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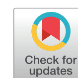
Development and validation of an LC-MS/MS method for the quantitative analysis of milciclib in human and mouse plasma, mouse tissue homogenates and tissue culture medium



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ABSTRACT

Milciclib is a promising cyclin-dependent kinase inhibitor currently in phase II clinical trials to treat several types of cancer. The first bioanalytical method for the quantitative analysis of milciclib in several biomatrices using liquid chromatography-tandem mass spectrometry is described here. This method was fully validated in human plasma according to FDA and EMA guidelines, and partially validated in mouse plasma, homogenates of mouse brain, kidney, liver, small intestine, spleen, and tissue culture medium. Palbociclib, an analog compound, was used as internal standard. A simple and fast sample pre-treatment by protein precipitation with acetonitrile was used, leading to efficient extraction of the analyte with recoveries between 95–100%. Chromatographic separation was achieved with a C_{18} analytical column and a gradient elution using 10 mM ammonium bicarbonate in water and 10 mM ammonium bicarbonate in water-methanol (1:9, v/v). This assay was selective, accurate, precise and linear in the concentration range of 1−1000 ng/mL. Moreover, samples above the upper limit of quantification can be integrally diluted up to 10-fold prior to analysis. The use of human plasma as a surrogate matrix to quantify milciclib in tissue culture medium and mouse matrices resulted in acceptable accuracy and precision, however tissue culture medium samples required a dilution with human plasma prior the pre-treatment. All performance parameters of the method complied with the acceptance criteria recommended by the guidelines, except for the carry-over, which was slightly above (22.9% of the lower limit of quantification) the recommended percentage (20%). Therefore, additional measures were taken to assure data integrity. Stability of milciclib in all matrices was evaluated, and in some matrices the analyte was unstable under the tested conditions. It is therefore recommended to keep these samples as briefly as possible at room temperature during the pre-treatment, and to store them at −70 °C to avoid analyte degradation. This method was successfully applied to support preclinical pharmacokinetic studies of milciclib.

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1. Introduction

Cyclin-dependent kinases (CDKs) are key complexes in the regulation of the cell cycle, that are activated when a cyclin (cyc) binds to them [1]. CDKs have different roles: they can control the entry of the cell cycle (CDK4/cycD and CDK6/cycD), the DNA replication (CDK2/cycE and cycA) and the initiation of mitosis (CDK1/cycA and cycB) [2]. CDKs, cyclins and the endogenous CDK inhibitors are frequently dysregulated in cancer cells, favoring tumorigenesis [1,3]. This has led to the development of small-molecule CDK inhibitors. So far, three selective CDK4/6 inhibitors, palbociclib, ribociclib and abemaciclib, have been approved by the FDA and EMA to treat cancer, and several other CDK inhibitors, including milciclib, are being investigated in preclinical or clinical studies.

Milciclib is a potent inhibitor mainly for CDK2 (IC_{50} CDK2/cycA 45 nM) and the tropomyosin receptor kinase A (TRKA, IC_{50} 53 nM), an enzyme that is activated in several types of cancer, which leads to the inhibition of apoptosis, inducing cell proliferation [4,5]. Milciclib also inhibits CDK1, CDK4, CDK5, and CDK7 with lower potency ($IC_{50} \geq 150$ nM) [4]. In addition to the cell cycle arrest *via*

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CDK2, it has recently been reported that milciclib reduces angiogenesis and inhibits glucose consumption in tumors by targeting CDK5 and CDK7, respectively [6,7]. Milciclib was developed for oral administration, and is currently being investigated in phase II clinical trials. Preclinical studies have shown the efficacy of milciclib against brain cancers in medulloblastoma and glioma models [8,9]. Milciclib also inhibited tumor growth in a transgenic mouse model for non-small cell lung cancer [10]. In xenografts models, milciclib has shown efficacy against melanoma, ovarian, colon, pancreatic, prostate and non-small cell lung cancer with a tumor growth inhibition from 64 to 91% [11]. In clinical studies, milciclib has been effective against thymic carcinoma and thymoma [12,13]. As a consequence, the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) granted the "orphan drug" designation to milciclib. In combination with gemcitabine, milciclib was well tolerated and showed clinical benefit in approximately 36% of the patients [14]. Furthermore, milciclib is currently being investigated in phase II clinical studies to treat hepatocellular carcinoma [6,15].

Despite (validated) bioanalytical methods using LC–MS/MS having been used to support some of the studies mentioned above, none of these have been described with respect to either the method settings or their performance [4,11,12,14]. In two of them, only the calibration range is mentioned [11,12]. In drug development, validated bioanalytical methods are pivotal to obtain reliable results and to make critical decisions supporting safety and efficacy of active compounds and/or pharmaceutical formulations [16,17].

The aim of this study is to provide a bioanalytical LC–MS/MS method that could support further pre-clinical and/or clinical research of milciclib. We developed an LC–MS/MS method and fully validated it in human plasma. It was also partially validated in tissue culture medium and in mouse matrices, including plasma and tissue homogenates of brain, kidney, liver, small intestine and spleen. A preclinical application of this method to pharmacokinetic and tissue distribution studies of milciclib in mice is also presented.

2. Material and methods

2.1. Chemicals and reagents

Reference standards of milciclib free base (99.5% pure) and palbociclib free base (100% pure) were obtained from Selleck Chemicals LLC (Houston, TX, USA) and AlsaChim (Illkirch-Graffenstaden, France), respectively. Methanol, water, and formic acid, all ULC-MS grade, and acetonitrile (Supra gradient grade) were supplied by Biosolve Ltd (Valkenswaard, The Netherlands). Ammonium bicarbonate (LC–MS grade) and dimethylsulfoxide (DMSO, Seccosolv) were purchased from Merck (Darmstadt, Germany).

2.2. Blank matrices

K_2EDTA control human plasma was obtained from BioIVT (Westbury, NY, USA). For the tissue culture medium, the Dulbecco's Modified Eagle Medium glutamax (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) was supplemented with 10% (v/v) Fetal Bovine Serum F7524 (Sigma-Aldrich, Darmstadt, Germany). Heparin plasma and organs from individual male and female mice were obtained from the animal laboratory of The Netherlands Cancer Institute (Amsterdam, The Netherlands, approval number from The Dutch Central Animal Testing Committee AVD3010020173825).

Blank tissue homogenates were prepared with 2% (w/v) bovine serum albumin (BSA, Roche Diagnostics GmbH, Mannheim, Germany) in water. A volume of 3 mL of this solution was added to liver and small intestine, 2 mL to kidneys and 1 mL to brain and spleen prior the homogenization using the Fast Prep-24^{TM} 5 G (MP Biomedicals Inc., Santa Ana, CA, USA). The organ weight to 2% BSA volume ratio (w/v) of the homogenates was approximately 0.4:1 for brain and liver, 0.25:1 for small intestine, 0.2:1 for kidneys, and 0.1:1 for spleen.

2.3. Stock and working solutions

Two independent stock solutions of milciclib with separate weighing were prepared at 1 mg/mL in DMSO. One stock solution was used for the preparation of eight working solutions of calibration standards in the concentration range from 20 to 20,000 ng/mL. The second stock solution was used to prepare working solutions of quality control (QC) samples at concentrations of 20, 60, 1000 and 15,000 ng/mL, and working solutions for tissue culture medium QC samples at 40, 120, 2000 and 30,000 ng/mL. All working solutions were prepared in water-acetonitrile (1:1, v/v).

For the internal standard (IS), a stock solution of palbociclib was prepared in 1% formic acid at 1 mg/mL. The IS-working solution was prepared by diluting this stock solution with water-acetonitrile (1:1, v/v) to obtain a concentration of 100 ng/mL. Stock and working solutions were stored at −20 °C.

2.4. Calibration standards and quality control samples

Eight calibration standards at 1, 2.5, 10, 25, 100, 250, 800 and 1000 ng/mL were prepared by diluting the corresponding milciclib working solutions 20-fold with control human plasma. The same dilution factor was used to prepare the QC samples, obtaining final concentrations of 1 (lower limit of quantification, LLOQ), 3 (LQC), 50 (MQC) and 750 ng/mL (HQC) in human and mouse plasma, and mouse tissue homogenates. QCs in tissue culture medium were prepared at 2 (LLOQ), 6 (LQC), 100 (MQC), and 1500 ng/mL (HQC).

2.5. Sample pre-treatment

For plasma samples, both from human and mouse, a volume of 50 μL was processed by adding 20 μL of the IS-working solution and 150 μL of acetonitrile. Then, samples were vortex mixed and centrifuged at 23,100g for 5 min. A volume of 150 μL of the supernatant was transferred to a glass vial with insert, and 1 μL was injected into the LC–MS/MS for analysis. When mouse plasma samples had a volume lower than the required (50 μL), 10 μL of mouse plasma was diluted with 40 μL of control human plasma to obtain the validated volume for the sample pre-treatment.

An aliquot of 100 μL of tissue homogenates was used for the pretreatment and the IS-working solution and acetonitrile were added proportionally based on the plasma samples. Tissue culture medium samples were diluted with control human plasma (1:1, v/v) prior to the pretreatment. Thereafter, the same procedure as described to treat the tissue homogenates was used.

2.6. Analytical instrumentation and conditions

An ultra-high performance liquid chromatograph (UHPLC) Nexera X2 (Shimadzu, Kyoto, Japan) was used, which consisted of two binary pumps (LC-30AD model), an autosampler (SIL-30AC_{MP}) and a column oven (CTO-20AC). Chromatographic separation was performed using a Gemini C_{18} column (50 × 2.0 mm ID, 5 μm, Phenomenex, Torrance, CA) protected by a Gemini C18 (4 × 2 mm) guard column, both maintained at 40 °C. For the elution of the analytes, a gradient was applied using 10 mM ammonium bicarbonate in water (mobile phase A) and 10 mM ammonium bicarbonate in water-methanol (1:9 v/v, mobile phase B) at a flow rate of 0.4 mL/min. Initially, from 0 to 0.5 min the percentage of B was 50%, from 0.5 to 1.0 min B increased to 100% and this percentage was

Table 1
MS settings.

Parameter	Setting	
Gas 1 (NEB)	50 au	
Gas 2 (Turbo)	50 au	
Curtain gas (CUR)	30 au	
Collision gas (CAD)	9 au	
Ion spray voltage (ISV):	5500 V	
Temperature (TEM)	600 °C	
Interface Heater	On	
Dwell time	200 msec	
Entrance potential	10 V	
Compound-dependent settings	**Milciclib**	**Palbociclib**
Mass transition (*m/z*)	461.1 → 430.2	448.1 → 380.1
Declustering potential (DP)	181 V	166 V
Collision energy (CE)	33 V	39 V
Collision cell exit potential (CXP)	26 V	24 V

kept until 2.5 min, at 2.6 min B decreased to 50% and was maintained until 3.1 min, at 3.6 min B increased again to 100% and was maintained until 4.6 min. At 4.7 min B decreased again to 50%, and the column was conditioned for 1.3 min resulting in a total run time of 6 min. The increase of 100% B from 3.6 to 4.6 min was included to decrease the memory effect. The temperature of the autosampler was maintained at 20 °C for the tissue culture medium samples, and at 4 °C for the rest of the samples.

The analyte and the internal standard were detected using a Triple Quad 6500+ mass spectrometer (Sciex, Foster City, CA, USA) with a turbo ion spray operated in positive ion mode. A divert valve was used to direct the flow from 1 to 3 min to the mass spectrometer and the remainder was directed to the waste. Selective responses were acquired *via* multiple reaction monitoring (MRM) using the settings described in Table 1. The Analyst software version 1.6.3 (Sciex) was used for data acquisition and processing.

2.7. Method validation

This method was fully validated for human plasma according to the FDA and the EMA guidelines for bioanalytical method validation [16,17]. Additionally, the method was partially validated for mouse plasma, mouse tissue homogenates (brain, kidney, liver, small intestine and spleen) and tissue culture medium, including selectivity, intra-run accuracy and precision, dilution integrity and stability, using calibration standards in human plasma.

2.7.1. Calibration model

Eight non-zero calibration standards (described in Section 2.4), a blank and a zero calibration standard (blank spiked with IS) were prepared in duplicate, processed according to Section 2.5 and analyzed in each validation run. The relationship between the analyte concentration and the ratio of the peak areas (milciclib/IS) was described by a linear model with a $1/x^2$ weighting factor, where x is the analyte concentration. The difference between the back calculated and nominal concentrations should be within ± 15%, and ± 20% for the LLOQ, in at least 75% of the calibration standards.

2.7.2. Accuracy and precision

Intra-assay accuracy and precision were assessed by analyzing five replicates of the QC samples at each concentration level (LLOQ, LQC, MQC, and HQC) in all the matrices, including human plasma, mouse plasma, mouse tissue homogenates, and tissue culture medium. Inter-assay accuracy and precision was determined in human plasma by analyzing five replicates of the above described QC samples in three separate analytical runs. The bias (%) between the nominal and the mean measured concentration was calculated to evaluate the accuracy. The intra- and inter-assay precision was determined by calculating the variability using the intra-assay coefficient of variation (%CV) and analysis of variance (ANOVA), respectively. Statistics analyses were performed using validated Microsoft Excel calculation sheets. The bias should be within ± 15% and the CV should be ≤ 15% for all tested concentration levels, except for the LLOQ, where ± 20% and ≤ 20% are accepted.

2.7.3. Selectivity

Blanks and LLOQ samples were prepared in six batches of human plasma, mouse plasma, and mouse tissue homogenates to evaluate the selectivity. For tissue culture medium, the selectivity was tested in one batch. The chromatograms of milciclib and palbociclib (IS) obtained in the blanks were compared with the chromatograms of milciclib in the LLOQ samples to determine potential interferences. The response of the interfering peaks in the blank sample should not exceed 20% of the response for milciclib and 5% of the response for the IS, in an LLOQ sample. Furthermore, at least 4 out of 6 milciclib LLOQ samples should be within ± 20% of the nominal concentration.

2.7.4. Lower limit of quantification

The LLOQ was set at the lowest calibration level, in which the signal-to-noise ratio should be at least 5. This was determined by comparing the signal of milciclib at this level to the noise obtained in a blank sample, in at least three validation runs.

2.7.5. Carry-over

Carry-over was investigated by injecting two blanks after the upper limit of quantification (ULOQ) sample in at least three analytical runs. The response at the retention time of milciclib in the first blank was compared with the response of milciclib in an LLOQ sample. Carry-over should not exceed 20% of the response at the LLOQ.

2.7.6. Matrix factor and recovery

The matrix effect was determined in six batches of control human plasma at LQC and HQC concentrations. For each batch and concentration level, matrix-present samples (MPS) were prepared. For this, each batch of blank plasma was processed until final extract and spiked with the corresponding QC working solution. The absolute matrix factor (MF) was determined by calculating the ratio of the peak area obtained in the MPS and in the matrix-absent sample (MAS), which was prepared by diluting the working solution with acetonitrile-water (3:1, v/v) to reach the same concentration as the MPS. Additionally, the IS-normalized MF was calculated by dividing the MF of the analyte by the MF of the IS.

The recovery was assessed by comparing the response of processed LQC and HQC samples with the MPS at the correspondent concentration levels. The CV of the IS-normalized MF should be ≤ 15%.

2.7.7. Dilution integrity

Dilution integrity was investigated in quintuplicate in human plasma, mouse tissue homogenates and tissue culture medium. For human and mouse matrices, a 10-fold dilution with control human plasma was tested at a milciclib concentration of 5000 ng/mL. For tissue culture medium, a 2-fold dilution with human plasma was tested at a concentration of 1500 ng/mL. The bias and CV of these samples should be ± 15% and ≤ 15%, respectively.

2.7.8. Stability

The stability of milciclib was investigated in all matrices. For human plasma it was evaluated at two concentration levels (LQC and HQC), while for the remaining matrices only at MQC concentration. For every matrix the following stability conditions were assessed: short-term at room temperature (RT), long-term at –20 °C (for all matrices) and at –70 °C (for mouse tissue homogenates),

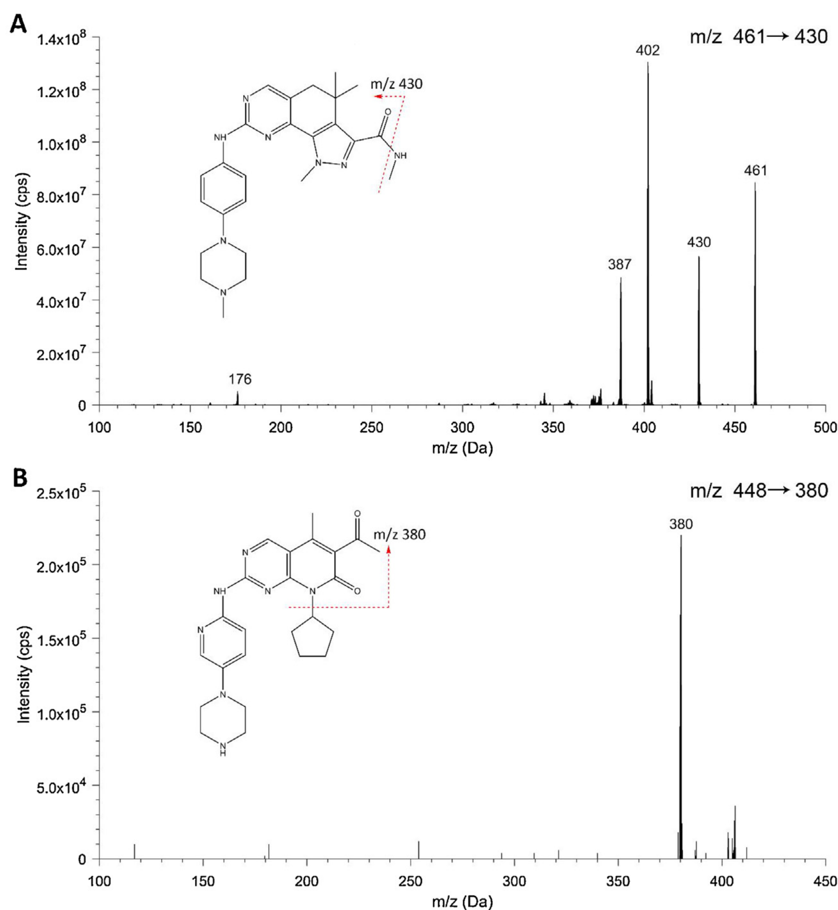

Fig. 1. Mass spectra with the proposed MS fragmentation pattern of milciclib (A) and the IS palbociclib (B). The red dotted line in the chemical structures indicates the proposed MS fragment selected for quantification.

and freeze-thaw (F/T) cycles from −20 °C and/or −70 °C to RT. The stability of the final extracts in every matrix at 2–8 °C was also determined. Solutions were considered stable if the measured concentration was between 85–115%. Finally, the stability of the stock and working solutions at −20 °C was evaluated, and they were considered stable if their response was within ± 5% compared to freshly prepared solutions.

2.8. Applicability of the method

This method was successfully used to support *in vitro* transport studies, as well as *in vivo* pharmacokinetic studies in mice with different genotypes (unpublished data). Mouse studies were conducted according to institutional guidelines complying with the Dutch and European legislation (approval number from The Dutch Central Animal Testing Committee: AVD301002016595). After at least of two hours fasting, 10 mg/kg of milciclib was orally administered to 9–16 weeks old aged female FVB mice (n = 6). Blood was collected from the tail tip (approximately 50 μL) at 0.5, 1, 2, 4, and 8 h. At 24 h, mice were anesthetized and blood was collected *via* cardiac puncture. All blood samples were centrifuged (9000*g*, 4 °C for 6 min) and plasma was obtained using sodium heparin as anticoagulant. Mice were sacrificed by cervical dislocation and organs were collected and weighed, including brain, kidney, liver, small intestine, and spleen. Tissue homogenates were prepared according to Section 2.2. Plasma and tissue homogenates were stored at −20 and −70 °C, respectively until analysis.

3. Results and discussion

3.1. Method development

Milciclib is an analog structure of other CDK inhibitors. It contains a piperazine ring and 2-aminopyrimidine which are also present in palbociclib, ribociclib and abemaciclib, although the substituents are different among them. Since it was not possible to obtain stable isotopically labeled milciclib, we tested these analog structures to choose a proper internal standard (results not shown). Palbociclib was the compound that showed the most similar chromatographic characteristics compared to milciclib, and was therefore chosen as internal standard.

Milciclib and palbociclib MS settings were obtained after performing a compound optimization in the mass spectrometer. Fig. 1 shows the MS spectrum for milciclib (A) and palbociclib (B). Despite the product ion of milciclib at *m/z* 402 being the most abundant, the product ion at *m/z* 430 was selected in the mass transition for this bioanalytical method, because the baseline obtained with the *m/z* 461→430 mass transition was more stable and the lower signal prevented detector saturation. For palbociclib the most abundant fragment with *m/z* 380 was selected as product ion.

We have previously described a bioanalytical method for the quantification of the CDK4/6 inhibitors abemaciclib, palbociclib and ribociclib [18]. Since milciclib is a basic compound with comparable chemical structure to these compounds, the chromatographic conditions selected were similar to this method. Due to the presence of memory-effect, after the elution of the analyte and the inter-

nal standard, an extra washing step was included in the gradient. With this cleaning step, the memory effect was reduced, decreasing the carry-over by approximately 60%. Although carry-over was still detected, the quantitative results were not substantially affected.

A very simple and fast protein precipitation was selected for the sample pre-treatment, considering the large number of samples that are generated during pharmacokinetic studies. Acetonitrile in a ratio 3:1 (v/v) to the sample volume was used as precipitation solvent based on the good recovery obtained with analogue compounds [18]. The supernatant was directly injected into the LC–MS/MS. Compared to the plasma aliquot volume used for the pre-treatment, a higher sample aliquot volume (100 μL) was used for tissue homogenates to obtain a more representative sample.

During method development, two particular issues were observed for the quantification of milciclib in tissue culture medium. Firstly, significant negative deviations were obtained in spiked tissue culture medium samples when quantified using human plasma calibration standards. This is most likely due to the matrix effect of the analyte, which was different from plasma. To solve this matter and considering that the expected concentration in real samples would be relatively high, all samples were diluted with human plasma (1:1, v/v) prior the pre-treatment. Secondly, when the final extract was maintained at low temperatures (*i.e.* 4 °C), solubility in the final extract decreased, leading to precipitation of the analyte. The accuracy was negatively affected, however this process was reversible. This issue was solved by maintaining the temperature of the autosampler at 20 °C when samples in tissue culture medium were analyzed.

3.2. Method validation

3.2.1. Calibration curve

This bioanalytical assay for the quantification of milciclib was linear in the concentration range from 1 to 1000 ng/mL, by using a linear regression with a weighting factor of $1/x^2$. Fig. 2 shows representative chromatograms of calibration standards at the LLOQ and ULOQ. Linearity was evaluated in twelve analytical runs where calibration curves were injected in duplicate (n = 24). Overall, the mean coefficient of correlation was 0.998, and the mean of all back-calculated concentrations was within ± 3.8% of the nominal concentrations (CV ≤ 10.2%). Calibration curves in all individual runs complied the acceptance criteria established by the bioanalysis guidelines.

3.2.2. Accuracy and precision

The results obtained to assess the accuracy and precision of the method are summarized in Table 2. For all matrices, the intra-assay bias and CV were within ± 15% and ≤ 15%, respectively, in all concentration levels including the LLOQ, complying with the FDA and EMA acceptance criteria for accuracy and precision. Additionally, the inter-assay accuracy and precision was assessed in human plasma, and the obtained data (Table 2) met the acceptance criteria previously indicated. These results also support the use of human plasma as a surrogate matrix for the quantification of milciclib in the non-human matrices used in this study.

3.2.3. Selectivity

This method was found to be selective for the analysis of milciclib in presence of endogenous compounds. The tested batch of tissue culture medium and the six batches of human plasma and mouse matrices were free from endogenous interferences. Representative chromatograms from blank matrices and samples of milciclib spiked at the LLOQ are depicted in Fig. 3 A-series and B-series, respectively, except for human plasma where chromatograms are presented in the A1 and A2 panels from Fig. 2. Additionally, the calculated concentrations of the LLOQ samples from all batches were within ± 20% of the nominal concentration for all matrices, except for spleen homogenate where at least 4 out of 6 batches were within this value. Thus, all the matrices complied with the acceptance criteria.

3.2.4. Lower limit of quantification

Based on the milciclib peak height at the LLOQ and the noise in the blank (Fig. 2), the calculated signal-to-noise ratio was at least 16.3. In addition, the bias and the CV for the quantification of milciclib at the LLOQ (1 ng/mL, Table 2) where within acceptance criteria: ± 20% and ≤ 20%, respectively. These results show that this concentration level has an adequate sensitivity, accuracy and precision for the LLOQ.

3.2.5. Carry-over

Despite the cleaning step in the gradient, this method showed carry-over for the analyte. For the internal standard, the mean calculated carry-over was 0.1%. Nevertheless, the response in the blank sample produced by the previous ULOQ sample injected was on average 22.9% of the response of the LLOQ. Since this value exceeds the recommendations from the FDA and EMA guidelines, some measures were taken when milciclib samples were analyzed. Samples were injected in ascending order of expected concentrations, and blanks were injected when a high difference between a high- and low-concentration sample was expected. In addition, the carry-over factor (CF) was calculated when preclinical samples were analyzed according to the following formula:

$$CF = \frac{\left(\frac{Response_X \times Mean\ response_{blank}}{Mean\ response_{ULOQ}}\right)}{Response_{X+1}} \times 100\%$$

where X represents any sample and X + 1 represents the consecutive injected sample. Based on the calculated CF value, a strategy was taken as follows: If CF was ≤ 5% it was concluded that the result of sample X + 1 was not significantly affected by sample X, therefore no action was taken. However, if CF > 5%, the response of sample X + 1 was influenced by sample X, as a consequence, it was necessary to re-inject again sample X + 1 at the end of the analytical batch. In this way, data integrity was insured.

3.2.6. Matrix factor and recovery

The mean matrix factor of milciclib and the internal standard was, respectively, 0.99 and 0.96 at the LQC, and 0.92 and 0.95 at the HQC concentration. The normalized matrix factors were 1.02 and 0.98 for the LQC and HQC concentration levels (CV ≤ 4.1%), respectively.

The recovery of milciclib was 104.7% and 103.0% at the low and high concentration levels, respectively.

3.2.7. Dilution integrity

Table 3 shows the accuracy and precision of samples above the ULOQ after dilution with human plasma. For all matrices, the bias between the measured concentration and the nominal concentration prior the dilution was within ± 13.3%, and the CV was ≤ 5.5%, complying with the acceptance criteria of the followed guidelines.

3.2.8. Stability

The stability of milciclib at relevant conditions in each matrix is summarized in Table 4. Short-term stability was tested at room temperature, where the analyte was stable in mouse and human plasma for 24 h. Milciclib was stable in tissue homogenates (excluding brain) for at least 4 h, and for 2 h in brain homogenate. In contrast, milciclib was unstable in tissue culture medium at room temperature even for 2 h, while immediate processing of spiked samples in this matrix showed a bias of −6.9% with respect to

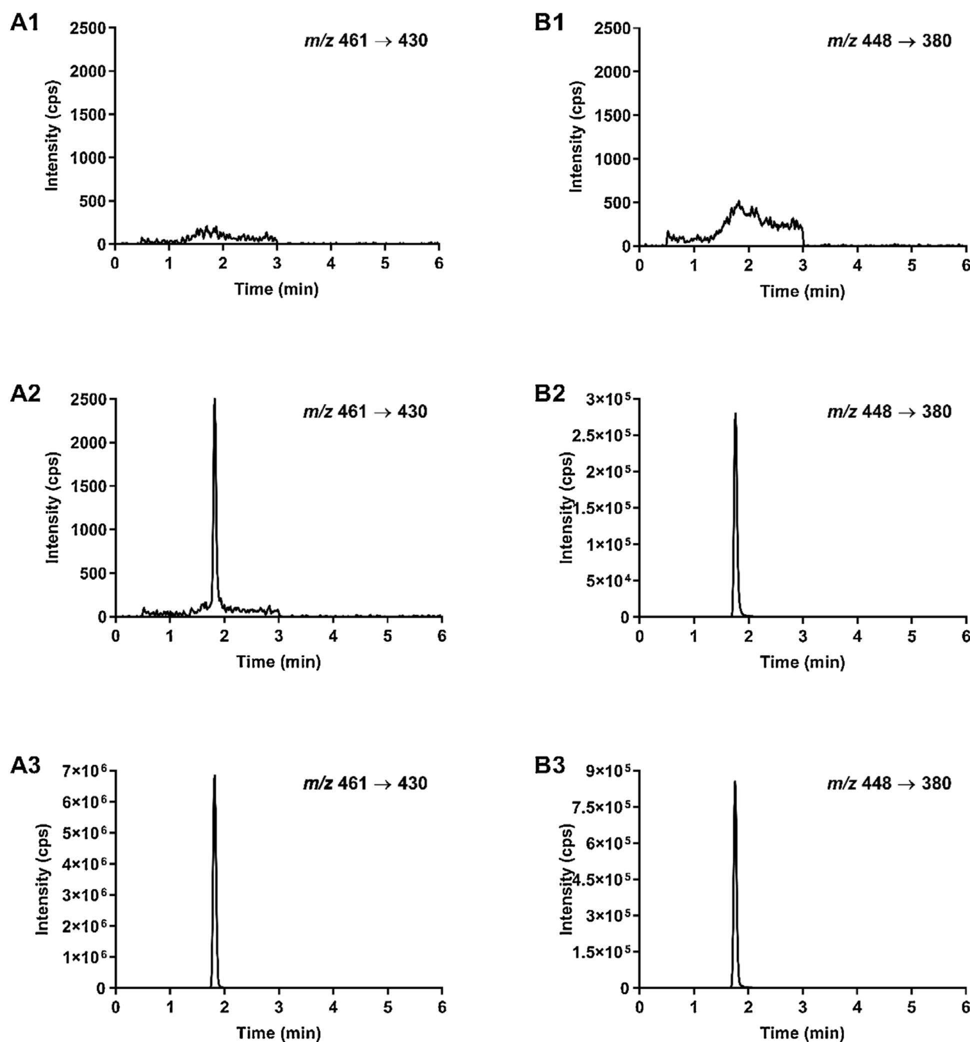

Fig. 2. Representative chromatograms at the mass transition of milciclib (A-series) and palbociclib (B-series) of a blank human plasma (A1 and B1), blank human plasma spiked at the LLOQ (containing 1 ng/mL of milciclib, A2 and B2) and at the ULOQ (containing 1000 ng/mL of milciclib, A3 and B3).

the nominal concentration. Therefore, it is recommended to perform the sample pre-treatment in a short period of time for tissue homogenates, especially brain, and tissue culture medium samples, in order to avoid that results are affected by any instability problem. To determine the appropriate storage conditions, long-term stability of milciclib was evaluated at –20 °C in all matrices. Moreover, since the stability in tissue homogenates is often compromised by the presence of enzymes, in these matrices the stability was also determined at –70 °C. At –20 °C, the analyte was stable for at least 104 days in plasma samples. After 56 days milciclib was also stable in tissue homogenates stored at –20 °C, except for kidney homogenate. In contrast, at –70 °C the analyte was stable in all tissue homogenates. Tissue culture medium samples were stable after 50 days stored at –20 °C. The freeze-thaw process from −20 °C to room temperature after 3 cycles did not affect the stability of milciclib in mouse and human matrices. In addition, this compound was stable in tissue homogenates after 3 cycles of freeze/thaw from −70 °C to room temperature. For tissue culture medium, stability of milciclib was evaluated in one freeze-thaw cycle, in which the analyte resulted stable. Finally, milciclib was stable at 4–8 °C for (at least) 5 days in the final extracts of human and mouse plasma, brain and liver homogenates, 9 days in kidney, small intestine, and spleen homogenates, and 15 days in tissue culture medium.

The stock and working solutions of milciclib were stable at –20 °C for at least 141 and 76 days, respectively. After these stability tests, it is recommended to store milciclib tissue samples at −70 °C, while plasma and tissue culture medium samples, stock and working solutions can be stored at –20 °C.

3.3. Preclinical application of the method

This bioanalytical assay was used to support preclinical *in vitro* and *in vivo* studies of milciclib. Fig. 3 C-series depicts representative chromatograms of samples generated in these preclinical studies. The *in vivo* studies aimed to assess the pharmacokinetics of milciclib in mice. As an example, the results obtained for the wild-type mouse strain are presented (Fig. 4). The semi-log plasma concentration-time curve of milciclib is presented in Fig. 4A, where the time to reach the maximum concentration (T_{max}) ranged from 1 to 8 h and the mean maximum concentration (C_{max}) was 624 ± 68 ng/mL. The mean area under the plasma concentration-time curve ($AUC_{0-24\,h}$) of milciclib was 7094 ± 122 h·ng/mL, due to the lack of points in the terminal phase, the half-life could only be determined in 4 mice, with a mean of 2.75 ± 0.13 h. Milciclib concentration in tissues was calculated in ng/g (Fig. 4B), considering the measured concentration in the homogenates, the volume of BSA added

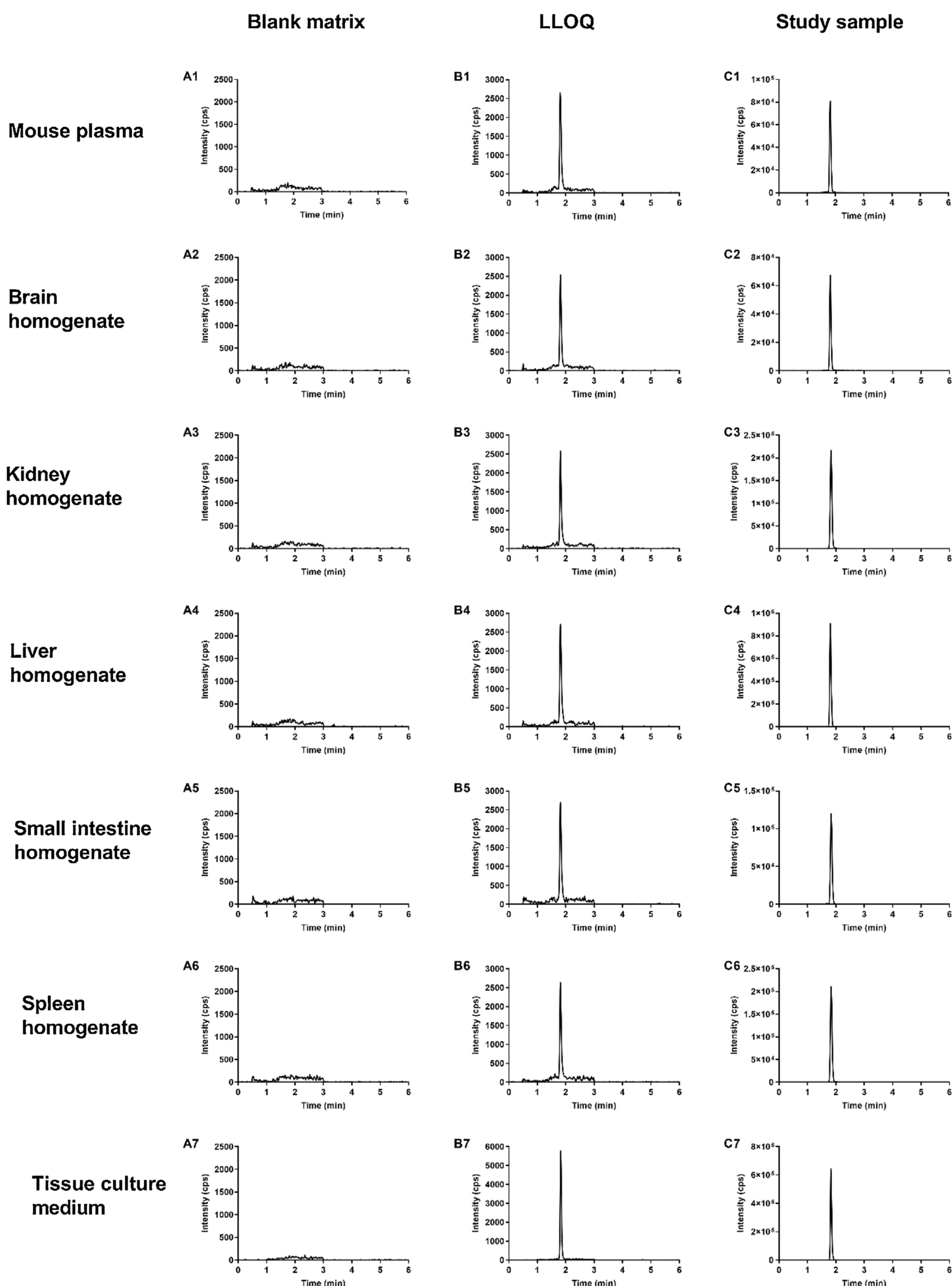

Fig. 3. Chromatograms at the mass transition of milciclib (*m/z* 461 → 430) of a blank matrix (A-series), a blank matrix spiked at the LLOQ (containing 1 ng/mL of milciclib, B-series), and representative samples from the preclinical studies (C-series). The matrix is indicated at the beginning of each row. Representative chromatograms of mouse samples (C1-6), which were collected 24 h after oral administration of milciclib 10 mg/kg. The representative chromatogram of a tissue culture medium sample C7 was collected during an *in vitro* transport experiment of milciclib using the parental MDCK-II cell line; sample was collected 2 h after the addition of 5 μM milciclib in one of the compartments. Measured concentrations in study samples (ng/mL): mouse plasma (C1), 9.95; brain homogenate (C2), 6.71; kidney homogenate (C3), 48.9; liver homogenate (C4), 70.3; small intestine homogenate (C5), 28.5; spleen homogenate (C6), 50.9; tissue culture medium (C7), 162.

Table 2
Accuracy and precision of milciclib in different matrices.

Matrix	Nominal concentration (ng/mL)	Intra-assay (n = 5)		Inter-assay (n = 15)[a]	
		Bias (%)	CV (%)	Bias (%)	CV (%)
Human plasma	1	±10.2	≤3.0	−0.7	9.6
	3	±7.0	≤2.9	−3.1	3.6
	50	±4.7	≤2.8	−1.3	3.5
	750	±5.9	≤2.2	−3.1	2.6
Mouse plasma	1	5.8	2.2		
	3	−0.4	2.0		
	50	−1.2	1.9		
	750	−0.1	3.1		
Brain homogenate	1	12.6	2.1		
	3	2.8	1.6		
	50	12.3	2.0		
	750	5.2	2.4		
Kidney homogenate	1	−3.4	2.5		
	3	−7.5	2.4		
	50	−7.7	2.9		
	750	−6.5	1.5		
Liver homogenate	1	13.2	3.8		
	3	3.6	1.8		
	50	1.0	3.0		
	750	−14.4	13.5		
Small intestine homogenate	1	14.4	3.4		
	3	7.5	4.3		
	50	10.2	2.4		
	750	2.8	5.0		
Spleen homogenate	1	−0.3	3.5		
	3	−9.0	2.9		
	50	−10.5	2.4		
	750	−11.5	2.1		
Tissue culture medium	2	−12.4	5.4		
	6	−5.7	2.2		
	100	−3.5	1.7		
	1500	−9.7	1.4		

[a] For inter-assay accuracy and precision five replicates of each concentration level in three different analytical runs were evaluated (n = 15).

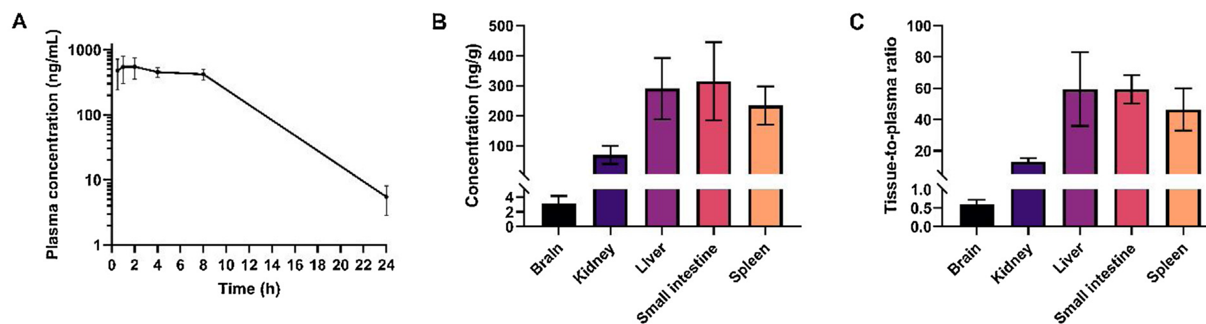

Fig. 4. Semilog plasma concentration-time curve (A), concentration in tissues (B) and tissue-to-plasma ratio (C) of milciclib over 24 h after oral administration of 10 mg/kg to female FVB mice (n = 6). Data are presented as mean ± SD.

to prepare the homogenate and the tissues weight. To determine the tissue distribution of this compound, the tissue-to-plasma concentration ratios were calculated and presented in Fig. 4C, where at 24 h milciclib was poorly distributed in brain, compared to the other tissues. Milciclib was also less distributed in kidneys with respect to liver, small intestine and spleen. All these measured samples where within the calibration range and show that this bioanalytical assay is applicable in preclinical studies of milciclib.

4. Conclusions

The first LC–MS/MS bioanalytical assay for the analysis of milciclib in several matrices was developed and validated following the FDA and EMA guidelines. This method was linear in the calibration range from 1 to 1000 ng/mL, and shown to be selective, accurate and precise for the quantification of milciclib in human plasma, mouse plasma, mouse tissue homogenates, and tissue culture medium. Ten-fold dilution of samples with concentrations above the ULOQ is appropriate for the measurement. Samples in tissue culture medium require special treatment, since a dilution 1:1 (v/v) in human plasma is needed to have an acceptable accuracy and precision. Furthermore, as final extracts of tissue culture

Table 3
Dilution integrity of milciclib samples.

Matrix	Nominal concentration (ng/mL)	Dilution factor	(n = 5)	
			Bias (%)	CV (%)
Human plasma	5000	10	−4.5	0.5
Brain homogenate	5000	10	−4.2	4.7
Kidney homogenate	5000	10	−4.8	4.3
Liver homogenate	5000	10	−13.3	4.0
Small intestine homogenate	5000	10	−3.0	5.5
Spleen homogenate	5000	10	−2.3	2.1
Tissue culture medium	1500	2	−3.3	3.6

Table 4
Stability of milciclib in each tested matrix (n = 3).

Matrix	Type of stability	Stability conditions	Nominal concentration (ng/mL)	Accuracy (% Bias)	Precision (% CV)
Human plasma	short-term	RT, 24 h	3	2.3	1.2
			750	0.8	0.6
	freeze/thaw cycles	−20 °C/RT, 3 cycles	3	−3.3	2.5
			750	−4.2	2.3
	long-term	−20 °C, 104 d	3	−5.5	2.9
			750	0.3	1.2
	final extract	4−8 °C, 5 d	3	−3.3	17.5
			750	1.7	0.9
Mouse plasma	short-term	RT, 24 h	50	−5.5	0.6
	freeze/thaw cycles	−20 °C/RT, 3 cycles	50	−7.1	1.4
	long-term	−20 °C, 104 d	50	−4.3	2.7
	final extract	4−8 °C, 5 d	50	−8.9	4.5
Brain homogenate	short-term	RT, 2 h	50	8.4	1.8
		RT, 4 h	50	−20.0	2.1
	freeze/thaw cycles	−20 °C/RT, 3 cycles	50	−1.3	0.3
		−70 °C/RT, 3 cycles	50	−0.2	3.0
	long-term	−20 °C, 56 d	50	−3.2	0.4
		−70 °C, 56 d	50	−0.5	2.4
	final extract	4−8 °C, 5 d	50	8.5	0.7
Kidney homogenate	short-term	RT, 4 h	50	1.2	3.5
	freeze/thaw cycles	−20 °C/RT, 3 cycles	50	−2.7	1.4
		−70 °C/RT, 3 cycles	50	1.3	3.0
	long-term	−20 °C, 56 d	50	−30.5	7.1
		−70 °C, 56 d	50	3.2	0.5
	final extract	4−8 °C, 9 d	50	3.5	4.6
Liver homogenate	short-term	RT, 4 h	50	4.2	6.1
	freeze/thaw cycles	−20 °C/RT, 3 cycles	50	2.4	1.1
		−70 °C/RT, 3 cycles	50	0.7	1.8
	long-term	−20 °C, 56 d	50	−5.9	0.9
		−70 °C, 56 d	50	−6.2	3.5
	final extract	4−8 °C, 5 d	50	7.3	4.1
Small intestine homogenate	short-term	RT, 4 h	50	6.9	1.1
	freeze/thaw cycles	−20 °C/RT, 3 cycles	50	0.3	2.2
		−70 °C/RT, 3 cycles	50	1.9	3.5
	long-term	−20 °C, 56 d	50	11.5	2.0
		−70 °C, 56 d	50	11.9	2.0
	final extract	4−8 °C, 9 d	50	−0.6	6.0
Spleen homogenate	short-term	RT, 4 h	50	−4.7	6.1
	freeze/thaw cycles	−20 °C/RT, 3 cycles	50	0.2	7.0
		−70 °C/RT, 3 cycles	50	1.1	2.3
	long-term	−20 °C, 56 d	50	−1.1	1.2
		−70 °C, 56 d	50	0.3	2.9
	final extract	4−8 °C, 9 d	50	3.6	5.2
Tissue culture medium	short-term	RT, T0	100	−6.9	3.6
		RT, 2 h	100	−44.9	6.3
	freeze/thaw cycles	−20 °C/RT, 1 cycle	100	−9.5	0.7
	long-term	−20 °C, 52 d	100	−14.9	1.8
	final extract	4−8 °C, 15 d	100	12.3	5.4

RT = room temperature, d = days, T0 = immediately processed sample.

medium samples should not be kept at cold temperatures, it is recommended to maintain the autosampler at 20 °C. The use of human plasma as a surrogate matrix to quantify milciclib in tissue culture medium and mouse matrices resulted in acceptable accuracy. To assure stability of milciclib in all tissue homogenates, samples must be stored at −70 °C and processed within 4 h, except brain samples which must be processed within 2 h. Samples in tissue culture medium must be processed immediately to avoid any degradation at room temperature. Plasma samples can be stored at −20 °C. This method has been successfully used to support preclinical studies of milciclib.

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CRediT authorship contribution statement

Alejandra Martínez-Chávez: Methodology, Investigation, Validation, Formal analysis, Visualization, Writing - original draft. **Matthijs M. Tibben:** Project administration, Conceptualization, Writing - review & editing. **Jelle Broeders:** Investigation, Validation, Writing - review & editing. **Hilde Rosing:** Project administration, Conceptualization, Supervision, Writing - review & editing. **Alfred H. Schinkel:** Supervision, Writing - review & editing. **Jos H. Beijnen:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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