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Challenges for drug studies in children: CYP3A phenotyping as example

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A paucity of data exists on the disposition and effect of drugs in young children. This information gap can be reduced by elucidating developmental principles of absorption, distribution, metabolism and excretion (ADME) *in vivo*. Such knowledge might enable the prediction of the disposition of individual drugs in children over the whole pediatric age range. CYP3A, the most abundant human drug metabolizing enzyme, is involved in the metabolism of more than 50% of all marketed drugs. Hence, elucidating the developmental pattern of CYP3A in relation to genetic background, disease and comedications might greatly enhance our knowledge on drug disposition in children. Several methods have been used to determine *in vivo* CYP3A activity in human adults, while similar studies in children face several ethical, practical and scientific challenges. The aim of this review is to identify these challenges and offer feasible solutions for studying drugs in young children, with an emphasis on CYP3A phenotyping as an example.

Introduction

Most drugs used in children are prescribed off-label because of a lack of studies in this age group and reluctance of manufacturers to invest in such studies [1]. Extrapolation of existing adult data to children is generally not warranted as most aspects of drug disposition and effects are different in children [2]. Hence, to facilitate rational drug therapy in children, the developmental patterns of pharmacokinetic and pharmacodynamic processes involved need to be elucidated.

One of the approaches taken is to study the *in vivo* development of drug disposition pathways. The underlying idea is that by elucidating the ontogeny of all aspects of absorption, distribution, metabolism and excretion (ADME), one will be able to predict the disposition of individual drugs over the whole pediatric age range.

Cytochrome P450 3A (CYP3A) is a subfamily of drug metabolizing enzymes involved in the metabolism of the largest group of currently marketed drugs [3,4]. Hepatic CYP3A enzyme activity shows a clear developmental pattern (as reviewed in Refs. [5,6]). CYP3A7 activity is high

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before birth, with a rapid decline thereafter. CYP3A4 activity is low at birth with a surge in the neonatal period to reach adult levels around one year of age, while activity of the polymorphically expressed CYP3A5 appears to be stable during the development [7–9]. The data on intestinal CYP3A ontogeny are less clear. Intestinal CYP3A4 protein content appears to mirror the hepatic developmental pattern. By contrast, intestinal CYP3A4 expression was shown to parallel the age-related surge in hepatic CYP3A4 expression in two studies, but not in another where CYP3A4 expression was the same in children aged 1 month to 17 years of age [10–12].

This developmental pattern of CYP3A, based on *in vitro* studies, is roughly supported by pharmacokinetic data of CYP3A substrate drugs [5]. Yet, the exact developmental pattern of CYP3A *in vivo* and its interplay with other factors that might affect CYP3A activity as disease state, genotype, diet and comedication, remains to be elucidated [13–15].

In adults, CYP3A activity has been extensively studied *in vivo* using several surrogate probes including midazolam clearance, ^{14}C-erythromycin breath test, β-OH-cortisol:cortisol urinary ratio, alfentanil pharmacodynamics and dextromethorphan urinary metabolite ratio [16–18]. Hence, it seems logic to use one of these probes to further elucidate the ontogeny of CYP3A *in vivo* and its interplay with other covariates. This approach was taken to study CYP3A activity in the pediatric population [15,19,20–23]. During this process, several roadblocks were encountered frequently associated with drug studies in young children [24]. These roadblocks has prevented the use of these probes the way they are validated in adults. Nevertheless, alternative solutions emerged, dealing with ethical, practical and scientific issues in children, which can provide valuable information on CYP3A ontogeny *in vivo*.

The aim of this review is to identify challenges encountered when translating adult drug study design to children, using CYP3A phenotyping with midazolam clearance and the erythromycin breath test, as an example (Table 1). The ultimate goal is to provide researchers of pediatric drugs with the tools to facilitate knowledge in pediatric pharmacology.

TABLE 1

Key concepts: challenges of pediatric drug studies: emphasis on CYP3A phenotyping

CYP3A probes of interest
Midazolam clearance rate
Erythromycin breath test
Possible combined CYP3A/p-gp probe?
Developmental challenges
Developmental changes in drug disposition pathways (e.g. metabolism)
Other patient factors (e.g. disease state)
Ethical challenges
Limitations of nontherapeutic (probe) drug studies
Sampling limitations
Challenges to adapt CYP3A phenotyping for use in children
Midazolam clearance rate
Administration as part of therapeutic use
Limited sampling schemes and urinary drug metabolite ratios: not useful?
Conclusion: feasible probe
Erythromycin breath test
Validity in adults
Stable isotope-labeled erythromycin to avoid radioactivity
Conclusion: probably not useful
Practical challenges
Probe drug administration
Adult i.v. formulations: risk for dosing mistakes in children
Lack of oral formulations: risk for GI damage or erratic absorption
Sampling
Need for sensitive analytical assays
Need for central line/urinary catheter: may impact patient recruitment
Need for age-appropriate sampling methods (e.g. urine and breath)

CYP3A probes of interest

Many CYP3A substrates have been used to phenotype CYP3A activity in adults and children, their validity and use in adults have been extensively reviewed by others and are outside the scope of this review [16,25].

Midazolam plasma clearance rate

Till date, the most validated and generally accepted probe of CYP3A activity *in vivo* is plasma clearance rate of the benzodiazepine midazolam. Hence, this probe has been used to study the ontogeny of midazolam in young children. Midazolam is almost exclusively metabolized by CYP3A4 and CYP3A5 to 1-OH-midazolam, 4-OH-midazolam and 1,4-OH-midazolam [26,27]. CYP3A4/5 is abundant in the liver and intestine, where it contributes significantly to the first-pass effect of oral midazolam [28,29]. In adult transplant patients, i.v. midazolam clearance rate is highly correlated with liver CYP3A4/5 content and activity [30]. Also, the inhibition and induction of CYP3A4/5 activity by known CYP3A inhibitors or inducers is adequately reflected by changes in midazolam clearance rate. Additionally, midazolam appears not to be appreciably transported by the drug transporter p-glycoprotein [31]. Hence, midazolam systemic clearance rate is generally accepted as *in vivo* probe of hepatic and combined hepatic/intestinal CYP3A activity after intravenous and oral administration, respectively [25,32].

^{13}C-erythromycin breath test (EBMT)

The ^{14}C-erythromycin breath test has been widely accepted and validated as CYP3A probe [16]. The rationale behind the erythromycin breath test is based on the CYP3A-mediated metabolism of erythromycin into CO_2 and a nonactive metabolite [33]. A single $^{14}CO_2$ measurement in a breath-sample collected after an intravenous or oral radioactive-labeled erythromycin dose is used to predict CYP3A activity *in vivo*. An important limitation of the EBMT is that the results not only reflect CYP3A4/5 activity, but also p-gp and other transporter activity [31,34,35]. Consequently, to elucidate the effect of age and other covariates, specifically, on CYP3A4/5 activity, it is a suboptimal probe. Yet, before rejecting this probe altogether, it might be a useful tool to predict the behavior of other drugs, which are combined CYP3A/p-gp substrates, such as the immunosuppressant tacrolimus. This possibility is supported by the observation that the EBMT results strongly correlate with tacrolimus dosing and occurrence of adverse events in kidney transplant patients [36,37].

Ontogeny in disposition pathways

No matter how perfect the probe used to determine CYP3A activity and its relevant covariates, when translating information gained

using this probe, a major limitation is the differential isoform specificity of different CYP3A drugs. Although some drugs might be catalyzed by CYP3A4 as their preferential isoform, other drugs might be substrates for the polymorphically expressed CYP3A5 [38]. Hence, differences in the developmental pattern of CYP3A4 and CYP3A5 might result in different developmental patterns of individual drug disposition. To our knowledge, the differential patterns of CYP3A4 and CYP3A5 ontogeny have not been fully elucidated, though limited data suggest that CYP3A5 protein content is stable throughout the development [38]. CYP3A5 immunoreactive protein was found in fetal liver with metabolic activity toward dehydroepiandrosterone (DHEA) metabolism [9,13]. Differences in substrate specificity for CYP3A7 and the other isoforms might, in theory, contribute to the different age-related changes in disposition of different drugs. For example, clarithromycin is a CYP3A4, CYP3A5 and CYP3A7 substrate [38]. Hence, theoretically, in newborns, clarithromycin clearance is expected to be less decreased than midazolam, which is a very weak CYP3A7 substrate, but *in vivo* data are lacking to support this hypothesis.

Also, drug metabolism pathways identified in adults might not necessarily be similar in children. For example, the contribution of CYP3A4 and CYP2D6 to dextromethorphan metabolism and hence excretion, changes considerably during the first year of life [39]. Similarly, the enzymes responsible for sirolimus metabolism appear to change significantly during early childhood [40]. Moreover, when drug metabolism is developmentally low, excretion pathways might alter from primary metabolism to renal excretion. For example, caffeine has been used in genotype studies for *N*-acetyltransferase 2 (NAT2) and CYP1A2 activity in adults [41]. As caffeine is frequently used in preterm infants to treat apnea of prematurity, elucidating factors that determine interindividual variation in this disposition might be important to improve therapy efficacy and safety [42]. In contrast to adults, caffeine is mainly eliminated unchanged by the kidney in the first weeks of life, because of developmentally low metabolic capacity [43,44]. Therefore, variation in caffeine clearance rate in neonates might be more related to variation in renal clearance and less to variation in drug metabolism. Hence, CYP1A2 phenotyping using urinary metabolic ratios (MRs) shown to reflect CYP1A2 activity in adults will not adequately reflect caffeine clearance. In addition, genotyping for CYP1A2 to explain interindividual variation in caffeine disposition in preterm infants does not seem useful.

Isoform specificity might further affect the influence of genetic polymorphisms on disposition of CYP3A substrates. For example, the CYP3A5*3 genotype does not appear to affect midazolam clearance rate, which is preferentially metabolized by CYP3A4 [45]. By contrast, the CYP3A5*3 genotype is associated with a lower clearance rate of tacrolimus than the CYP3A5*1 (wild-type) genotype [46]. It is unclear if this genetic difference persists in very young children, who have developmentally low CYP3A4/5 activity. Obviously, data gained from studies using midazolam as CYP3A4/5 probe cannot be used to predict, for example, tacrolimus disposition in relation to CYP3A5 genotype.

Other patient factors

Additionally, other cofounders might interfere with age-related changes in drug metabolism [5,6]. The most important factors are comedication and disease state. It is well established that certain drugs and other chemicals can inhibit or induce CYP3A activity. It is less well acknowledged, that infectious and noninfectious inflammation, as well as chronic renal failure, are associated with downregulation of CYP3A activity [47–50]. Of interest, aminopyrine clearance rate, as an overall measure of drug metabolism by cytochrome P450 was decreased by approximately 50% in children with severe sepsis [14]. This might partially explain the lower mean midazolam clearance rate in our pediatric intensive care unit (PICU) patients when compared to the mean clearance rate in age-matched relatively healthy children, [19,51]. Additionally, at the present time the effect of variability in hepatic blood flow secondary to disease on midazolam clearance rate cannot be excluded.

Hence, when using pharmacokinetic data or phenotyping studies to design dosing schedules for other drugs in children, differences in isoform specificity, disposition mechanisms and population characteristics, in addition to age, need to be addressed.

Ethical challenges

Before embarking on a clinical drug study in children, ethical issues pertaining to the involvement of children need to be addressed [52]. The participation of either ill or healthy children in clinical drug trials is restricted by the ethical regulations. Most countries have adopted the International Conference on Harmonisation (ICH) guidance on good clinical practice for clinical trials (see: http://emea.europa.eu/pdfs/human/ich/013595en.pdf). In the context of phenotyping studies, this guidance contains a specific section (4.8.14) on nontherapeutic trials in subjects who cannot give their own informed consent.

'Nontherapeutic trials might be conducted in subjects with consent of a legally acceptable representative provided the following conditions are fulfilled:

(a) The objectives of the trial cannot be met by means of a trial in subjects who can give informed consent personally.
(b) The foreseeable risks to the subjects are low.
(c) The negative impact on the subject's well-being is minimized and low.
(d) The trial is not prohibited by law.
(e) The approval/favorable opinion of the Institutional Review Board (IRB) is expressly sought on the inclusion of such subjects, and the written approval/favorable opinion covers this aspect. Such trials, unless an exception is justified, should be conducted in patients having a disease or condition for which the investigational product is intended. Subjects in these trials should be particularly closely monitored and should be withdrawn if they appear to be unduly distressed.'

Hence, the administration of a drug for nontherapeutic reasons, as is the case when a medication is solely given as probe drug, followed by repeated blood sampling might be perceived and interpreted differently by different IRBs and ethical roadblocks might be encountered.

A possible solution is to study a drug that is already prescribed as part of clinical care or to study the pharmacokinetics of a drug as part of a therapeutic trial. Alternatively, the administration of over-the-counter drugs or food products combined with urine

or breath-sample collections might be considered 'minimal impact' and, therefore, pose an interesting alternative. The cough syrup dextromethorphan and the beverage constituent caffeine were approved to be given nontherapeutically to study drug metabolism in children by at least one IRB [15,39]. Finally, a stable isotope-labeled drug can be administered as part of therapeutic drug therapy.

To estimate pharmacokinetic parameters, blood and/or urine sampling is necessary

For obvious concerns of anemia caused by excessive blood sampling in young children, most IRBs have institution-specific guidelines for the maximum blood volume that can be sampled for research purposes. A general guideline is a maximum of 3–5% of circulating total blood volume (TBV) over a three-month period. In a 1-kg baby this translates to a total of 2.5–4 ml (3–5% of TBV 80 ml/kg). This limitation might be overcome by the use of limited sampling schedules, the population pharmacokinetic study design or urine collections.

Specific challenges to CYP3A phenotyping in children

Midazolam as a probe in children

The validated method for using midazolam as CYP3A probe utilizes subtherapeutic doses of midazolam (0.015–0.025 mg/kg i.v. or orally) [30]. Non-therapeutic midazolam administration was considered unethical in preterm infants, therefore midazolam pharmacokinetics was studied in the context of a therapeutic trial comparing oral with intravenous midazolam, using a therapeutic midazolam dose (max 0.1 mg/kg) which is higher than used in most CYP3A phenotyping studies [21,53]. As midazolam does not appear to show saturation kinetics at low therapeutic doses, this should not affect the validity of midazolam clearance rate as CYP3A probe in this situation [54–56]. Midazolam as CYP3A probe has not been validated using this higher dose. In addition, midazolam clearance calculated from a continuous infusion of midazolam as alternative method for CYP3A phenotyping has not been validated. If, for sedation in pediatric intensive care patients, doses higher than 0.1 mg/kg are given nonlinear pharmacokinetics might occur, which might impact the reliability of midazolam clearance rate as CYP3A probe. Since midazolam is a medium extraction drug, variation in clearance rate at higher doses may also be affected by changes in liver blood flow, which are not infrequent in critically ill patients [45].

Limited sampling schedule or 1-OHM/M ratio as an alternative probe

The feasibility of a minimal sampling schedule (e.g. max 3 sampling time points per patients) was studied in preterm infant. Different sample time combinations were studied, but there was no correlation between any minimal sampling schedule and midazolam clearance rate [23]. This failure was attributed to the large interindividual variation in midazolam pharmacokinetics in young children, a finding also reported by others [57]. Similarly, no single time point could be identified at which the plasma 1-OH-M/M ratio correlated with midazolam clearance rate, which is in line with adult data [58,59].

Urinary ratio as an alternative probe

Urinary MRs have also been used as surrogate markers for drug metabolism *in vivo* [44]. The midazolam MR could be an interesting candidate as noninvasive probe of CYP3A activity [60]. No significant correlation between midazolam clearance rate and different urinary MRs was found, when studied in healthy volunteers during baseline CYP3A activity or after CYP3A inhibition.

Of potential interest, the MR decreased similarly to the decrease in midazolam clearance (−33.6% versus −42.4%, $P > 0.05$) after the administration of a CYP3A inhibitor. This finding suggests that though urinary MR is not very reflective of baseline CYP3A activity, it might have potential as an indicator of CYP3A inhibition, but further validation in both adults and children is needed before it can be used as such. By contrast, as a marker to study interindividual variation in CYP3A activity, it appears not useful.

Outcome of pediatric studies with midazolam

Notwithstanding, pharmacokinetic data, based on continuous infusions and therapeutic single doses, are largely in line with the *in vitro* developmental pattern of CYP3A4/5 [7,13,22,57]. Hence, we believe that midazolam plasma clearance rate using therapeutic doses might serve as a surrogate marker of CYP3A activity in children.

EBMT as a probe in children

Because the EBMT, in contrast to midazolam systemic clearance rate, is relatively less invasive, the usefulness of this test was studied as surrogate marker of CYP3A4/5 activity in young children. By labeling erythromycin with a radioactive ^{14}C, $^{14}CO_2$ formation can be easily measured in exhaled breath using scintillation counting. Obviously, to administer to a child a radioactive compound nontherapeutically, no matter how small the dose is, will not be considered 'minimal risk' in general. Instead, stable labeled, nonradioactive, erythromycin is available and can substitute the radioactive variant [34]. To overcome the ethical barriers associated with nontherapeutic drug administration in children, in one study one dose of regular erythromycin, during the treatment for ureaplasma infection, was replaced with a dose of stable labeled erythromycin. Stable labeled isotopes have been used frequently in nutritional studies in adults and children to study the disposition of food components, gastric emptying and to diagnose *Helicobacter pylori* infection. In the context of this review, the administration of stable isotope-labeled drugs to study the ontogeny and the induction of pharmacokinetics is of great potential as an ethical alternative for radioactive-labeled drugs [61].

One limitation of stable isotope-labeled drugs is the high cost in comparison to radioactive-labeled drug. For example, 500 g of stable labeled erythromycin costs around US$ 400 (see http://www.isotope.com), but ^{14}C-erythromycin costs around US$ 80 (see http://las.perkinelmer.ca/enCA/Catalog/ProductInfoPage.htm?ProductID=NEC777250C).

Outcome of validation studies in children

In a pilot study in preterm infants, not significant change in $^{13}CO_2$ in exhaled breath was found after oral ^{13}C-erythromycin. Assuming that oral absorption was adequate, we speculate that this lack of change was because of developmentally low CYP3A4/5 activity.

The decreased metabolism by CYP3A4/5 in these neonates probably does not exceed the baseline variation in $^{13}CO_2$ excretion in these children. Hence, this probe does not seem useful in preterm infants to study the effects of covariates on *in vivo* CYP3A4/5 and p-gp activity [20]. In a similar study, using stable labeled caffeine to study CYP1A2 ontogeny *in vivo*, also no change in exhaled $^{13}CO_2$ was found in preterm infants, while in older children exhaled $^{13}CO_2$ clearly did increase after ^{13}C-caffeine [43]. Of interest, in a study where the ^{13}C-erythromycin breath-test was given to preterm infants and older children, again the same pattern was seen. $^{13}CO_2$ excretion was also very low to not measurable in preterm infants, but was measurable in older children [62]. Yet, because of the large interindividual variation in $^{13}CO_2$ excretion and the lack of a consistent rise after CYP3A induction with carbamazepine in these older children, the authors concluded that the stable isotope EBMT is a suboptimal *in vivo* probe for CYP3A4 activity in children, in addition to the other limitations for the use of the EBMT as CYP3A4/5 probe.

Practical challenges

Probe drug administration

Intravenous administration

For many intravenously administered drugs, there is a lack of age-appropriate stock concentrations available for children. Most drug vials are aimed at adults and preparing an i.v. solution for a 1-kg baby requires repeated dilutions. For example, midazolam (Versed®) was initially only available in 5 mg/ml vials. To give a 1-kg baby a 0.1 mg/kg dose would require 0.1 mg, which is 0.02 ml of this stock solution. Obviously, a second dilution step is necessary to modulate a practical volume. Hence, the risks of inaccurate dosing are large. Even in the controlled setting of a study on drug preparation accuracy, one-third of infusion solutions prepared by nurses contained errors [63]. Larger magnitude errors were associated with the use of more concentrated solutions ($P < 0.001$) and preparation of smaller infusion doses ($P < 0.001$). Such errors not only impose unnecessary risks on the health of the child, but also lead to the wrong interpretation of pharmacokinetic data. A possible solution to reduce these drug preparation issues is for example batch preparation of age-appropriate drug vials by the local hospital pharmacy.

To predict disposition of an intravenous CYP3A substrate accurately, ideally, the phenotyping probe, for example, ^{13}C-erythromycin, should be given intravenously to ensure complete bioavailability. Oral administration would result in variation introduced by drug metabolism and transport in intestine and liver. Yet, ^{13}C-erythromycin and other stable labeled drugs are only available as chemical grade and not as a pharmaceutical product. Hence, the administration of this compound was restricted by one pharmacy to the oral route, for fear of impurities in the product [20]. Interestingly, others have resolved this issue by adding a micropore filter in the intravenous fluid line [34]. Optimally, this should be done only after ensuring that the active drug is not retained in the filter.

Oral administration

The lack of age-appropriate pediatric oral formulations for many drugs is a major roadblock to study drug metabolism and first-pass effect in children adequately. When oral midazolam pharmacokinetics was studied in preterm infants, the manufacturer was working on an oral formulation, but it was not available at the time. Hence, the i.v. formulation and given orally to determine oral midazolam pharmacokinetics in preterm infants, with the ultimate goal to elucidate the combined developmental pattern of intestinal and hepatic CYP3A activity. Also, ^{13}C-erythromycin was not available in an oral formulation for neonates. Concerns regarding the administration of an i.v. formulation orally are high osmolality and very acidic or basic pH of the solution, both might be caustic to gastrointestinal tract. Also, dependent on the oral formulation used, absorption of erythromycin is significantly impaired when gastric pH is low [64,65]. Hence, to ensure optimal oral absorption, this probe can to be administered with an antacid [62]. This imposes new challenges, such as the administration of another 'nontherapeutic' drug for nontherapeutic reasons to a child, though this approach was allowed by the IRB in one study [62].

Alternatively, gastric pH was measured in our patients before ^{13}C-erythromycin was given. As the patient population consisted of preterm infants who already had a gastric tube in place for clinical reasons, this could easily be done [20]. All patients had a neutral gastric pH, which might probably be explained by the age-appropriate, high frequency of alkaline feedings (i.e. milk formulas) they were receiving.

To ensure adequate absorption of a stable labeled drug, plasma levels can be determined, but this undoes the noninvasive advantages of this probe. Alternatively, using the properties of the stable labeled drug, excretion of the drug and metabolites in urine, breath and feces can be easily measured by gas chromatography–mass spectrometry (GC–MS). This might help to distinguish low $^{13}CO_2$ in exhaled breath because of possible impaired drug metabolism from reduced gastrointestinal absorption [34].

Challenges in sampling

Blood sampling

Obviously, the restriction on total sample volume leads to a restriction in the individual sample volume. This highlights the need for very sensitive analytical methods in these studies, which are currently often lacking because of the limited need for such specific sensitivity in adult studies, where sample volume is usually not an issue. Also, the availability of specific equipment such as LC–MS or GC–MS, often needed for such sensitive analytical methods, is still limited in most institutions, because of the high purchase and maintenance costs.

In a midazolam study in preterm infants, where sample volume was restricted to 0.2-ml blood, a laboratory was found that was able to analyze midazolam and 1-OH metabolite with excellent lower limits of detection. A limitation of the assay was that in sufficient plasma volume was available to also measure the 1-OH-M-glucuronide, which would have given additional valuable information on both CYP3A and glucuronidation ontogeny [22]. By contrast, when such assays are available, parent drug and all metabolites can be determined in small volume samples, enabling the study of drug metabolism in small infants.

Additionally, in small children, blood sampling might be challenging for several reasons. Even a 'large' bore peripheral intravenous catheter might not allow sufficient blood sampling in

preterm and term neonates. The risks associated with the insertion of a central catheter (e.g. infection, bleeding and catheter displacement) cannot be considered 'minimal'. Consequently, the insertion of such a line, solely for the purpose of research, is generally no option in pediatric research. Therefore, patient inclusion will often be restricted to young children, who already have a central venous or arterial line in place for clinical purposes, as was encountered in a midazolam trial in preterms [21]. Almost all of these infants undergo stressful procedures as part of routine clinical care, which made them eligible for midazolam administration in the context of the trial. Initially full pharmacokinetic (PK) sampling curves for each patient were obtained, hence requiring a central line for repeated sampling. The number of patients eligible for the study was significantly impacted by the prerequisite for a central line (from approximately 120 to 40 over a two-year period), as only the sickest preterm infants already had central lines in place [21]. In addition to the recruitment problems, this also meant that the data obtained might not be applicable to a larger group of relatively healthier children.

Instead, to circumvent the need for a central line, small volume of blood samples can be taken during regular blood work by heel prick. A major disadvantage of this approach is that multiple timed samples are not really feasible for practical and ethical reasons. Also, from our personal experience, many parents refuse informed consent for the reasons of prolonged heel prick blood sampling time, which is painful and stressful, where they would approve blood sampling from a central catheter.

Urine sampling

Urinary drug and metabolite clearance rates have been used as alternative noninvasive surrogate markers of drug metabolism *in vivo* [23,60,66]. Although the collection of urine over a prolonged period of time (>12 h) is difficult even in healthy adults, the situation in children is even more problematic. Alternatively, if a catheter is not available, other methods can be considered. One option is the use of a urine collection bag that adheres to the skin around the urogenital region [23]. Although noninvasive and frequently used in clinical practice, this method has several disadvantages. First, more often than not, the bag's adhesive is not sufficiently strong to ensure complete attachment to the skin and frequent leakage occurs, for anatomical reasons more in female than in male neonates. Second, the skin is extremely fragile, especially in very young preterm infants and repeated use of an adhesive collection bag can cause serious skin damage. This is not only painful, but is also a risk for opportunistic infections.

The 'gauze/cotton ball method' is another method used to collect urine in the clinical and research setting. A small gauze with cling-film (the latter facing the diaper material to prevent urine absorption in the diaper) is put in the diaper and urine is collected by expressing the urine from the gauze [67]. As well, nonabsorbent diapers can be used in a similar way [68]. Bag and gauze/diaper collection methods have a disadvantage in that complete urine recovery is virtually impossible because of leakage around the opening of the bag or loss of urine in the gauze/diaper material. Yet, this limitation might be overcome by weighing the diaper before and after use to determine urine loss. Next, total drug and metabolite excretion can be calculated from total urine volume and drug and metabolite concentration.

Breath sampling

Collection of breath samples for $^{13}CO_2$ recovery in studying drug metabolism in young children is challenging, but very feasible. The original collection method of respiratory CO_2 occurs via trapping of CO_2 in sodium hydroxide. This method involves a tight-fitting facemask and passing of the expired air through a condenser containing sodium hydroxide [43]. This is impractical and difficult in preterm infants. Therefore, recently, a direct nasopharyngeal sampling technique was developed [69]. This technique permits direct sampling from the nasopharynx using a gastric tube attached to a syringe or direct attachment of a syringe to a side-port of the endotracheal tube. During observed expiration, the researcher collects air by pulling the syringe. The collected air is then transferred to a vacuum tube for laboratory analysis. This validated method is obviously much easier and more patient-friendly, than the original method.

Possible solutions

Insight was obtained in midazolam clearance and 1-OH-midazolam formation in intensive care patients over the whole pediatric age range. In line with *in vitro* data, it was shown that midazolam clearance rate increases with age [19,21,22]. It was also shown that the ^{13}C-erythromycin breath-test in preterm infants can be performed ethically and practically, but that it does not appear to be an useful probe to study CYP3A4/5 activity in preterm infants and older children [20,62].

Yet, the exact developmental pattern of CYP3A activity and the factors that govern its ontogeny have not been elucidated till date, which is not only unique for drug metabolizing enzymes alone, but also for other pathways of drug disposition as renal and biliary clearance. Interestingly, several novel approaches in addition to the phenotyping studies described have emerged to study the drug disposition in children (Table 2).

Population pharmacokinetic modeling

An interesting and very feasible approach in children, is the use of population pharmacokinetic modeling to determine drug clearance rate of midazolam or other drug phenotyping substrates [70]. Population pharmacokinetic modeling to predict drug clearance rate has several advantages. First, blood samples do not have to be collected at specified times. Second, data from patients with

TABLE 2

Key concepts: possible solutions for drug studies in children, emphasis on CYP3A phenotyping

Population pharmacokinetics
Limited sampling per patient
Uses data from real-life situation
Strong to study factors that determine variation
Physiology-based pharmacokinetic modeling
Based on physiological, *in vitro* and *in vivo* data
Limitation: lack of *in vivo* data of children <5 years of age
Other CYP3A probes, using urinary drug metabolite ratio
Dextromethorphan, tramadol, 6β-OH cortisol/cortisol, omeprazole
Affected by renal function, urinary pH, nonspecificity for CYP3A
Microdosing
Limits risks associated with nontherapeutic dosing
Possible underestimation of toxicity

missing or limited samples can still be used. Blood samples can be collected from patients who already receive the drug for clinical reasons and the sampling schedule can accommodate the clinical setting. These are large advantages, which accommodate studies in the clinical situation much better than rigid pharmacokinetic, full washout sampling designs. In addition to collecting new data, data from already published studies, covering a wide age range, can be combined to study drug metabolism and other drug disposition pathways *in vivo*. Moreover, population pharmacokinetic modeling is a strong tool to determine the relevance of other covariates in the context of the development of drug disposition.

Pediatric physiologically based pharmacokinetic (PBPK) modeling

To overcome many of the limitations discussed above in predicting the disposition of CYP3A substrates for groups of patients, PBPK modeling appears to be a promising solution. Johnson *et al.* [71] combined demographic, genetic and physiological data from adults and children with *in vitro* data on human drug metabolism from children to predict drug disposition of 11 drugs over the whole pediatric age range. They were successful in predicting drug disposition for these substrates when validated against available pediatric pharmacokinetic data. An important limitation till date is the scarcity of pharmacokinetic data in the zero to five years age range, needed to further validate this model. This model does not negate the need for further data collection on disposition of drugs in this age range, which can be accomplished using the other methods described here (e.g. population PK modeling and urinary MRs).

Other CYP3A probes, using urinary drug metabolite ratio

Interesting results have been obtained using the MR of dextromethorphan and tramadol and respective metabolites as markers of drug metabolism by CYP3A4 and CYP2D6 [23,39]. After correction for age-related changes in renal function, the developmental pattern of the drug/metabolite MR is in line with *in vitro* and PK data of CYP3A4/5 [72]. By contrast, the 6β-OH-cortisol/cortisol urinary ratio appears as a suboptimal marker to determine the ontogeny of CYP3A activity. The cortisol to 6β-OH-cortisol conversion is also mediated by CYP3A7, which might explain why a higher ratio is found directly after birth than in adults and in older children [73,74]. Interestingly, these data are not supported by *in vitro* data on CYP3A7 substrate specificity and activity [7], since testosterone hydroxylation, which has a higher affinity for CYP3A7 than cortisol, is not increased in the first weeks of life.

Urinary drug and metabolite ratios have been widely used as phenotyping probes in adult and pediatric populations to study genetic and age-related variation in drug metabolism, not only for CYP3A but also for other drug metabolizing enzymes [23,25,66]. In addition to the practical issues regarding urinary collections in children, potential limitations of this method are the effect of renal function, urinary pH, extrahepatic drug metabolism and alternative excretion pathways (e.g. biliary) on the measured ratios [66].

Urinary drug:metabolite ratios are a combined marker of unbound renal clearance rate of parent drug and unbound clearance rate of the drug by the relevant enzyme: MR = CLuR/CLuint [66]. Hence, the drug:metabolite ratio might be importantly affected by the changes in renal function. The effect of renal function on MR can be overcome by collecting urine for parent drug and metabolite sufficiently long so that all administered drug is recovered. Otherwise, the MR might be corrected for by renal function to more adequately reflect drug metabolism and not renal function.

The urinary metabolite ratio might also be affected by urinary pH as has been shown for dextromethorphan and metoprolol when used as CYP2D6 probe drugs. For these drugs, a correction factor for pH has been determined [75]. It is unknown how the MR is affected by urinary pH for midazolam or tramadol [76]. Ideally, the influence of urinary pH on the MR of these probes should be determined.

Also, differential expression of renal and hepatic CYP3A5 and CYP3A4 might further contribute to a discrepancy between CYP3A4/5 substrate plasma clearance and urinary MR [77].

Both cortisol and dextromethorphan show a poor correlation with other CYP3A probes, probably consequent to a combination of these limitations [25,78]. Additionally, dextromethorphan and tramadol are also metabolized by CYP2D6. Hence, changes in the CYP3A resulting metabolite:drug ratio might reflect a change in CYP2D6 activity instead of CYP3A activity [24]. Nevertheless, despite the lack of well-validated noninvasive probes of CYP3A activity for use in children, urinary MRs might still generate valuable information on the developmental pattern of drug metabolism, taking the mentioned limitations into account.

Microdosing studies

To minimize possible harm from nontherapeutic drug administration, microdosing of a probe drug might be an interesting alternative [79]. Microdosing entails the administration of a dose that is considerably lower than (often 10% of) the therapeutic dose. Recently, the validity of this approach was tested for five different probe drugs in adults [80]. For three drugs, one of them being midazolam, microdosing pharmacokinetics seemed to reflect therapeutic dose disposition adequately. Of potential importance, for two other drugs, pharmacokinetics after microdosing or therapeutic dosing were significantly different. One of the reasons for this discrepancy might be nonlinear pharmacokinetics because of differences in the saturation of different ADME processes. This might be even more relevant for pediatrics. In the case of developmentally low metabolic enzyme activity, metabolism might not be saturated at very low drug doses. By contrast, with therapeutic doses, pathways might become easily saturated, with alternative pathways for excretion partially or fully compensating. Hence, if therapeutic dosing is based on microdosing pharmacokinetics, children might be exposed to toxicity because of unanticipated impaired clearance. This important limitation needs to be taken into account when using this method to study ontogeny of drug metabolism.

Future directions

Information gaps continue to exist in drug disposition and effects in children. It is obvious from this review and from other information sources (Box 1) that multiple ethical, practical and scientific challenges hamper our attempts to increase our knowledge on drugs during the development. Using alternative solutions to overcome these challenges, we have been able to confirm globally

BOX 1

Key resources

Books

Pediatric Pharmacology and Pharmacokinetics
"*This book is an important resource for physicians, nurses, clinical pharmacologists, researchers and healthcare professionals to understand the physiological, pharmacological, and PK differences between the pediatric population and adults*
"Author: Iftekhar Mahmood, Ph.D.
ISBN 0976643839, First Edition, 2008

Paediatric Drug Handling
"*Written for new pharmaceutical scientists, this book aims to give a background of paediatric pharmacy*
"Series editors: AT Florence, Tony Moffat,
ISBN: 0853696861, First Edition, 2006

Paediatric Clinical Pharmacology
"*This textbook will help pediatric health professionals select the most appropriate medication to effectively treat children and ensure minimal side effects*"
Editors: Evelyne Jacqz-Aigrain, Imti Choonara
ISBN: 0824721896, First Edition, 2006

Neonatal and Pediatric Pharmacology Therapeutic Principles in Practice
"*Written by experts at the forefront of current research and clinical practice, this volume provides evidence-based guidelines for safe, effective, and rational drug therapy in newborns, children, and adolescents*"
Authors: Jacob V. Aranda, Sumner J. Yaffe
ISBN: 9780781741859, Third Edition, 2004

URLs

Pediatric Drug Development
FDA's 'pediatric drug development' homepage with links to information on, that is, FDA Amendments Act of 2007 (FDAAA), Best Pharmaceuticals for Children Act of 2007 (BPCA of 2007), regulations, guidance for industry for performing drug studies in children http://www.fda.gov/cder/pediatric/

EMEA-Medicines in Children
Extensive section of the EMEA website created to provide convenient access to all information relating to the Agency's work in the area of paediatric medicines http://www.emea.europa.eu/htms/human/paediatrics/introduction.htm

PharmGKB
Pharm GKB website: PharmGKB curates information that establishes knowledge about the relationships among drugs, diseases and genes, including their variations and gene products. The mission is to catalyze pharmacogenomics research http://www.pharmgkb.org/

Directory of P450-containing Systems
The goal of this website is to facilitate access to electronic resources world-wide for all researchers working in the field of P450 proteins and P450-containing systems
http://www.icgeb.org/~p450srv/

the developmental pattern of CYP3A activity *in vivo*. We have not, however, been able to identify crucial time points in ontogeny of CYP3A activity. It is still unclear when in the first year of life CYP3A activity reaches adult levels. Similarly, the effect of gestational age on ontogeny of CYP3A activity is only partially known [22,81]. The reasons for the apparent increased clearance rate of many CYP3A substrates in infants have not been elucidated. Can the polymorphic expression of CYP3A5 and CYP3A7 explain the large interindividual variation of CYP3A substrates in neonates? How does infant formula affect ontogeny of drug metabolism in newborns [15,82]? Ultimately, it is crucial to identify how these changes in CYP3A activity affect pharmacokinetics and relate to pharmacodynamics. We still do not know if the supposedly higher clearance rate of CYP3A4/5 substrates in infants explains the higher failure rate of midazolam as sedative in pediatric intensive care patients [5,83]. Similarly, is the observed difference in sedative effect to midazolam in preterms associated with developmental changes in CYP3A4/5 or with age-related pharmacodynamics [53]? These questions not only remain for CYP3A and midazolam but also for many other drugs prescribed to children.

In view of the described limitations for drug studies in children, wider use of population pharmacokinetic–pharmacodynamic and PBPK modeling, urinary MRs, stable labeled drugs and microdosing combined with age-appropriate biomarkers for drug effect might provide great opportunities to facilitate knowledge and more effective use of drug therapy in children.

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