Placental Passage of Humulone and Protopine in an 
Ex Vivo Human Perfusion System# 

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ABSTRACT
The placental passage of humulone and protopine was investigated with a human ex vivo placental perfusion model. The model was first validated with diazepam and citalopram, 2 compounds known to cross the placental barrier, and antipyrene as a positive control. All compounds were quantified by partially validated U(H)PLC-MS/MS bioanalytical methods. Only a small portion of humulone initially present in the maternal circuit reached the fetal circuit. The humulone concentration in the maternal circuit rapidly decreased, likely due to metabolism in the placenta. Protopine was transferred from the maternal to the fetal circuit, with a steady-state reached after 90 min. None of the study compounds affected placental viability or functionality, as glucose consumption, lactate production, beta-human chorionic gonadotropin, and leptin release remained constant. Histopathological evaluation of all placental specimens showed unremarkable, age-appropriate parenchymal maturation with no pathologic findings.

Introduction
During pregnancy, a large number of women need medical care. Pharmacotherapy in pregnant women is challenging, given that adverse effects on the embryo/fetus have to be considered [1].

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The situation is exacerbated by the fact that pregnant women are, in most cases, actively excluded from clinical drug development trials. This severely reduces the number of medications labeled for use during pregnancy [2, 3]. As a consequence, clinicians often make use of the so-called off-label prescribing (i.e., they advise the use of medications in a way that diverges from the approved product information [e.g., indication, application, dosage, patient categories]) [4]. Probably for all these reasons, expectant mothers often perceive synthetic medications as potentially dangerous. They try to reduce their consumption (compare with [5, 6]) and seek supposedly safe alternatives, such as phytomedicines. In a multinational study, an average of 28.9% of pregnant women reported using herbal medicines during pregnancy, with an even higher proportion of 40.6% in Switzerland [7].

Some phytomedicines are used to treat NMDs in pregnancy, such as sleep disorders, restlessness, anxiety, and mild depression. A recent prevalence estimate in Switzerland reported that 16.7% of perinatal women used mental healthcare [8].

The perception of phytomedicines as safe in pregnancy [9] contradicts that studies on their safety in pregnancy are essentially lacking. For example, how much phytochemicals can pass across the placental barrier to reach the fetus is unknown. We are currently investigating the transplacental transfer of selected compounds from medicinal plants used to treat mild NMDs in pregnancy to shed some light on the matter.

According to the German Commission E Monograph [10], hops (Humulus lupulus L., Cannabaceae) can treat mood disturbances such as restlessness, anxiety, and sleep disturbances. Various phytomedicines and food supplements containing hops are available in the EU and Switzerland [11, 12]. However, these products always contain additional plant extracts, and the content in bitter acids is not specified. Analytical data reported content of 2% to 12% (w/w) of alpha-acids in hops cones, with humulone as the major compound (35% to 70%) [13]. Hops extracts reportedly activate melatonin receptors in vivo [14], which may explain, at least in part, the sleep-inducing properties. Humulone was found to act as a positive allosteric modulator of recombinant GABA<sub>A</sub> receptors expressed in HEK-293 cells [15]. Due to a lack of sufficient safety data, the CHMP of the EMA does not recommend the use of hops during pregnancy [16].

California poppy (Eschscholzia californica Cham., Papaveraceae) has a long tradition among indigenous people in the USA [17]. The CHMP has classified it for traditional use as a sleeping aid and the relief of mild symptoms of mental stress [18]. Various Eschscholzia products are on the market, ranging from approved phytomedicines to food supplements available via the internet. They contain either powdered herbal drug or extract as the active ingredient, sometimes combined with other herbs. Very few products have been standardized for their content in total alkaloids [11, 19, 20]. California poppy contains 0.5% to 1.2% of total alkaloids, with protopine being one of the major compounds [21–23]. Extracts of California poppy have shown sedative and anxiolytic effects in vitro [17], and these properties have been attributed to the isoquinoline alkaloids [24]. Several in vitro studies suggest that protopine is a CNS-active compound. Protopine was found to bind with GABA<sub>A</sub> receptors in rat synaptic membrane preparations [25, 26]. Protopine was also shown to be a ligand at 5-HT<sub>1A</sub> receptors expressed in human CHO cell membranes [27]. The alkaloid is also an inhibitor of serotonin and noradrenaline transporters expressed in murine S6 and N1 cells, respectively [28]. As for hops, the CHMP does not recommend using California poppy during pregnancy due to a lack of sufficient safety data [18].

A broad range of in vitro and in vivo models have been used to assess fetal exposure to exogenous compounds. Chronically cannulated sheep have been used extensively, and in situ placental perfusion techniques in rodents (guinea pigs, rabbits) have been established [29]. Ex vivo perfusion models with rats [30] and mice [31] have been used for an early screening of substance transfer across the placental barrier. However, with all animal models, the extrapolation of results to humans is limited due to functionally and anatomically large interspecies differences [29, 32, 33]. In vitro models utilizing well-established human placental cell lines (e.g., BeWo, Jar, JEG-3 cells) or human placental primary cells (villous trophoblasts) and explant tissue have been employed. These latter models enable the study of various factors affecting the transplacental transport, such as uptake, efflux, and metabolism. BeWo b30 cells (a clone of BeWo cells) form confluent monolayers on the semipermeable membrane of Transwell inserts and can be used as an in vitro model for the placental barrier. However, all cell-based placental models lack the cellular organization, compartmentalization, and 3-dimensional structure of intact, physiologically active placentae [34]. The current gold standard among the placental transfer models is the ex vivo perfusion utilizing human placentae obtained immediately after delivery [35–37]. Here, the structure of the cotyledon as a functional unit of the placenta is fully preserved [38], and data obtained are highly predictive of the in vivo transfer [39].
We here determined the transplacental transfer of humulone, a characteristic and pharmacologically active compound in hops [40], and protopine, a major alkaloid in California poppy [41], side by side with compounds known to cross the placental barrier as citalopram, diazepam, and antipyrine (Fig. 1). The effects of humulone and protopine on the viability of placental tissue and the production of placental hormones were also investigated.

Results

In a first step, the ex vivo placental perfusion model—with which we wanted to study the transfer of humulone and protopine—was validated with 3 placenta-permeable compounds, namely citalopram, diazepam, and antipyrine (Fig. 2). The latter compound served as a connectivity (positive) control in all placental perfusions to verify the overlap of the maternal and the fetal side.

Citalopram and diazepam crossed the human placental barrier as expected [42, 43]. With citalopram, a steady-state concentration that corresponds to approximately 11% of the initially present citalopram at the maternal side was reached on the fetal side after 60 min, with a concentration of approximately 22% on the maternal side. Concentrations did not change during the next 300 min (Fig. 2a). For diazepam, an equilibrium (same concentrations in both reservoirs) between maternal and fetal circuits was reached after 45 min, with 24% of initially analyzed concentrations and a slow decrease until 240 min (Fig. 2c). Antipyrine also readily crossed the placenta barrier and reached equilibrium after approximately 120 min (Fig. 2b and d). These findings aligned with previous work and confirmed that antipyrine was a suitable connectivity control [44]. The transplacental transfer of humulone revealed a marked and rapid decrease in the maternal circuit but little to the fetal circuit. After 45 min of perfusion, only 9.7% of the humulone was detected in the maternal side, and a maximum
of 8.4% in the fetal compartment (▶ Fig. 2e). At the same time, the connectivity control showed a normal pattern (▶ Fig. 2f). Protopine was also transferred from the maternal to the fetal circuit. A gradual decrease of protopine in the maternal compartment and a concomitant increase in the fetal compartment were observed. Already after 60 min, a steady-state was established in the 2 circuits, with virtually no change over the remaining 300 min (approximately 27% [maternal] vs. 20% [fetal]) of initially analyzed concentration (except t = 300 and 360 min, in which only 2 values are included).

If the fetal-maternal concentration ratio (FM ratio; ▶ Fig. 3) of humulone is considered, the fetal and maternal concentrations reached an FM ratio of 1.0 after 60 min but at very low concentrations. During the remaining 300 min, the (low) concentration in the fetal circuit was always higher than that in the maternal circuit (FM ratio ≥ 1.0). For protopine, no concentration equilibrium was apparent in the fetal and maternal compartments at any point of the placental perfusion (FM ratio of 0.75 after 360 min). The profiles of the 3 synthetic compounds are shown for comparison. In citalopram, an equilibrium between fetal and maternal concentrations was never reached (FM ratio of 0.63 after 360 min). As for diazepam, the FM ratio was comparable to antipyrine (0.98 vs. 0.95 at 360 min).

Several aspects must be considered for the recovery calculations (▶ Fig. 5, Table 1S, Supporting Information) of study compounds during placental perfusions. Looking at the final distribution of the compounds in the 2 compartments (fetal and maternal circuit) after 360 min of the perfusion, we found they all passed the placental barrier and were distributed in the following propor-
tions (fetal vs. maternal): antipyrine (19.8% vs. 22.0%), humulone (1.0% vs. 0.8%), protopine (14.0% vs. 23.1%), citalopram (6.4% vs. 11.4%), and diazepam (7.9% vs. 7.9%). At least one-sixth to one-third of the final recovery corresponded to the substance removed by sampling during the perfusion. In addition, it is crucial to include the results from the 360 min system adherence test (empty perfusion). While only small losses were seen for antipyrine, humulone, and protopine, considerable amounts of citalopram and diazepam adhered to the equipment and tubing after 360 min. Using the system adherence test to assess the final recovery, we obtained the following values for antipyrine (71.2 ± 7.2%), humulone (14.4 ± 0.8%), protopine (71.4 ± 8.6%), citalopram (71.9 ± 5.2%), and diazepam (73.6 ± 4.8%). Finally, the fraction unbound to homogenates (f_{u,hom}) of placental tissue was assessed to account for potential loss of compound in the placenta itself (Table 2S, Supporting Information). The f_{u,hom} was equal to 1.0 for antipyrine and humulone, followed by protopine (0.48 ± 0.04), citalopram (0.21 ± 0.01), and diazepam (0.09 ± 0.007). Thus, f_{u,hom} followed the pattern of the system adherence test, reflecting the lipophilic nature of the compounds.

A f_{u,hom} of 1.0 (completely unbound) did not explain the loss of humulone, and therefore the stability of study compounds was tested over 360 min in 3 different matrices (PBS, PM, and placental homogenates [Donors 1–3]) at 2 temperatures (4 °C, 37 °C). The stability data of antipyrine, humulone, and citalopram were very comparable in the 2 matrices PBS and PM, while protopine (in PM) and diazepam (in PBS) were slightly less stable over 360 min at 4 °C and 37 °C (Fig. 6). A significant loss of humulone was observed in the presence of placental homogenate with only 29.3% (Donor 1), 41.7% (Donor 2), and 55.3% (Donor 3) remaining after 360 min. Degradation in homogenate at 37 °C was also observed with diazepam (66.7%, 68.3%, and 73.3%, respectively). The use of 3 different placental homogenates (donors) resulted in comparable values for all test substances. Differences due to matrix effects were excluded in a separate experiment (see Fig. 2S, Supporting Information).

The placental perfusion model can be used to characterize the transplacental compound transfer and investigate the possible effects of the compounds on placental viability and hormonal production. All placentae viability and metabolic activity were constant in our case, as neither glucose consumption nor lactate production was affected by the study compounds (Fig. 7a). With antipyrine (from control perfusions, Fig. 3S, Supporting Information), the total glucose consumption and lactate production during 360 min were 0.39 and 0.27 μmol/g/min, respectively. Perfusion with humulone, protopine, citalopram, and diazepam showed comparable values, indicating that they did not impair placental viability and metabolic activity. As an additional measure for placental function, the production of β-hCG and leptin was determined and expressed as the release rate per min and weight of cotyledon (g) (Fig. 7b). The tissue of all placentae retained its functionality throughout the ex vivo perfusion period. A β-hCG production of 95.7 mU/g/min and leptin production of 334.8 pg/g/min were observed in control perfusions with antipyrine (from control perfusions, Fig. 3S, Supporting Information). Neither humulone nor protopine inhibited β-hCG and leptin production in a statistically significant way, even though leptin pro-

Fig. 6 Stability data of study compounds (a–e) expressed as a percentage (%) of the initial concentration (C0) in PBS. The stability test was performed for 360 min at 2 different temperatures (4 °C and 37 °C) and 3 different matrices (PBS, PM, and placental homogenates from 3 different donors). Differences due to matrix effects were excluded in a separate experiment (see Fig. 2S, Supporting Information). Samples were processed via solid-phase extraction or protein precipitation before analysis. All data are represented as mean ± standard deviation (SD).
duction was somewhat lower in the presence of all study compounds.

To establish the human ex vivo placental perfusion model, we introduced a detailed histopathological examination of the perfused tissue. Representative macroscopic and microscopic images of the transition between perfused and nonperfused tissue and details of the perfused area and an individual villus with an outer layer of trophoblast cells are shown (Fig. 8). The most important histopathological criteria for QC were: (i) macroscopically, an evident effect of the perfusion on the cotyledon, in contrast to the nonperfused tissue (Fig. 8a); (ii) a difference between perfused and nonperfused tissue, the latter being characterized by a narrow intervillous space and blood-filled capillaries (Fig. 8b); (iii) the perfused area had dilated intervillous space, bloodless capillaries, and regular (mature) villi (Fig. 8c); and (iv) the proportion of vacuolated (degenerated) trophoblast cells was between 0–20% (Fig. 8d). Initial histopathological examinations showed that, in our hands, perfusion was effectively taking place and did not seriously damage the perfused cotyledon. These examinations were then performed after every perfusion experiment with study compounds to demonstrate successful perfusion (including connection of the fetal and maternalperfused area) and assess possible deleterious effects of the compound on the tissue compared to nonperfused tissue.

The histopathological evaluation of the placental specimens showed that the only macroscopically discernible effect of the perfusion was the pale tissue, which was apparent in all placenta. In all cases, the microscopic examination (Table 1) of the tissue sections revealed a clear transition between perfused and nonperfused tissue. The villous vessels of the perfused side were mostly dilated and ≥ 80% empty (the nonperfused area was ≥ 70% blood-filled). The intervillous space of the perfused tissue was also ≥ 85% bloodless (the nonperfused area was ≤ 50% blood-filled) and mostly dilated (40–80%), in contrast to the nonperfused side (10–80%). Hydropic villous changes were formed more often in perfused (0–20%) than nonperfused areas (0–1%). Histopathological examinations showed that the endothelium in perfused and nonperfused tissue was still viable after 360 min of perfusion. There were also no ruptures of villous vessels or extravasation into villous stroma in perfused and nonperfused areas. In addition, no thrombi could be detected in vessels of stem villi. The percentage of thrombi in villous vessels (0% or 1%) was the same in perfused and control tissue (nonperfused), whereas 2 cotyledons perfused with protopine showed a slightly higher proportion of villous thrombi compared to control (5% vs. 1%). Trophoblast vacuolization in the perfused areas occurred in a small proportion of 0–20% and was substance independent. Overall, humulone and protopine did not cause apparent damage to placental tissue according to the assessment of endothelium, vascular rupture, thrombi, and trophoblast vacuolization. In addition, no signs of inflammation were found in any of the perfused areas of the placentae examined, as neither bacteria nor neutrophils were present in the villous vessels and intervillous spaces. The assessment of global placental pathology was also inconspicuous, with no signs of fetal or maternal malperfusion, an absence of villous immaturity, chronic/acute villitis, chronic deciduitis, and chorioamnionitis, and no bacteria in the nonperfused area of the placenta.

Discussion

The present data show that humulone and protopine are differentially transported across the human placental barrier. Humulone concentrations rapidly decreased in the maternal circuit, but only a small portion of the compound appeared in the fetal circulation. After 45 min, the same amount of humulone was found in the maternal and fetal circuit (9.7% and 8.4% of the initial concentration measured in the maternal circuit, FM ratio ~ 1.0). As humulone did not significantly adsorb to the perfusion setup, the principal metabolization was found. However, the FM ratio of protopine was lower than that of antipyrine (0.75 vs. 0.96), and no equilibri-
The difference between maternal and fetal concentrations was reached. This finding was similar to the results obtained with citalopram. Whether the absence of equilibrium is due to active transplacental transport of protopine deserves further investigation. In our experiments, the transplacental transfer of citalopram, diazepam, and antipyrine was comparable with previously reported data [42–44].

Placental perfusion is the gold standard when studying the transfer of compounds from the mother to the fetus. Nevertheless, results obtained with term placentae have to be interpreted with caution. The thickness of the barrier between the maternal and fetal circuits decreases after 16 wk of gestation, leading to a higher permeability. Thus, the substance transfer may be overestimated compared to that in the premature placenta of early pregnancy [33]. Moreover, the model cannot mimic the mother’s drug absorption, distribution, metabolism, and excretion. It is crucial to work with very low concentrations to determine the transfer of compounds from (multicomponent) extracts. The U(H) PLC-MS/MS methods were developed accordingly to be as close as possible to clinically relevant concentrations.

Ex vivo placental perfusions allow determining the transfer of study compounds and assessing placental viability and function. Control perfusions with antipyrine showed similar values for the placental viability (glucose consumption, lactate production) and functionality (β-hCG and leptin production) as previously reported for this model [37]. None of the study compounds altered the glucose consumption, lactate production, and β-hCG accumulation, and only a statistically nonsignificant decrease in leptin production was observed. These results indicated that none of the compounds impaired placental performance. The histopathological evaluation of perfused tissues (cotyledons) was in line with these results. No pathological findings were observed, and all placental specimens showed only unremarkable, age-appropriate parenchymal maturation. Trophoblastic vacuolization of villi was the only perfusion-induced change observed, and it was not dependent on the substance tested. Despite the ischemic periods up to
60 min, no increase in placental tissue damage could be observed with time (Table 1 and Table 3S, Supporting Information). Neither the number of thrombi in villous vessels nor the vacuolization of trophoblasts in villi was increased.

It was crucial to calculate the recovery of study compounds in the best possible way to validate the findings. The amounts in the fetal and maternal compartments, the amounts removed by sampling, and the loss due to adsorption in the maternal circuit could be considered. Study compounds may also be taken up by cells/membranes and may be metabolized by placental enzymes. To minimize adsorption to the perfusion system, we used only tubes recommended for pharmaceutical and medical applications, and we shortened the tubing in the model. Moreover, in placental homogenates, fraction unbound and stability of compounds were determined.

Nevertheless, the calculated recoveries are likely to be underestimated, given that adsorption in the fetal circuit could not be measured. The final recoveries were 71.2% for antipyrine, 71.4% for protopine, 71.9% for citalopram, and 73.6% for diazepam. The recovery of humulone (14.4%) was substantially lower. Given that humulone did not accumulate in the membranes of the placental tissue (fu, hom = 1.0), our results point to an active uptake into cells and/or possible metabolization of humulone in the placenta. Moreover, the stability of humulone in the presence of homogenate was much lower at 37 °C compared to 4 °C, indicating the involvement of active processes. Phase I metabolism of humulone in liver microsomes has been investigated, whereby hydroxylation at the different isoprenoidal sides chains was reported [45]. We are currently investigating in more detail the hepatic metabolization of humulone. It appears thus reasonable to assume that humulone is being metabolized in the placenta via similar processes, as it is known that CYP450 enzymes are expressed in placental tissue [46].

To further assess California poppy and hops, additional phytochemicals in these plants have to be investigated, together with testing in additional models. We are currently using a range of in vitro assays to characterize intestinal absorption of the various compounds, their cytotoxic and genotoxic effects, and their influence on metabolic and differentiation processes. From a safety perspective, these results will be particularly relevant for compounds that can cross the placental barrier and, therefore, have access to fetal circulation, as is the case for protopine. In addition, we are investigating the intestinal and hepatic metabolization of these phytochemicals. As the example of humulone shows, also metabolization by the placenta needs to be investigated.

The human placental ex vivo perfusion model was successfully implemented and used for the first time with phytochemicals. These first findings illustrate that placental transfer and the fate of phytochemicals can substantially differ. The ex vivo placental perfusion model will now be used for transport studies with relevant phytochemicals in other medicinal plants used to treat mild NMDs in pregnancy.

### Table 1

| Experiment number | 1    | 2    | 3    | 1    | 2    | 3    | 4    |
|-------------------|------|------|------|------|------|------|------|
| Microscopic effects of perfusion (in perfused tissue) in % | | | | | | | |
| Emptyness of villous vessels | 80   | 95   | 95   | 90   | 80   | 95   | 95   |
| Blood-filled villous vessels in nonperfused | 90   | 100  | 70   | 90   | 95   | 100  | 95   |
| Dilated (recognizable) villous vessels | 10*  | 60   | 40   | 95   | 50   | 60   | ND   |
| Intervillous spaces without blood | 95   | 100  | 85   | 100  | 95   | 95   | 95   |
| Blood-filled intervillous space in nonperfused | 20   | 10   | 10   | 50   | 30   | 5    | 20   |
| Dilated intervillous space | 40   | 60   | 50   | 80   | 40   | 80   | 50   |
| Dilated intervillous space in nonperfused | 80   | 30   | 30   | 30   | 10   | 30   | 40   |
| Hydropic changes in perfused | 0    | 0    | 20   | 15   | 10   | 5    | 5    |
| Hydropic changes in nonperfused | 0    | 0    | 0    | 1    | 0    | 0    | 0    |
| Sharp transition perfused/nonperfused | Y    | Y    | Y    | Y    | Y    | Y    | Y    |
| Damage of placental tissue (in perfused tissue) in % | | | | | | | |
| Thrombi in villous vessels | 1    | 1    | 1    | 1    | 5    | 0    | 5    |
| ▪ If so: in nonperfused too? | 1    | 1    | 1    | 1    | 1    | –    | 1    |
| Thrombi in vessels of stem villi | 0    | 0    | 0    | 0    | 0    | 0    | 0    |
| ▪ If so: in nonperfused too? | –    | –    | –    | –    | –    | –    | –    |
| Vacuolated trophoblast in villi | 0    | 0    | 5    | 0    | 20   | 1    | 1    |
| ▪ If so: in nonperfused too? | –    | –    | 0    | –    | 0    | 0    | 0    |

* Mainly intermediate villi; ND = not determined; Y = yes.
Materials and Methods

Chemicals, reagents, and study compounds

All solvents were of U(H)PLC grade. Merck KGaA supplied MeCN, and MeOH was purchased from Reuss-Chemie AG. Purified water was obtained from a Milli-Q integral water purification system. Scharlau supplied DMSO, and formic acid was from BioSolve. Antipyrine and BSA were purchased from Sigma-Aldrich and antipyrine-D3 from HPC Standards GmbH. Protopine HCl was purchased from Extrasynthese SAS, humulone from Carbosynth Limited, and verapamil HCl from Sigma-Aldrich. Citalopram HBr, diazepam, and diazepam-D5 were purchased from Lipomed AG, and citalopram-D4 HBr was obtained from CDN Isotopes.

Ex vivo human placental perfusion

Placentae collection. Placentae were collected in collaboration with the Department of Obstetrics of the University Hospital of Zurich, Switzerland. Only placentae from women undergoing elective cesarean section from uncomplicated term pregnancies (37–41 wk) were considered. Each pregnant woman signed informed consent before delivery for the use of placentae for research. This procedure (including consent form) was approved by the Ethics Committee of the Canton of Zurich (KEK-StV73 Nr. 07/07; March 21, 2007). Exclusion criteria included: twin and/or complicated pregnancy, smoking, substance abuse, and patients positive for HIV, HBV, and SARS-CoV-2.

Equipment and experimental procedure of perfusion. A slightly modified ex vivo human placental perfusion model [37, 44] was used to study the transfer of the study compounds across the placental barrier (Fig. 9). A cotyledon of the placenta (lobule) was perfused with 2 reconstructed circuits representing the maternal and fetal sides. Both sides consisted of an artery transporting the perfusate to the cotyledon and a vein that returns the perfusate to the original reservoir. Two heating magnetic stirrers were added to ensure physiological conditions and prevent the uneven distribution of study compounds.

The time course of a perfusion experiment, including the preparatory phases, checkpoints, and samplings, is shown in Fig. 10. After obtaining a suitable placenta (checkpoint 1), the fetal artery and associated vein of a selected cotyledon were cannulated and mounted in a perfusion chamber within 60 min after delivery. The selection of a suitable cotyledon for perfusion was based on a thorough visual examination of the villous structures and associated fetal vessels. Cotyledons with a ragged maternal surface (visible disruptions; macroscopic tissue trauma), evidence of basal plate fibrin deposition (on the maternal surface), suspected placental infarction, or too little fetal membrane (on the disk of the placenta) were not considered for perfusion. The chorionic artery was cannulated (∅ 1.2 mm cannula) first, following
the chorionic vein cannulation (Ø 1.5–1.8 mm cannula). A surgical suture (orange or green PremiCron HR17, USP3/0, B. Braun Medical AG) was used to fix the cannulas. The experiment was performed using PM (modified composition [37] using cell culture medium 199 from Sigma-Aldrich) circulating through the fetal vessels (checkpoint 2), leading to a 20 min open preliminary (pre-) phase (nonrecirculating), followed by a 20 min closed pre-phase (recirculating). During the latter, the volumes of maternal and fetal perfusates have to remain stable to begin the main experiment (checkpoint 3). The main experiment started with circulating PM containing the connectivity control (antipyrine) and study compound at the desired concentration (○ denoting the experiment’s beginning and end). At defined timepoints (0, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 min), samples were taken from the maternal and fetal reservoir for analysis. At the beginning of the experiment and after each hour, the pH and the FM leak were measured.

In the following recirculating closed pre-phase (20 min; with the venous outflow leading back to the corresponding reservoir), the perfusate volumes on the maternal and fetal sides were monitored. Stability of volumes (50 mL PM in each reservoir) ensured the integrity of the circuits and the absence of leaking in the fetal-to-maternal circuit (checkpoint 2). The main experiment was then initiated by replacing the fetal and maternal perfusate simultaneously with equal starting volumes of 100 mL fresh PM, with the addition of the study compound and antipyrine (as a connectivity control) to the perfusate of the maternal circuit, both at a final concentration of 5 µM (941 ng/mL antipyrine; 1812 ng/mL humulone; 1767 ng/mL protopine; 1622 ng/mL citalopram; and 1424 ng/mL diazepam). In both reservoirs, the pH was adjusted to a physiological range (7.3 ± 0.1) at the beginning of the experiment (and adjusted after every hour of perfusion), and the FM leak was not allowed to exceed a value of 4 mL/h (checkpoints 4). Samples (3–4 mL) for analysis were taken at defined timepoints over a 360 min period (0, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 min). Samples were stored at −80 °C for bioanalytical analysis in 0.8 mL glass micro-inserts (VWR) immediately after centrifugation at 800 rpm/1837 rcf (Centrifuge Sigma 6–16; 4 °C; 10 min) to remove residual erythrocytes. Additional QC measures included a fetal perfusion pressure of ≤ 70 mmHg throughout the perfusion and an antipyrine equilibrium between the fetal and maternal circuit after 240–360 min. For an overview of the experimental conditions and characteristics of the placentae used, see Table 3S (Supporting Information).

Fetal capillary integrity marker analysis. For establishing and validating the ex vivo placental perfusion model, initial experiments were performed using FITC-dextran (40 kDa, Sigma-Aldrich) as a test substance for assessing the integrity of fetal capillaries (Fig. 4S, Supporting Information). FITC-dextran was added to the fetal circuit at a final concentration of 200 µg/mL. The concentration in the fetal and maternal samples was detected by a Cytation 3 fluorescence microplate reader (BioTek Instruments; excitation wavelength 490 nm; emission wavelength 520 nm). The samples (undiluted) were added to black Nunc MaxiSorp microtiter plates.

Perfusion system adherence test (empty perfusion). Before starting the perfusions with human placentae, a system adherence test (circuit of study compounds through an empty perfusion chamber comprising only the maternal circuit) was performed for a total of 360 min. This test was used to assess the dissolution and adherence of new yet unknown compounds to the perfusion equipment and mainly to the tubing system to evaluate the final recovery better. Study compounds were, therefore, directly dissolved in PM at a final concentration of 5 µM. All study compounds were tested individually in at least 3 independent experiments (n = 3).

Viability and functionality of placental tissue. We measured glucose and lactate concentration in fetal and maternal samples.
at the beginning and end of every perfusion. This was done to determine glucose consumption and lactate production throughout a perfusion as indicators of tissue viability and metabolic activity using an automated blood gas system (ABL800 FLEX). The production of 2 placental hormones—β-hCG and leptin—was monitored to assess tissue functionality ex vivo by standard EUSA as described previously by Malek et al. [47, 48]. The only deviation following the protocol was using a dilution solution consisting of 1% BSA instead of 2% BSA.

**Histopathological evaluation.** Each placental specimen was pathologically examined as an additional QC. For this purpose, representative tissue sections—each from the perfused and non-perfused placental portion and the transitional area—were removed immediately after each perfusion, fixed in 4% paraformaldehyde for at least 24 h, and then processed according to the standards of routine histopathological diagnosis of the Department of Pathology and Molecular Pathology (University Hospital of Zurich). Briefly, the fixed tissue sections were embedded in paraffin, cut into 2–3 µm thick sections, then stained with standard hematoxylin and eosin stain and with a modified Gram stain (according to Braun-Brenn). The latter was used to test for bacterial contamination in the perfused area [49]. Tissue from the nonperfused specimens was examined for general placental pathologies described in routine diagnostics [50, 51]. The quality of perfusion was correlated based on the blood void and width of the intervillous (maternal) space and fetal blood vessels in the chorionic villi, with particular attention to the presence of intravascular thrombi. To test whether tissue damage might have occurred due to perfusion, we sought and compared general signs of degeneration such as vacuolization of the cytotrophoblast, the viability of the villous vascular endothelium, and the formation of hydropic villous changes with the tissue condition of the nonperfused area. All microscopic effects studied and damage to placental tissue in the perfused area are reported in relative amounts (%) compared to nonperfused tissue.

**LC-MS/MS analysis**

**Instrument and chromatographic conditions.** U(H)PLC-MS/MS analyses were performed on an Agilent 6460 Triple Quadrupole MS system connected to a 1290 Infinity LC system consisting of a binary capillary pump G4220A, column oven G1316C, and multipumper G7167B. Quantitative analysis by MS/MS was performed with ESI in MRM mode. Desolvation and nebulization gas was nitrogen. The auto- sampler was set at 10 °C. An Acquity UPLC HSS T3 column (100 mm × 2.1 mm; 1.8 µm) (Waters Corp.) was used for separa- tion of the analyte and the IS, except for diazepam and its IS diaze-epam-D5 where a Kinetex column (100 mm × 2.1 mm; 1.7 µm) (Phenomenex) was used for separa- tion. Within- and between-series imprecision and inaccuracy. As mentioned above.

**Method qualification.** The bioanalytical fit-for-purpose methods had been developed and qualified only based on some validation tests (within- and between-series imprecision and inaccuracy, as well as carry-over, had been assessed to validate the methods), following the current guidelines for industry [52, 53].

**Within- and between-series imprecision and inaccuracy.** Six replicates of 6 QCs (LLOQ [5 ng/mL], QCL, QCM, QCH, ULOQ [500 ng/mL]) were processed and injected into the U(H)PLC-MS/MS. Three validation runs on 3 different days were performed to ensure reproducibility. In each run, the imprecision (CV%) of each QC series had to be below 15% (20% for LLOQ) within the series. The inaccuracy (RE%) had to be within ±15% of the nominal val-
ues (± 20% at the LLOQ). After these 3 runs, CV% and RE% were calculated between the series by determining the overall mean ± standard deviation (SD) for each QC level. The acceptance criteria were the same as for within-series acceptance criteria.

**Carry-over.** The carry-over of analyte and IS in each analytical run was determined by injecting a blank sample immediately after ULOQ in both sets of calibrators. The mean carry-over in the blank sample from the 2 calibrators sets should not exceed 20% of the signal of the LLOQ for the analyte and 5% for the IS [53]. Details of the carry-over assessment of all study compounds can be found in Tables 14S–18S (Supporting Information).

**Recovery/mass balance of study compounds in the placental perfusion system.**

The final recovery of each study compound after the perfusion was calculated with the following equations (Eqs. 1 and 2):

\[
\text{Final recovery} (\%) = \frac{[C_{\text{Mfinal}} \times V_{\text{Mfinal}} + C_{\text{Ffinal}} \times V_{\text{Ffinal}} + \sum_{d} (C_{\text{SI}} \times V_{\text{SI}})]}{C_{\text{Mt0}} \times V_{\text{Mf}}} \times 100 + (100 - \text{EP})
\]

where \(\text{EP} = \frac{C_{\text{Pfinal}} \times V_{\text{Pfinal}} + \sum_{d} (C_{\text{SI}} \times V_{\text{SI}})}{C_{\text{Pf}} \times V_{\text{Pf}}} \times 100\) (2)

in which \(C_{\text{M(f)}}\) is maternal/fetal/sample concentration (ng/mL), \(V_{\text{M(f)}}\) is maternal/fetal/sample volume (mL), and \(t_0\) and \(t_{\text{end}}\) are the beginning and end of the perfusion. The final recovery (%) is the sum of the amount of study compound in maternal and fetal perfusates at the end of a perfusion, and samples (S) collected in relation to the initial amount of study compound measured in the maternal perfusate, including the mean amount adhered from at least 3 system adherence tests (empty perfusion, EP, in %).

**Fraction Unbound and Stability Assay in Placental Homogenate.**

**Preparation of placental homogenate.** Placental homogenates were prepared on a Precellys 24 Tissue Homogenizer (cycle: 5000 rpm, 2 × 20 sec) in 2 mL tubes containing 1.4 mm zirconium oxide beads (Precellys). To 1 g placental tissue, 4 mL PBS (without Ca\(^{2+}\) or Mg\(^{2+}\); Dominique Deutscher) were added, resulting in a 5-fold dilution (v/v). After homogenization, the tubes were centrifuged for 5 min at 4 °C (1000 rpm), and the supernatant was collected. Samples were kept on ice throughout the whole procedure.

**Determination of fraction unbound in the placental homogenate.** Fraction unbound was determined by membrane dialysis on a RED device (ThermoFisher) with membranes of a 6–8 kDA molecular weight cut-off. A 100-fold concentrated DMSO SS of test compounds was added to the placental homogenates, yielding a final concentration of 2 µM of the compound and 1% DMSO. According to the manufacturer’s instructions, 200 µL of the spiked homogenates were added to the donor chamber, and 350 µL of blank buffer were added to the receiver chamber. Samples were collected after equilibration (240 min on an orbital shaker at 600 rpm, 37°C). Samples were analyzed by U(H)PLC-MS/MS. Fraction unbound was calculated as follows [54]:

\[
\text{Diluted} \ f_u = \frac{\text{Receiver Area Ratio}}{\text{Donor Area Ratio}}
\]

and

\[
\text{Undiluted} \ f_u = \frac{1}{D} \left(1 + \frac{1}{D}\right)
\]

where D is the dilution factor of 5 as stated above.

**Stability of compounds in the placental homogenate.** The stability of the compounds was assessed over 360 min (to match the time of placental perfusion experiments) in PBS, PM, and placental homogenates. Homogenates spiked with study compounds were prepared as described above. After compound spiking in the different matrixes, samples were either immediately processed for U(H)PLC-MS/MS analysis (C0) or kept at 4 °C and 37°C for 360 min on an orbital shaker (600 rpm) before processing for U(H)PLC-MS/MS analysis. Stability was expressed as follows [54]:

\[
\text{Stability as % remaining} = \left(\frac{\text{Area Ratio at 4°C or 37°C at 6h}}{\text{Area Ratio at C0}}\right) \times 100\%
\]

**Data processing and calculations.**

Concentrations in placental perfusion profiles (Fig. 2) and system adherence (Fig. 4) are expressed as a percentage (%) of the initial analyzed concentration in the maternal sample at the beginning of the perfusion. Note that the recovery values were calculated differently (see, Eqs. 1 and 2).

The FM ratio (Eq. 6; Fig. 3) was calculated for each point and plotted against the perfusion time (min). Glucose consumption (Eq. 7) and lactate production (Eq. 8) are displayed as the sum of changes (from the perfusion beginning \(t_0\) to the end \(t_{\text{end}}\)) of total content (µmol) in both fetal and maternal circuits, normalized by the total perfusion time (min) and perfused cotyledon weight (W\(_{\text{cot}}\); g). The net release rate of placental hormones β-hCG (mU) and leptin (pg) (Eq. 9) during the placental perfusion was normalized by the total perfusion time (min) and perfused cotyledon weight (g) as well.

\[
\text{FM ratio, } t = \frac{C_{\text{Ft}}}{C_{\text{Mt}}}\)

\[
\text{Glucose consumption} = \left(\frac{[C_{\text{Mf}} \times V_{\text{Mf}} - C_{\text{Mf0}} \times V_{\text{Mf0}}]}{t_{\text{end}} \times W_{\text{cot}}} + \frac{(C_{\text{Ff}} \times V_{\text{Ff}} - C_{\text{Ff0}} \times V_{\text{Ff0}}]}{t_{\text{end}} \times W_{\text{cot}}}ight)
\]

\[
\text{Lactate production} = \left(\frac{[C_{\text{Mf}} \times V_{\text{Mf}} - C_{\text{Mf0}} \times V_{\text{Mf0}}]}{t_{\text{end}} \times W_{\text{cot}}} + \frac{(C_{\text{Ff}} \times V_{\text{Ff}} - C_{\text{Ff0}} \times V_{\text{Ff0}}]}{t_{\text{end}} \times W_{\text{cot}}}ight)
\]
Hormone production = \( \frac{CM_{end}}{W_{cot}} \times \frac{V_{M_{r}}}{W_{end}} + \frac{CM_{end}}{W_{cot}} \times \frac{V_{F_{r}}}{W_{end}} \) (9)

Statistical analysis

For glucose consumption, lactate production, β-hCG, and leptin production, multiple group comparisons were performed using the Brown-Forsythe and Welch ANOVA tests, followed by the Dunnett’s T3 multiple comparisons posthoc test (with individual variances computed for each comparison) with GraphPad Prism (version 9.1.0 for macOS; GraphPad Software). Data are expressed as mean ± SD of at least 3 independent experiments (if not otherwise indicated). Probability values *p ≤ 0.05 were considered statistically significant. The asterisks represent significant differences from the control group.

Supporting Information

Perfusion profiles of all study compounds with absolute concentrations (ng/mL), compound recoveries, fraction unbound of compounds to the placental homogenate, homogenate matrix effects, perfusion profile of antipyrine from control perfusions, characteristics of placentae used, data from individual perfusions in detail, assessment of the suitability of a fetal capillary integrity marker, and details on the U(H)PLC in detail, assessment of the suitability of a fetal capillary integrity marker, and details on the U(H)PLC MS/MS bioanalytical methods are available as Supporting Information.

Contributors’ Statement

APSW, MH, and OP designed the study. DS established, validated, and conducted the placental perfusion experiments, performed data analysis/interpretation, and wrote the first complete version of the manuscript under the supervision of APSW. VFA and AC developed and validated the bioanalytical methods, and VFA performed all analyses. MO and AT supervised method development, and AT performed stability testing and determination of fraction unbound. MR performed the histopathological examinations. ED was assisting in placental perfusions with protopine. All authors were involved in data interpretation and reviewing of the manuscript. All authors agreed with the final version.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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