Received: 11 August 2020 | Revised: 6 October 2020 | Accepted: 8 October 2020

DOI: 10.1111/epi.16746

FULL LENGTH ORIGINAL RESEARCH

Epilepsia®

Hydrolytic biotransformation of the bumetanide ester prodrug DIMAEB to bumetanide by esterases in neonatal human and rat serum and neonatal rat brain—A new treatment strategy for neonatal seizures?

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Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: 274/15-1

Abstract

Objectives: The loop diuretic bumetanide has been proposed previously as an adjunct treatment for neonatal seizures because bumetanide is thought to potentiate the action of γ-aminobutyric acid (GABA)ergic drugs such as phenobarbital by preventing abnormal intracellular accumulation of chloride and the subsequent "GABA shift." However, a clinical trial in neonates failed to demonstrate such a synergistic effect of bumetanide, most likely because this drug only poorly penetrates into the brain. This prompted us to develop lipophilic prodrugs of bumetanide, such as the *N*,*N*-dimethylaminoethyl ester of bumetanide (DIMAEB), which rapidly enter the brain where they are hydrolyzed by esterases to the parent compound, as demonstrated previously by us in adult rodents. However, it is not known whether esterase activity in neonates is sufficient to hydrolyze ester prodrugs such as DIMAEB.

Methods: In the present study, we examined whether esterases in neonatal serum of healthy term infants are capable of hydrolyzing DIMAEB to bumetanide and whether this activity is different from the serum of adults. Furthermore, to extrapolate the findings to brain tissue, we performed experiments with brain tissue and serum of neonatal and adult rats.

Results: Serum from 1- to 2-day-old infants was capable of hydrolyzing DIMAEB to bumetanide at a rate similar to that of serum from adult individuals. Similarly, serum and brain tissue of neonatal rats rapidly hydrolyzed DIMAEB to bumetanide.

Significance: These data provide a prerequisite for further evaluating the potential of bumetanide prodrugs as add-on therapy to phenobarbital and other antiseizure drugs as a new strategy for improving pharmacotherapy of neonatal seizures.

KEYWORDS

blood-brain barrier, BUM5, carboxylesterase, NKCC1, ontogenesis, phenobarbital

1 | INTRODUCTION

Neonatal seizures represent the most common neurological emergency in newborn infants and are associated with poor neurodevelopmental outcomes.[1-4] Neonatal seizures result from an underlying cerebral pathology, most frequently hypoxic-ischemic encephalopathy (HIE) in term infants. Neonatal seizures must be treated promptly because they may exacerbate neuronal injury in the immature brain.[5] However, currently used medications, such as phenobarbital or benzodiazepines, are often ineffective, so new treatment strategies are urgently needed.[1-4] Among newly emerging therapies, bumetanide, which has been used safely as a diuretic in neonates, has been proposed to potentiate the antiseizure efficacy of phenobarbital in neonates by an effect on neuronal chloride concentrations in the brain.[4,6] However, a clinical trial failed to demonstrate that bumetanide as an add-on to phenobarbital improves seizure control in newborn infants with HIE.[7]

One likely explanation for this negative clinical trial is the poor penetration of bumetanide into the brain.[8,9] Preclinical experiments on plasma and brain levels of bumetanide in neonatal and adult rodents have shown that less than 2% of the systemic concentration of bumetanide enters the brain parenchyma.[8,9] This is a consequence of bumetanide's high ionization rate in the blood, its extensive binding to plasma proteins, and active efflux at the blood-brain barrier (BBB). This prompted us to develop lipophilic prodrugs of bumetanide, which easily cross the BBB and are cleaved to bumetanide by carboxylesterases in the brain.[10]

One of these prodrugs, the *N*,*N*-dimethylaminoethylester of bumetanide (DIMAEB; see Figure 1) was shown to enhance the antiseizure efficacy of phenobarbital in adult rodent models of seizures, whereas bumetanide was ineffective.[10,11] In line with our hypothesis, pharmacokinetic experiments in mice and rats showed that bumetanide levels in brain tissue following systemic administration of DIMAEB are significantly higher compared to administration of the parent drug.[10]

In vitro experiments with brain homogenates demonstrated that DIMAEB is rapidly cleaved to bumetanide.[10] Because carboxylesterases and other esterases that hydrolyze ester prodrugs such as DIMAEB to the parent drug are present not only in the brain parenchyma but also in a wide variety of tissues and serum[12]; serum of different species was used as a surrogate for DIMAEB cleavage experiments.[10] As a result of the marked species differences in serum esterase activity,[12-14] DIMAEB was much more rapidly hydrolyzed to bumetanide in adult rat and mouse serum than in serum of adult humans, which may indicate that the half-life of DIMAEB in humans will be much longer than in rodents.[10]

However, these findings in adults cannot be extrapolated directly to neonates because of ontogenetic differences in the expression and functional activity of carboxylesterases.[14-16]

Key Points

- Neonatal seizures are the most common neurological emergency in the neonatal period and only respond poorly to antiseizure drugs
- In rodents, bumetanide was reported to potentiate phenobarbital, but was ineffective in a clinical trial in neonates
- Bumetanide penetrates into the brain only poorly, so lipophilic prodrugs such as *N*,*N*-dimethylaminoethylester of bumetanide (DIMAEB [BUM5]) may be advantageous
- Here we demonstrate that DIMAEB is cleaved to bumetanide by neonatal human and rat serum and neonatal rat brain tissue
- These data provide a prerequisite for further evaluating the potential of bumetanide prodrugs as add-on therapy to phenobarbital in neonates

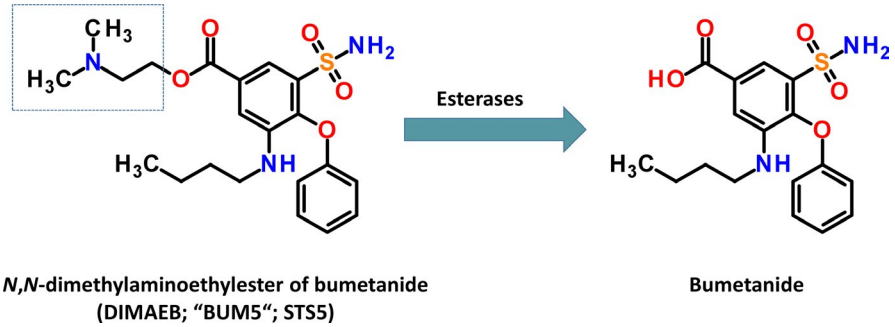

FIGURE 1 Chemical structure of bumetanide and its *N*,*N*-dimethylaminoethylester (DIMAEB) and hydrolysis of DIMAEB to bumetanide by esterases such as carboxylesterase in serum and tissues, including the brain. Whereas bumetanide is highly ionized at physiologic pH (pK_a 3.6) because of its carboxyl group, this is not the case with DIMAEB, thus favoring the rapid distribution of this prodrug into the brain

For instance, the hydrolytic activity of two major human carboxylesterases hCE1 and hCE2 in liver microsomes of adults is about four times higher compared to child microsomes.[17] Similarly, the carboxylesterase activity in liver, plasma, and lung of rats is more than three times higher in adult than neonatal (7 day) rats.[18] On the other hand, compared to fetal tissues, there is a postnatal surge in carboxylesterases hCE1 and hCE2 expression to ensure that the hydrolytic capacity rapidly increases after birth.[17-20] This has been demonstrated by experiments with prodrugs such as oseltamivir (Tamiflu), a widely used anti-influenza drug, which is hydrolytically activated by carboxylesterase.[19]

Preliminary experiments in a rat model of birth asphyxia–induced neonatal seizures indicate that parenteral administration of DIMAEB potentiates the antiseizure efficacy of phenobarbital, whereas bumetanide is ineffective in this regard (M. Johne, B. Gailus, W. Löscher, unpublished data). These data would indicate that DIMAEB enters the neonatal brain and is hydrolyzed to bumetanide; however, we did not yet study whether DIMAEB is hydrolyzed by neonatal brain tissue and serum and whether such hydrolysis can be extrapolated to human neonates. Thus the aim of the present study was to examine whether human carboxylesterases or other esterases in neonatal serum are capable of hydrolyzing DIMAEB to bumetanide and whether this activity is different from the activity in adults. Furthermore, to study the activity of esterases directly in brain tissue, we performed experiments with brain tissue and serum of neonatal and adult rats. For the experiments with human neonatal serum, we sampled blood at day 1 or 2 after birth, because neonatal seizures as a result of HIE often occur in the first 1-2 days of life.[4] Because of the species differences in postnatal brain development, this roughly corresponds to postnatal day 10 in rats,[21] which was therefore used for the present rat experiments.

2 | MATERIALS AND METHODS

2.1 | Subjects and blood samples

Venous blood was sampled from 20 healthy term male and female newborns at day 1 (n = 1) or 2 (n = 19) after birth. Blood samples were taken within a venipuncture for routine newborn mass screening in the maternity ward of the Women's Hospital of Hannover Medical School. Declaration of consent for the study was given by the parents after comprehensive information. The study was approved by the ethics committee of Hannover Medical School (no. 7877_BO_S_2018).

Blood samples (1 mL per neonate) were stored at room temperature for 15-60 minutes in S-Monovette serum tubes (Sarstedt) and then centrifuged for 15 minutes at 550 *g* at room temperature. Serum was stored at −20°C for up to 1 week prior to the in vitro experiments (see below). For comparison with the data on neonatal serum, venous blood was sampled from two adult female human volunteers (WT, CB).

2.2 | Blood and brain samples from animals

Pregnant Sprague-Dawley rats were obtained from Janvier (Le Genest-St-Isle). Animals were housed under controlled conditions (ambient temperature 22-24°C, humidity 30%-50%, lights on from 6:00 AM to 6:00 PM); food (Altromin 1324 standard diet) and water were freely available. The rats' offspring were sacrificed at postnatal day 10 (P10), which in terms of brain development roughly corresponds to that in human term babies shortly after birth.[21-23] For comparison with neonatal data, some experiments were done in adult rats. Blood and brain were freshly sampled and serum and brain tissue homogenates were used for the in vitro experiments described below. All animal experiments were performed according to the EU council directive 2010/63/EU and were formally approved by the animal subjects review board of our institution. All efforts were made to minimize both the suffering and the number of studied animals.

2.3 | Synthesis of the *N,N*-dimethylaminoethylester of bumetanide

As described previously,[10] synthesis of the *N,N*-dimethylaminoethylester (or DIMAEB; previously also termed "BUM5" or "STS5") was initiated by penethamate, the diethylaminoethyl ester prodrug of benzylpenicillin that results in enhanced tissue distribution of this highly ionized antibiotic.[24] Synthesis of DIMAEB was based on our previous description[10] and performed as described in the following.

2.3.1 | General methods

All reactions were carried out under positive pressure of argon, with oven-dried glassware using standard Schlenk techniques. Dry solvents were obtained from Acros Organics in Acroseal bottles and used without further purification. Bumetanide was purchased from Alfa Aesar and used without further purification. Reactions were monitored by thin-layer chromatography (TLC; 0.2 mm, silica gel 60, F_{254}, aluminium-backed; Machery-Nagel), with detection by UV light (254 nm). Flash chromatography was performed on Merck silica (60M). Nuclear magnetic resonance (NMR) spectra were recorded on an AMX-400 instrument (Bruker) at 400 MHz or at 100 MHz for ^{1}H and ^{13}C, respectively.

Deuterated methanol and chloroform were used as solvent, and spectra were calibrated against the residual solvent peak. Data are presented as follows: chemical shift (δ, ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet), coupling constant (reported in Hertz [Hz]), and integration.

2.3.2 | Synthesis of DIMAEB

First, the methylester of bumetanide was synthesized (Figure 2A). Bumetanide (1.82 g, 5 mmol) was suspended and 13 mL of dry MeOH and $SOCl_2$ (800 μL, 11 mmol) was added. Reaction mixture was stirred overnight under argon atmosphere. After complete conversion (monitored by TLC toluene-to-EtOAc ratio, 3:2; Bromocresol green as stain), methanol was removed under reduced pressure and residue was dissolved in EtOAc. The organic layer was washed with saturated aqueous $NaHCO_3$, H_2O, and brine. The organic layer was then dried over $MgSO_4$, filtered, and solvent removed under reduced pressure. The crude solid was purified by recrystallization from EtOH to yield the title compound (1.59 g, 4.2 mmol) in 84% yield as a white solid.

NMR spectra of methyl 3-(butylamino)-4-phenoxy-5-sulfamoylbenzoate: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (d, J = 1.9 Hz, 1H), 7.59 (d, J = 1.9 Hz, 1H), 7.36-7.28 (m, 3H), 7.11 (t, J = 7.4 Hz, 1H), 6.93 (d, J = 7.8 Hz, 2H), 4.87 (s, 2H), 4.01-3.95 (m, 1H), 3.94 (s, 3H), 3.11 (t, J = 6.9 Hz, 2H), 1.48-1.37 (m, 2H), 1.22-1.10 (m, 2H), 0.82 (t, J = 7.3 Hz, 3H).

For synthesis of 2-(dimethylamino)ethyl 3-(butylamino)-4-phenoxy-5-sulfamoylbenzoate hydrochloride (DIMAEB), the methyl ester of bumetanide (222 mg, 0.59 mmol) was dissolved in 3 mL of *N*,*N*-dimethyl-2-aminoethanol and a catalytic amount of sodium was added (Figure 2B). The reaction mixture was heated at 75°C for 4 hours until no more starting material was present. Volatiles were removed under reduced pressure, residue taken up in EtOAc, and washed 3 times with H_2O, once with brine and dried over Na_2SO_4. The crude product was purified by column chromatography (EtOAc:Et_3N, 4:1), dried thoroughly under vacuum, and dissolved in 1:1 mixture of Et_2O and H_2O. One hundred microliters of 1 M aqueous HCl was added and volatiles were removed under reduced pressure, giving title compound (118 mg, 0.25 mmol) in 42% yield as a white solid.

NMR spectra of 2-(dimethylamino)ethyl 3-(butylamino)-4-phenoxy-5-sulfamoylbenzoate hydrochloride (DIMAEB): ^{1}H NMR (400 MHz, MeOD) δ 7.94 (d, J = 2.0 Hz, 1H), 7.62 (d, J = 2.0 Hz, 1H), 7.35-7.25 (m, 2H), 7.08 (dd, J = 10.6, 4.2 Hz, 1H), 6.96-6.86 (m, 2H), 4.77-4.67

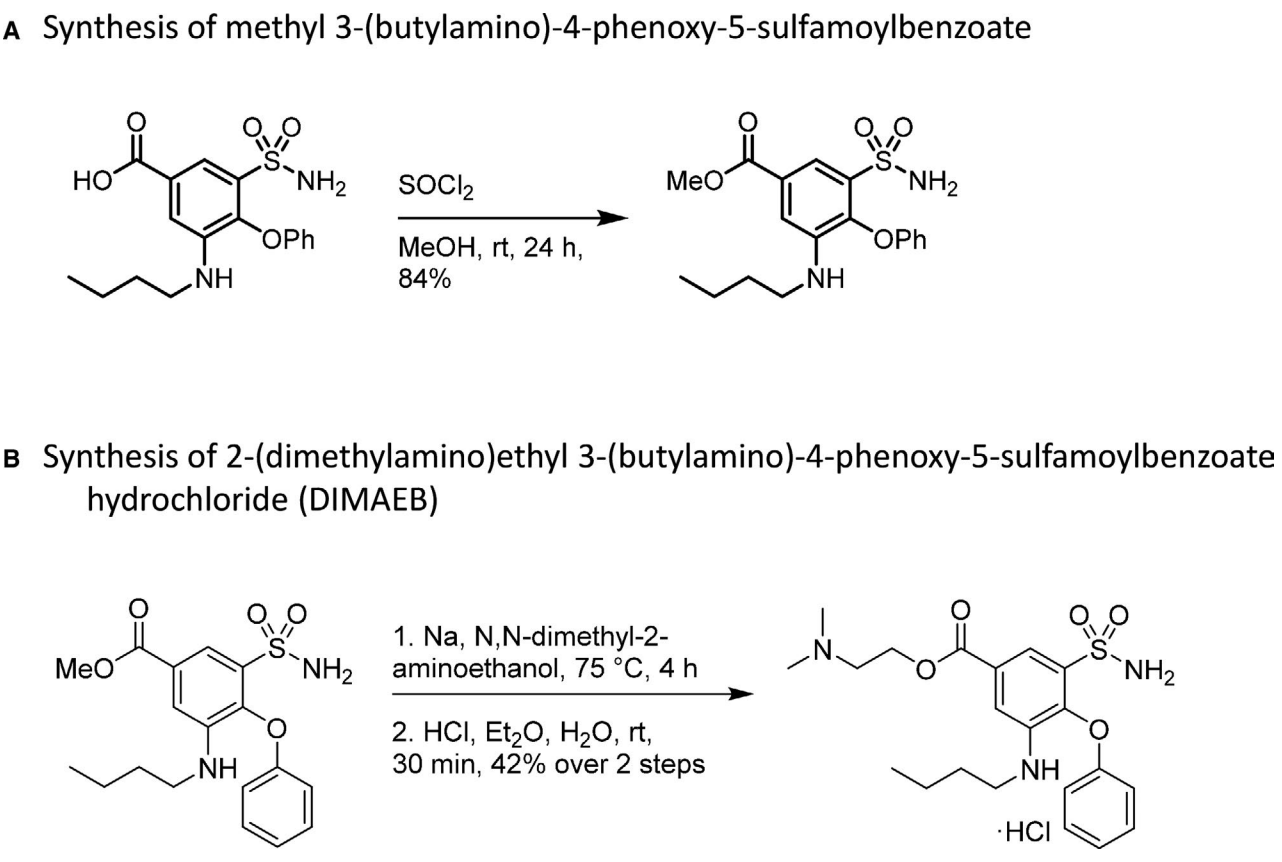

FIGURE 2 Two-step synthesis of DIMAEB. (See text for details)

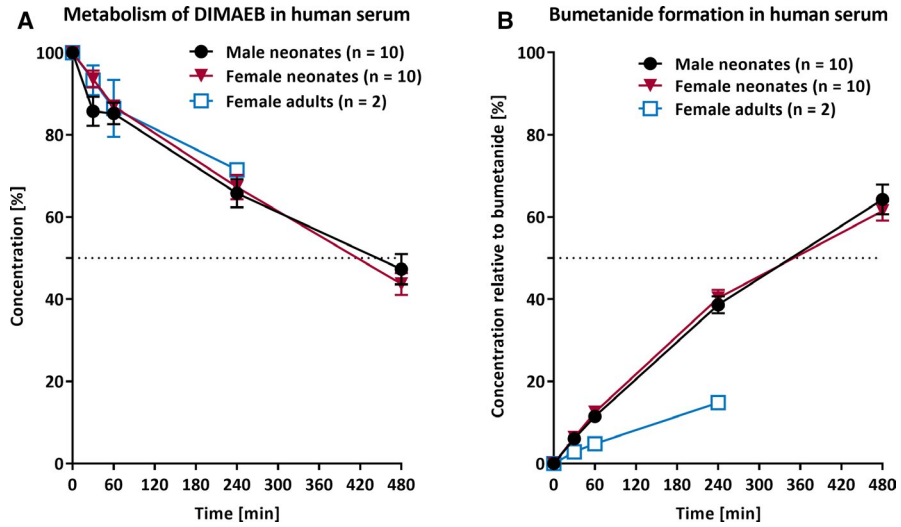

FIGURE 3 In vitro hydrolysis of the bumetanide ester DIMAEB to bumetanide in human serum. Data for neonates (age 1-2 d) are separately shown as means ± standard error of the mean (SEM) of 10 male and 10 female neonates, respectively. For comparison, the average data of two female adults are shown. (A) Degradation of DMAEB in human serum, illustrated as percent decline from the initial concentration. (B) Formation of bumetanide from DIMAEB in human serum, illustrated as percent relative to the initial concentration of DIMAEB

(m, 2H), 3.70-3.59 (m, 2H), 3.14 (t, $J = 6.8$ Hz, 2H), 3.03 (s, 6H), 1.50-1.37 (m, 3H), 1.15 (dq, $J = 14.6$, 7.4 Hz, 2H), 0.82 (t, $J = 7.4$ Hz, 3H).

2.4 | In vitro cleavage of DIMAEB in the serum of humans and rats

Experiments were performed essentially as described previously for cleavage of DIMAEB in serum of adult humans and rats.[10] DIMAEB was dissolved in hydroxypropyl-ß-cyclodextrin (Roquette-Pharma; Frankfurt, Germany), and aliquots of 20 µL were added to serum aliquots (0.2-0.3 mL) to obtain a drug concentration of 20-40 µg/mL serum. The serum aliquots were intermittently stirred and maintained at 37°C for up to 60 minutes (rats) or 480 minutes (humans), respectively. Following the onset of exposure, intermittent 50 µL samples were taken for analysis of the intact ester and cleaved bumetanide as described below.

In pilot experiments, we examined whether storage of human serum samples at −20°C has an effect on the activity of carboxylesterases. In these tests, it could be confirmed that storage had no effect on the cleavage of DIMAEB to bumetanide (data not shown).

2.5 | In vitro cleavage of DIMAEB in brain tissue homogenates of rats

Similar to serum, adult brain tissue contains high activities of esterases,[12] so brain tissue homogenates can be used to study whether the esterase activities of neonatal brain are already sufficient to cleave DIMAEB. Brains were freshly obtained from P10 rats as described above and homogenized in water (about 500 mg tissue in 800 µL). A total of 100 µL of the dissolved DIMAEB was added to brain homogenate to obtain a drug concentration of 40 µg/g brain tissue. The brain homogenate was stirred intermittently and maintained at 37°C for 60 minutes. At 0, 0.5, 5, 15, 30, and 60 minutes following onset of exposure, 100 µL samples were taken for analysis of cleaved bumetanide as described below.

2.6 | Spontaneous (nonenzymatic) hydrolysis of DIMAEB in water

Carboxylic esters can hydrolyze spontaneously in water with increasing temperature.[25] Thus we performed a control experiment in which we added DIMAEB to water (pH adjusted to 7.35) and exposed the solution at 37°C for 480 minutes. All details were as described above for serum.

2.7 | Analysis of DIMAEB and bumetanide in water, serum and brain homogenates

Bumetanide was analyzed in water, serum, and brain by high-performance liquid chromatography (HPLC) with ultraviolet detection (UV) as recently described in detail.[10,26] The same method was used with a modified composition of the mobile phase (0.05 M phosphate buffer/acetonitrile 30:70 instead of 70:30; see below) for determination of DIMAEB. As is standard for HPLC analysis, we used a commercial solution

(0.5 mg/mL) of the sodium salt of bumetanide (Burinex), which was kindly provided by Leo Pharma.

In short, 50 µL of serum was mixed with 150 µL methanol and then centrifuged for 3 minutes at 13 400 *g*. Aliquots of the supernatant were injected into the HPLC apparatus equipped with a Nucleosil 120-5 C18 pre-column (60 × 4 mm; Knauer), a Nucleosil 120-5 C18 column (250 × 4.6 mm), and a UV detector (at 210 nm). The mobile phase, which was pumped with a flow rate of 1 mL/min, was composited of 0.05 M phosphate buffer and acetonitrile in a proportion of 70:30 (for DIMAEB 30:70); the pH was adjusted at 5.6. The same HPLC conditions were used for analysis of DIMAEB and bumetanide in brain tissue samples.

For analysis of DIMAEB and bumetanide in brain tissue, brain tissue homogenate aliquots were centrifuged for 20 minutes at 21 881 *g*, and the supernatant was purified by solid phase extraction using a Chromabond HR-X column (Macherey-Nagel; Düren; Germany). Compounds were extracted from the column by methanol, the extract was evaporated to dryness by nitrogen, the residue was dissolved in 100 µL buffer, and 20 µL was used for HPLC analysis. The detection limit for bumetanide was about 50 ng/mL in plasma and 100 ng/g (=0.28 µM) in brain tissue.

3 | RESULTS

3.1 | Experiments with human serum

Data from the one infant in whom blood was sampled at day 1 after birth did not differ from those in whom blood was taken 2 days after birth, so data were analyzed together and illustrated in Figure 3. Because gender differences in the hydrolysis of ester prodrugs by carboxylesterases have been reported,[27] data on hydrolysis of DIMAEB in neonatal serum samples are illustrated separately for both genders (Figure 3). As shown in Figure 3A, DIMAEB was metabolized in neonatal serum at about the same rate in both genders; 50% degradation was obtained at about 7 hours. In parallel to the reduction in DIMAEB levels, bumetanide levels increased in the neonatal serum of both genders (Figure 3B). Unexpectedly, the rate of DIMAEB hydrolysis in neonatal serum was not slower than the rate in adult serum (Figure 3A). Furthermore, formation of bumetanide was considerably faster and much more pronounced in neonatal serum compared to adult serum (Figure 3B).

3.2 | Experiments with rat serum

In serum of neonatal (postnatal day 10) rats, DIMAEB was much more rapidly metabolized (Figure 4A) than in human neonatal serum (Figure 3A). Indeed, complete (100%) degradation occurred within 15-30 minutes in neonatal rat serum without any obvious sex difference. As shown in Figure 4B, DIMAEB was rapidly hydrolyzed to bumetanide in neonatal rat serum. Compared to neonatal rat serum, hydrolysis of DIMAEB to bumetanide was even more rapid in adult rat serum (Figure 4A,B), confirming previous data.[10]

3.3 | Experiments with rat brain homogenates

In neonatal rat brain homogenates, bumetanide formation from DIMAEB (Figure 5) was less rapid than in neonatal rat serum (Figure 4). About 50%–70% of DIMAEB was hydrolyzed to bumetanide within 60 minutes. In comparison to neonatal rat brain homogenates, hydrolysis of DIMAEB to bumetanide

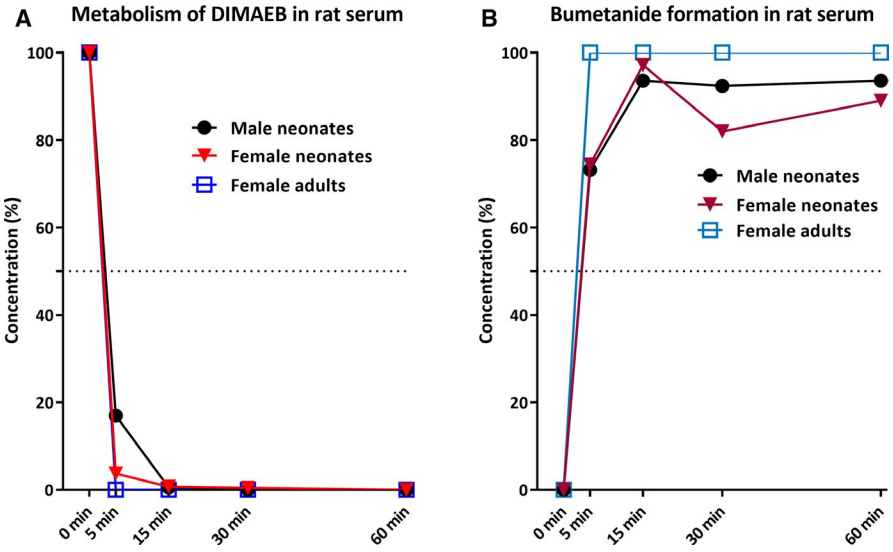

FIGURE 4 In vitro hydrolysis of the bumetanide ester DIMAEB to bumetanide in rat serum. Data for neonatal rats (age 10 d) are shown separately for male and female animals as means from pooled samples. For comparison, average data of six female adult rats are shown. (A) Degradation of DMAEB in rat serum, illustrated as percent decline from the initial concentration. (B) Formation of bumetanide from DIMAEB in rat serum, illustrated as percent relative to the initial concentration of DIMAEB

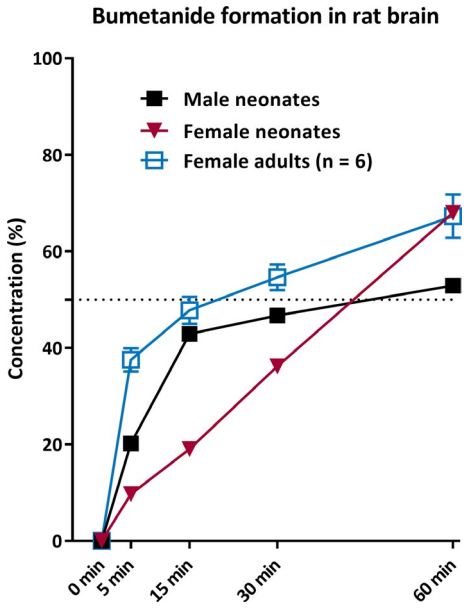

FIGURE 5 In vitro hydrolysis of the bumetanide ester DIMAEB to bumetanide in rat brain homogenates. Data for neonatal rats (age 10 d) are shown separately for male and female animals as means from pooled samples. For comparison, average data (±SEM) of six female adult rats are shown. Data are illustrated in percent bumetanide relative to the initial concentration of DIMAEB

appeared to be more rapid in brain homogenates from adult animals, although the difference was only moderate.

3.4 | Control experiment with water

Spontaneous (nonenzymatic) hydrolysis of DIMAEB at 37°C was determined in water at pH 7.35. Within 480 minutes, the DIMAEB concentration was reduced by 34% (±0.84%; n = 3), on average, because of spontaneous hydrolysis to bumetanide (data not shown). As shown in Figure 3, respective figures for neonatal human serum were 53% (male) and 56% (female), indicating that ~60% of the hydrolysis observed in human serum was nonenzymatic. Nonenzymatic hydrolysis did obviously not play any significant role for neonatal rat serum, because almost complete hydrolysis of DIMAEB occurred within 5-15 minutes (Figure 4A). In contrast to nonenzymatic hydrolysis of DIMAEB in water at 37°C, such neutral hydrolysis was not observed when solutions were kept at low temperatures.

4 | DISCUSSION

Carboxylesterases (or carboxylic-ester hydrolases; EC 3.1.1.1) are major hydrolytic enzymes responsible for the metabolism of numerous carboxylic acid esters, carbamates, thioesters, and amide agents.[28] In humans, three carboxylesterases have been identified, although human carboxylesterase1 (hCE1) and human carboxylesterase 2 (hCE2) are the two extensively studied isoenzymes involved in xenobiotic metabolism.[28] Both isoenzymes play crucial roles in the hydrolysis and bioactivation of various ester prodrugs such as oseltamivir, clopidogrel, irinotecan, and capecitabine.[28] When designing DIMAEB, we thought that this lipophilic prodrug of bumetanide would rapidly cross the BBB and would be hydrolyzed by carboxylesterases in the brain to bumetanide. This assumption was subsequently confirmed by in vivo and in vitro experiments in adult rodents.[10] In the current study we demonstrate that neonatal brain tissue is also capable of hydrolyzing DIMAEB to bumetanide to a an extent similar to that in adult brain tissue. Furthermore, by using human neonatal serum as a surrogate for brain tissue, we show that serum from 1- to 2-day-old infants is capable of hydrolyzing DIMAEB to bumetanide at a rate similar to that of serum from adult individuals.

The latter finding was unexpected, because experiments with liver tissue samples and liver microsomes have indicated that the hydrolytic activity of carboxylesterases of adults is higher than that of children and neonates when using prodrugs such as oseltamivir as a substrate.[17,19] However, the hydrolytic activity of carboxylesterases is dependent on the substrate,[28] so ontogenetic differences in drug hydrolysis cannot be extrapolated to different prodrugs. Furthermore, Shi et al[19] and Chen et al[20] reported a postnatal surge in carboxylesterase hCE1 and hCE2 expression and activity in human neonates, which may be important to enable the infant to detoxify potentially harmful endogenous and exogenous compounds early after birth. As shown by Shi et al,[19] the postnatal surge in hCE1 can be used to hydrolytically activate ester prodrugs such as the widely used anti-influenza drug oseltamivir. The present data indicate that the postnatal surge of carboxylesterases can also be used to activate the ester prodrug DIMAEB. Thus this new compound might constitute as a novel strategy for potentiating the efficacy of phenobarbital in treatment of neonatal seizures. In this respect, it is interesting to note that phenobarbital has been reported to induce neonatal carboxylesterases.[29] As mentioned in the Introduction, promising data from preliminary experiments in a rat model of birth asphyxia–induced neonatal seizures indicate that parenteral administration of DIMAEB potentiates the antiseizure efficacy of phenobarbital, whereas bumetanide is not effective (M. Johne, B. Gailus, W. Löscher, unpublished data).

We cannot exclude that DIMAEB was hydrolyzed by esterases other than carboxylesterases in human and rat serum and rat brain. Esterases, that is, hydrolases that split ester bonds in human blood, have an important role in the disposition of drugs. They participate in the activation of ester prodrugs

but may also inactivate drugs and detoxify natural and synthetic ester–containing poisons.[30-32] Blood serum or plasma contains different esterases, including butyrylcholinesterase (EC 3.1.1.8), paraoxonase (EC 3.1.8.1), acetylcholinesterase (EC 3.1.1.7) and other cholinesterases, carboxylesterase (EC 3.1.1.1) and albumin, which has been proven to act as an esterase.[30,31] Data on the relative activities of these esterases in serum or plasma of humans and experimental animals are contradictory. However, more recent studies consistently found that carboxylesterase activity in human plasma or serum is low, whereas it is high in rodent serum.[12,32-40] Nevertheless, significant carboxylester-hydrolyzing enzyme activity is present in human serum or plasma, both in neonates and adults,[12,33-35] which is also demonstrated by the current study.

In addition to enzymatic hydrolysis in serum and brain homogenates, we found that DIMAEB is also hydrolyzed spontaneously in water at body temperature. "Neutral" hydrolysis of simple carboxylic esters in water at increased temperatures is a well-known phenomenon, but typically is much slower than enzymatic hydrolysis.[25] This was also observed in the present study. Nevertheless, particularly in case of neonatal human serum, nonenzymatic hydrolysis certainly contributed to the overall hydrolysis of DIMAEB to bumetanide determined in the present study.

One potential disadvantage of lipophilic ester prodrugs such as DIMAEB is that although increased lipophilicity may improve movement across the BBB, it also tends to increase uptake into other tissues, causing an increased tissue burden.[41,42] However, by comparing diverse prodrugs of bumetanide, DIMAEB was shown to achieve the highest bumetanide brain levels, whereas diuretic activity was lower than that of bumetanide, a finding that would argue against unspecific tissue distribution.[10] There are several promising examples of ester-linked prodrugs for enhancing CNS delivery, which is the most commonly employed approach for increasing lipophilicity of polar molecules exhibiting limited CNS penetration.[42,43] The high CNS delivery of lipophilic ester prodrugs is a consequence of the high cerebral blood flow, which represents about 15%–20% of the cardiac output, even though the brain represents only 2% of the body weight.[44,45] Thus relative brain perfusion is higher than perfusion of most other organs and tissues, which enables small, lipophilic prodrugs to penetrate into the brain parenchyma rapidly.

In summary, the present study shows that neonatal serum and rat brain tissue are capable of rapidly hydrolyzing the bumetanide ester prodrug DIMAEB to bumetanide. These data are a prerequisite for further evaluation of the potential of bumetanide ester prodrugs as add-on to phenobarbital and other antiseizure drugs as a new strategy for improving pharmacotherapy of neonatal seizures. The positive interaction between bumetanide and GABAergic drugs such as phenobarbital is thought to be a consequence of the inhibitory effect of bumetanide on the sodium/potassium/chloride cotransporter NKCC1 in brain neurons, although other mechanisms may contribute to the interaction.[4,6,9] An advantage of prodrugs of clinically approved drugs such as bumetanide is that the drug development pipeline to first use in humans is much shorter compared to new chemical entities.[42,46] Currently, about 10% of pharmaceutical products are used as prodrugs, nearly half of them being converted to the active form by hydrolysis, mainly by ester hydrolysis, which demonstrates the success of this strategy.[47] DIMAEB provides a useful tool to further improve the bumetanide prodrug strategy for neurological indications, such as neonatal seizures, in which NKCC1 inhibitors are thought to be useful.

ACKNOWLEDGMENTS

We are grateful to all parents who participated in our study. The study was supported in part by a grant (Lo 274/15-1) from the Deutsche Forschungsgemeinschaft (Bonn, Germany). The open access publication was supported by the Deutsche Forschungsgemeinschaft and University of Veterinary Medicine Hannover, Foundation, within the funding program Open Access Publishing enabled and organized by ProjektDEAL.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL PUBLICATION STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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How to cite this article: Theilmann W, Brandt C, Bohnhorst B, et al. Hydrolytic biotransformation of the bumetanide ester prodrug DIMAEB to bumetanide by esterases in neonatal human and rat serum and neonatal rat brain—A new treatment strategy for neonatal seizures? *Epilepsia*. 2021;62:269–278. https://doi.org/10.1111/epi.16746