer) equipped with Elite-5MS capillary column (30 m, 0.32 mm, 0.25 μm. Perkin Elmer) connected to Clarus 600C mass spectrometer (Perkin Elmer). The oven temperature program was as follows: initial temperature of 180°C was held for 1 min, followed by a linear ramp of 20°C/min to 270°C and then a linear ramp of 5°C/min to 290°C, which was held for 10 min. Helium was used as carrier gas at a flow rate of 1 mL/min and 1 μL of sample was injected in splitless mode. Mass spectrometric data were acquired in selected ion monitoring mode (OTMSi-ethers) at m/z = 462 for 27-hydroxycholesterol-d₆ and at m/z = 456 for 27OHC.

### Statistical analysis

The concentration of 27OHC recovered in plasma and different organs was expressed as mean ± s.d. of four different values. The time 0 mean values were subtracted from the mean values obtained at time 1, 4, 8 and 24 h, and the corresponding area under the time/concentration curve (AUCs) was calculated adopting the trapezoidal method.

### Results and discussion

The data of 27OHC recovery in plasma and examined tissues after the administration p.o. to CD1 male mice of a single dose of the oxysterol (25 mg/kg/b/wt) are reported in Table 1, together with the basal endogenous values determined at time 0.

The physicochemical properties of 27OHC (AlogP 5.9, polar surface area 40.4 Å², one Lipinski violation) suggested a relatively poor solubility, moderate oral bioavailability and a significant volume of distribution. As reported in Fig. 1, the absorption of the oxysterol by the small intestine was moderate as expected but still relevant and rapid, showing a peak 1 h after supplementation and being almost completed 24 h after treatment. The 27OHC basal content in the mouse small intestine was moderate as expected but still relevant being almost completed 24 h after treatment. The 27OHC concentration in the other tissues (Table 1) were about 0.18 μg/g of tissue (Fig. 1). Apparently there are no data in the literature with which to compare our finding. Further, an AUC of 77.88 µg/g/h was calculated according to the trapezoidal rule.
Table 1  Recovery of 27OHC in small intestine, plasma, liver, brain, and lungs of CD1 male mice at different time points after the administration by gavage. Values are means ± s.d. of four different samples taken at time 0, 1, 4 and 8 h and mean of two samples taken at 24 h.

| Hours | Intestine | Plasma | Liver | Brain | Lungs |
|-------|-----------|--------|-------|-------|-------|
| 0     | 0.21 ± 0.03 | 0.04 ± 0.01 | 0.61 ± 0.09 | 0.19 ± 0.02 | 0.35 ± 0.05 |
| 1     | 11.16 ± 5.82 | 0.97 ± 0.21 | 10.51 ± 0.66 | 0.20 ± 0.01 | 0.82 ± 0.04 |
| 4     | 5.90 ± 0.34 | 0.34 ± 0.15 | 12.31 ± 0.75 | 0.31 ± 0.02 | 0.64 ± 0.08 |
| 8     | 2.09 ± 0.52 | 0.28 ± 0.12 | 5.64 ± 0.64 | 0.21 ± 0.02 | 0.41 ± 0.08 |
| 24    | 0.50 ± 0.08 | 0.09 ± 0.07 | 3.26 ± 0.18 | 0.21 ± 0.02 | 0.29 ± 0.07 |

In the plasma compartment, 27OHC basal level resulted to be 0.04 µg/mL (Table 1), in line with the mean values reported by Musman et al. (0.04 µg/mL – female C57BL/6J) (Musman et al. 2016) and by Lütjohann et al. (0.06 µg/mL – male C57BL/6) (Lütjohann et al. 2018). Following the oral dosing, plasma levels of 27OHC appeared to increase rapidly (Fig. 2), as in the case of the intestinal absorption, and reach a C<sub>max</sub> of 928 ng/mL (2.3 µM), followed by elimination with a total AUC of 6.22 µg/mL/h.

The predominant role of liver in metabolizing 27OHC was confirmed in this pharmacokinetic analysis, which showed a bell-shaped curve up to 8 h after dosing and an AUC of 146.90 µg/g/h (Fig. 3). The basal hepatic concentration measured 0.61 µg/g of tissue (Table 1) and was in line with the values available in literature (Musman et al. 2016, Lütjohann et al. 2018). 27OHC is known to be mainly metabolized in the liver, by the cytochrome P450 enzyme, oxysterol 7α-hydroxylase (CYP7B1), the resulting 7α-27-dihydroxycholesterol being utilized for bile acid synthesis (Wu et al. 1999). Contrary to cholesterol 7α-hydroxylase (CYP7A1), which is liver specific, CYP7B1 protein is expressed in various tissues other than liver, including small intestine, lung and brain (The Human Protein Atlas b), which are the organs considered in the present investigation. Hence, the obtained data indicate that 27OHC is a high-extraction drug, with a certain extrahepatic component contributing to its total clearance.

In the lungs, the pharmacokinetic analysis showed a relatively low 27OHC concentration, with a maximum level at 4 h after dosing, an AUC of 10.39 µg/g/h and a complete return after 24 h dosing to the physiological basal level, the latter measuring 0.35 µg/g of tissue (Table 1) (no data available in the literature for comparison) (Fig. 4).

Figure 5 reports the trend of 27OHC concentration in the brain during the 24 h of monitoring. Only a modest and transient increase above the basal tissue level of about 0.18 µg/g was observed 4 h after oral supplementation with the oxysterol, with a measured AUC of 5.27 µg/g/h. The basal brain level of 27OHC observed in this study (Table 1) well matches those reported in the literature (Valenza et al. 2015, Musman et al. 2016, Lütjohann et al. 2018). The ability of 27OHC to cross the blood–brain barrier, first described by the group of Björkhem (Heverin et al. 2005), was then confirmed by the pharmacokinetics approach, but the extent of this passage into the brain appeared quite modest, as already outlined by the original discoverers (Heverin et al. 2005).

Finally, Fig. 6 provides an overall picture of the 27OHC levels recovered in plasma and various organs of CD1 male mice after 1, 4, 8 and 24 hours after a single administration.
Conclusions

This report most likely provides the first useful information about absorption and organ distribution of a natural oxysterol administered p.o. Information could guide non-clinical studies aiming at investigating possible therapeutic use of 27OHC or its analogs, both of natural (e.g. 25OHC) or of synthetic origin. Certainly of interest in the case of cerebrotendinous xanthomatosis, the oral absorption of 27OHC, complexed with 2HPβCD. Indeed, by this way, one should have a better idea of the actual distribution of the oxysterol in the in vivo experimental model adopted.
administration of 27OHC and related oxysterols appears attractive because of their experimentally proven broad-spectrum antiviral effect (Lembo et al. 2016). In relation to this, it is noteworthy that the reported 25 mg/kg dose in the mouse provides systemic steady-state concentrations similar to the in vitro EC50 values of 27OHC as in rotavirus and rhinovirus infections (Civra et al. 2014).

In the absence of pharmacokinetic data from other species, allometric scaling based on generic allometric constants suggests that the human equivalent dose corresponding to the 25 mg/kg in mice would be about 2.0 mg/kg, or about 140 mg, in a 70 kg subject. As a consequence, the human dose range corresponding to the active therapeutic dose range would be 40–1000 mg/day, assuming linear pharmacokinetics.

Another quite different but equally interesting application of pharmacokinetic analyses focused on oxysterols is the toxicology of the non-enzymatic cholesterol oxidation products in food are lacking simply because no solid information is available about their actual absorption in the gut and their systemic distribution. This is a further incentive to expand the pharmacokinetic approach in the study of oxysterol pathophysiology.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the study reported.

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**Author contribution statement**
GP, DR and RB conceived and supervised the overall study; GP wrote the manuscript; VL, CC and FV performed the pharmacokinetic analyses, under the expert monitoring of RC and MA; DL and AC provided significant contribution to the discussion of the results.

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