Differences in Sustained Cellular Effects of MET inhibitors Are Driven by Prolonged Target Engagement and Lysosomal Retention

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ABSTRACT

Intracellular distribution of drug compounds is dependent on physicochemical characteristics and may have a significant bearing on the extent of target occupancy and, ultimately, drug efficacy. We assessed differences in the physicochemical profiles of MET inhibitors capmatinib, crizotinib, savolitinib, and tepotinib and their effects on cell viability and MET phosphorylation under steady-state and washout conditions (to mimic an open organic system) in a human lung cancer cell line. To examine the differences of the underlying molecular mechanisms at the receptor level, we investigated the residence time at the kinase domain and the cellular target engagement. We found that the ranking of the drugs for cell viability was different under steady-state and washout conditions and that under washout conditions, tepotinib displayed the most potent inhibition of phosphorylated MET. Postwashout effects were correlated with the partitioning of the drug into acidic subcellular compartments such as lysosomes, and the tested MET inhibitors were grouped according to their ability to access lysosomes (crizotinib and tepotinib) or not (capmatinib and savolitinib). Reversible lysosomal retention may represent a valuable intracellular storage mechanism for MET inhibitors, enabling prolonged receptor occupancy in dynamic, open-physiologic systems and may act as a local drug reservoir. The use of washout conditions to simulate open systems and investigate intracellular drug distribution is a useful characterization step that deserves further investigation.

SIGNIFICANCE STATEMENT

Generally, determination of potency and receptor occupancy is performed under steady-state conditions. In vivo conditions are more complex due to concentration differences between compartments and equilibrium processes. Experiments under steady state cannot explore effects such as sustained target inhibition. This study has shown that differences between MET inhibitors are observable by applying washout conditions to in vitro assays. This important finding applies to most compound classes and may inspire readers to rethink their assay designs in the future.

Introduction

The mesenchymal-epithelial transition factor kinase [MET; also called the hepatocyte growth factor (HGF) receptor] is a tyrosine kinase proto-oncogene that is preferentially expressed in epithelial cells. Although the physiologic role of MET is not fully understood, it is involved in several developmental stages and is normally strictly regulated. MET signaling can be dysregulated by a variety of MET alterations, leading to oncogenesis through hyperactive or otherwise aberrant MET signaling.

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ABBREVIATIONS: ABBREVIATIONS: ACN, acetonitrile; koff, dissociation rate constants; LAMP1, lysosomal-associated membrane protein 1; LC-MS/MS, liquid chromatography–tandem mass spectrometry; logP, lipophilicity; MEM, Minimum Essential Medium; MET, mesenchymal-epithelial transition factor; PCR, polymerase chain reaction; P-gp, P-glycoprotein; pMET, phosphorylated MET.
et al., 2020; Hong et al., 2021), and savolitinib has been approved more recently in China (Lu et al., 2021).

In clinical settings, MET inhibitors can be differentiated by pharmacokinetic characteristics such as volume of distribution and half-life, which not only influence drug dosage (Jones et al., 2021; Xiong et al., 2021) but also potentially modulate primary pharmacology and the risk-benefit ratio of therapeutic agents. In particular, volume of distribution is largely driven by specific and unspecific binding of the therapeutic agent to intracellular proteins and membranes and, to a lesser extent, by the stability of the drug-target complex and lysosomal retention on a subcellular level (Hamid and Kruse, 2016; Schmitt et al., 2021). Complex pharmacokinetic effects cannot be detected in standard closed-vial, steady-state in vitro viability assays. Washout conditions, which mimic an open system (Copeland, 2016), may detect more subtle differences, as described for the cyclin-dependent kinase (CDK)-4/6 inhibitor palbociclib (Llanos et al., 2019). To date, MET inhibitors have been studied in closed in vitro systems (Henry et al., 2016; Baltschukat et al., 2019), which do not appropriately reflect differences in pharmacokinetic profiles. For oncogenic signaling targets, complete and sustained target inhibition is often required to induce a phenotypic response (Zou et al., 2007). Consequently, target modulation and phenotypic effects from in vitro washout experiments may identify pharmacokinetic profile differences that could help guide drug development.

We characterized the MET inhibitors crizotinib, capmatinib, savolitinib, and tepotinib under washout conditions and investigated how pharmacologic differences affected cellular target inhibition and efficacy. We also extensively assessed the underlying mode of action, comparing physicochemical profiles, differences in on-target residence time using surface plasmon resonance, NanoBRET techniques, and lysosomal retention.

Materials and Methods

Capmatinib (part number: 104838) and crizotinib (part number: 100966) were obtained from ChemShuttle (Hayward, CA), savolitinib was purchased from Enamine (part number: 95D948; Kyiv, Ukraine), and tepotinib was synthesized in-house (2015). For all cell lines and experiments, cell passages did not exceed 25.

Cell Viability Assay and Washout Procedure

Non–small cell lung cancer cell line EBC-1 (with MET amplification) was obtained by Health Science Research Resources Bank (Japan Health Sciences Foundation, Osaka, Japan) and cultured in Minimum Essential Medium (MEM) Eagle media (Sigma-Aldrich, Germany) supplemented with 10% (v/v) FBS (part number: P30-1502, PAN-Biotech, Aidenbach, Germany) Medium (MEM) Eagle media (Sigma-Aldrich, Germany) supplemented was obtained by Health Science Research Resources Bank (Japan Health Sciences Research Resources Bank, Japan) without phenol red and without FBS. 10,000 cells per well in 100 μl medium and treated with tepotinib, capmatinib, crizotinib, and salvotinib using a Tecan D300 dispenser (Männedorf, Switzerland). All wells were normalized to 0.3% (v/v) DMSO. After a 1-hour incubation, cells were centrifuged at 400 g for 5 minutes, incubation media were removed by flicking the plate, and fresh media were added. The washing step was repeated three times with fresh cell culture media. Viability was measured with CellTiter-Glo 3D Cell Viability Assay (Promega, Madison, WI) after 72 hours. Before reading luminescence with an EnVision plate reader (PerkinElmer, Waltham, MA), all samples were transferred to black 384-well plates (part number: 781090, Greiner Bio-One).

Detection of Phosphorylated MET

EBC-1 cells were seeded at 20,000 cells per well, incubated and washed as described above. After 24-hour incubation, inhibition of MET phosphorylation was measured using the Lumit Immunoassay for Cellular Systems Kit (Promega) (Hwang et al., 2020). Rabbit anti–phosphorylated MET (pMET) (Y1234/Y1235) clone D26 XP (part number: 0777S, batch number: 9) and mouse anti-MET clone L141G3 primary antibodies (part number: 3148S, batch number: 9) from Cell Signaling Technology (Danvers, MA) were used in combination with anti-mouse SmBiT (part number: W104B, batch number: 438405) and anti-rabbit LgBit secondary antibodies (part number: W104B, batch number: 419317) from Promega. Samples were transferred to white Greiner Bio-One 384-well plates (part number: 781080) before reading luminescence. The Lumit immunoassay was multiplexed with viability measurement of glycy-leucyl-benzylamine (Henry et al., 2016; Baltschukat et al., 2019), which do not approach the activity measured by the Lumit system. The Lumit assay was used to measure the activities recovered from inhibited surfaces.

Surface Plasmon Resonance to Monitor Drug-Target Complexes and Residence Time

Dissociation Kinetics. The human MET kinase domain (amino acids 1048–1348, including a C-terminal hexahistidine-tag, part number: PR-0038, batch number: 84, Proteros Biotecuses, Munich, Germany) was immobilized by standard amine coupling to a CM5 chip with surface densities of 1300–1600 Rus on a Biacore 8K+ (part number: 29149603, Cytiva, Marlborough, MA). The reference surface was prepared in the same way, but the protein solution was simply replaced by running buffer (20 mM HEPES; 150 mM NaCl; 1 mM DTT; 2 mM MgCl₂; 0.1 mM EGTA; 0.05% Tween 20; 2% DMSO, pH 7.40). Protein and reference surfaces were thoroughly washed for 4 hours in running buffer. MET inhibitors diluted to 0.5 μM in running buffer were injected for 1 minute to achieve complete surface saturation of immobilized MET with the inhibitor. Dissociation of the MET-inhibitor complexes were then tracked for 16 hours at 25°C and plotted after subtraction of the reference surface signal.

Recovery of Surface Activities. The MET kinase domain [amino acids 1051–1349, including a C-terminal hexahistidine tag, part number: PR-0040 (batch 21), Proteros Biotecuses, Munich, Germany] was immobilized by standard amine coupling (Amine Coupling Kit, part number: BR-1000-50, GE Healthcare, United Kingdom) to a CM5 chip on a Biacore 4000 (Cytiva). Using the same running buffer and temperature as above, the maximum binding levels of MK-8033 (batch number: J233H-JUB004-324, Jubilant Chemsys, Noida, India) (Northrup et al., 2013), a chaser molecule with unique on- and off-kinetics (Quinn et al., 2018), on each MET surface were determined, and the surfaces were saturated with MET inhibitor. At timepoints as close as possible to 0.5, 1.5, 3, 6, 12, 24, 48, and 72 hours, the surfaces were probed for the recovery of free binding sites by injection of saturating concentrations of MK-8033 (2 μM). The long-term stability of noninhibited MET surfaces were followed on a parallel flow channel and used to correct the activities recovered from inhibited surfaces.

Dissociation rate constants (koff) of the binary complexes were calculated from recovery values (A) at the different timepoints (t) using

\[ k_{\text{off}} = -1 \times \ln \left( \frac{1 - A}{100} \right)/t, \text{and residence time } (\tau) \text{ was calculated by} \]

\[ \tau = 1/k_{\text{off}} \]  

NANO BRET Cellular Target Engagement Assay

HEK293T cells were obtained from DSMZ (Braunschweig, Germany) and cultured at 37°C/5% CO₂ in Dulbecco’s modified Eagle medium high glucose supplemented with 10% FBS (PAA Laboratories), 2 mM
L-glutamine, 1 mM sodium pyruvate, and 1x Non-Essential Amino Acids Solution (all Gibco, Life Technologies, Carlsbad, CA). All reagents for the NanoBRET assay and the C-terminal NanoLuc MET plasmid were obtained from Promega (Madison, WI), including transient transfected cell batch MET TE HEK293 (part number: CS1810E18, batch number: 433041) and the NanoBRET TE in-cell Kinase Kit (part number: N2501, batch number: 288446).

The NanoBRET tracer displacement assay was used to measure cellular target engagement. The bioluminescence resonance energy transfer signal occurs between the chemiluminescence signal of the product of the NanoLuc-tagged MET and the tracer molecule (NanoBRET Kinase Tracer K5) labeled with BODIPY fluorophore (Promega) in live cells transiently transfected by the Fugene method 24 hours prior to the assay. The tested MET inhibitor competes with tracer for MET-NanoLuc protein binding and reduces the bioluminescence resonance energy transfer signal in a concentration-dependent manner.

The NanoBRET assay was performed according to the manufacturer’s instructions with some adaptations: 120 nl serially diluted inhibitor (in DMSO, final concentration 30 μM to 1 nM respectively). DMSO control was dispensed to 384-well plates (Greiner Bio, PP, F-Bottom, 781201) by Labcyte Echo (Beckman Coulter, Brea, CA). Forty microliters of a suspension of MET-NanoLuc-transfected cells were added, reseeded at a density of 20,000 cells per ml after trypsination, and resuspended in Opti-MEM without phenol red (Life Technologies). The compounds and cells were incubated for 1 hour at 5% CO2 and 37°C. For the washout NanoBRET assay, cells were handled as described above. For HEK293T cells, centrifugation was adapted to 4 minutes at 300g. After washout, cells were resuspended in 40 μl Opti-MEM without phenol red, and 20 μl were transferred to 384-well plates (Greiner Bio-One, white, F-bottom, 781080). The washout cells were incubated for 4 hours at 5% CO2 and 37°C to allow equilibration.

For the NanoBRET assay, 750 nM NanoBRET Kinase Tracer K5 (in DMSO) was added to the cell suspension by a Tecan D300e dispenser, after a short mix, the plates were incubated for 2 hours at 5% CO2 and 37°C for the standard NanoBRET assay. The plates were equilibrated to room temperature for 30 minutes, and Nano-Glo Substrate and Extracellular NanoLuc Inhibitor were added according to the manufacturer’s instructions to measure the NanoBRET signal. After a 1-hour incubation in the dark at room temperature, luminescence was measured at 450 nm and fluorescence at 610 nm (acceptor) on a PerkinElmer EnVision multimode reader, and the NanoBRET ratio signal was calculated. Four-parameter curve fitting was performed with Genedata screenr (Genedata, Basel, Switzerland), with normalization based on DMSO control and background values in the absence of tracer.

Bioanalytical Quantification by Liquid Chromatography–Tandem Mass Spectrometry

For bioanalysis, ultrapure water was prepared in-house (Milli-Q IQ 7000 purification system). Acetonitrile (ACN) (LiChrosolv, hypergrade for LC-MS), formic acid (ACS reagent grade), DMSO for spectroscopy, ammonia solution 25% (LC-MS LiChropur), and ammonium formate (LiChropur) were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Ammonium bicarbonate was obtained from Fluka, Honeywell Specialty Chemicals Seelze GmbH, Germany. Internal standards for tepotinib (2H3-tepotinib) and savolitinib (structural analog) were synthesized in-house, and [13C2,2H3]-capmatinib, and [13C2,2H5]-crizotinib were purchased from Alschim (Ilkirch, France). For quantification of tepotinib, capmatinib, crizotinib, and savolitinib, EBC-1 cells were treated as described above (cell viability assay), with cell count and volumes adapted for 96-well plates (part number: 651281, Greiner Bio-One). The treatment concentrations were 0.01, 0.1, 1.0, and 5 μM for tepotinib and 1.0, 5.0, 7.5, and 10 μM for capmatinib, crizotinib, and savolitinib. The difference in the concentration ranges is based on different sensitivities in analyte measurement via liquid chromatography–tandem mass spectrometry (LC-MS/MS). After the treatment period, the incubation media were transferred to a fresh polypropylene polymerase chain reaction (PCR) plate and diluted 1:100 to 1:10 with MEM (supplemented with 10% FBS), respectively. Cell pellets were covered with 100 μl ACN and incubated for 1 hour at 300 rpm for lysis. Tepotinib samples were prepared for LC-MS/MS analysis (ACQUITY UPLC I-Class (Waters Corporation, Milford, MA) coupled to SCIEX 6500+ (SCIEX, Framingham, MA)) via protein precipitation. Capmatinib, crizotinib, and savolitinib samples were prepared via liquid-liquid extraction with methyl tert-butyl ether (MTBE, HiPerSolv CHROMANORM, VWR International, PA). The measured test item amounts from cell lysate were normalized to 10,000 cells to compensate variabilities in cell numbers. The experiment was performed in six replicates on 3 independent days for each compound.

Preparation of Calibration Standards. Calibration standards were prepared with Tegan D300e by dispensing DMSO-based 10 mM stock solutions to 100 μl MEM media with 10% FBS (composition described in the EBC-1 culture section). The final concentration range was 0.4–200 ng/ml for tepotinib, 5.5–200 ng/ml for crizotinib, and 0.8–20 ng/ml for capmatinib, and 5.0–200 ng/ml for savolitinib. Calibration standards for the quantitation of cell lysate samples were prepared by dispensing a 10 mM DMSO-based stock solution to 100 μl ACN with a Tegan D300e. Calibration curves were calculated for each compound via linear regression with 1/x2 weighting.

Sample Preparation for Tepotinib: Protein Precipitation. Supernatant samples from the incubation of EBC-1 cells with tepotinib were diluted with MEM (supplemented with 10% FBS) to a final dilution of 1:100, or 1:10 to match the calibration range. Washed cell pellets from the compound incubation step were lysed by adding 100 μl ACN. After incubation with organic solvent for 1 hour at 300 rpm and room temperature, the cell lysates were diluted with ACN in the same manner as supernatant samples. For tepotinib, 30 μl from each calibrator and sample were transferred to a polypropylene PCR plate with 90 μl internal standard solution (ACN with 4 ng/ml stable isotope-labeled tepotinib). The PCR plate was mixed for 10 minutes at 1200 rpm and subsequently centrifuged at 1700g for 10 minutes at 20°C. To adjust the organic solvent ratio in the final samples, 50 μl supernatants were transferred to 100 μl ammonium bicarbonate buffer (20 mM, pH 6.9). Afterward, 7.5 μl was injected into the LC-MS/MS system.

Sample Preparation for Capmatinib, Crizotinib, and Savolitinib: Liquid-Liquid Extraction. Capmatinib, crizotinib, and savolitinib samples were purified by liquid-liquid extraction due to high matrix effects from MEM. A volume of 50 μl standard solution, diluted supernatant, or cell lysate sample was transferred to a 1 ml deep-well plate. For pH adjustment, 50 μl buffer (ammonium bicarbonate buffer 20 mM (pH 6.9) for capmatinib and crizotinib and ultrapure water with 0.025% ammonia (pH 12.0) for savolitinib) were added. For extraction, 600 μl MTBE with internal standard (stable isotope-labeled forms with 2.5 ng/ml for capmatinib, 0.25 ng/ml for crizotinib, and 0.5 ng/ml of a structural analog as internal standard for savolitinib) were added to each well. Afterward, the plate was incubated for 10 minutes at 800 rpm and room temperature. Then, the plate was centrifuged at 4700g for 10 minutes at 15°C. In the final step, 200 μl supernatant was transferred to a 300-μl PCR plate. The solvent phase was evaporated by TurboVap LV (Zymark, Hopkinton, MA) for 30 minutes at 40°C. Finally, the dried extract was reconstituted with 100 μl Eluent A (ultrapure water with 0.025% ammonia) and ACN [80:20 (v/v)], and 7.5 μl aliquots were injected into the LC-MS/MS system.

LC-MS/MS Conditions. For the separation of tepotinib, capmatinib, and crizotinib, a Waters ACQUITY UPLC CSH C18 (130 Å, 1.7 μm particle size, 2.1 × 50 mm) column was used. A gradient method at 40°C was applied with a flow of 0.75 ml/min using two eluents: Eluent A (ultrapure water, 0.1% formic acid, and 10 mM ammonium formate) and Eluent B (acetonitrile). The initial eluent composition of the gradient was 80% Eluent A and 20% Eluent B (0–1.5 minutes), followed by a linear gradient to 60% Eluent B (1.5–2.8 minutes). Eluent B was then increased to 95% (2.8–
2.9 minutes), followed by a plateau until 3.0 minutes. From minute 3.0 to 5.0, starting conditions were applied (80% Eluent A and 20% Eluent B).

The separation of savolitinib was carried out on a Waters ACQUITY UPLC BEH C18 column (130 Å, 1.7 μM particle size, 2.1 × 50 mm). A gradient was applied with Eluent A [ultrapure water with 0.025% ammonia (v) and a pH value of 12.0] and Eluent B (acetonitrile) with a flow of 0.6 ml/min. The initial Eluent composition was 90% Eluent A and 10% B. Eluent B increased to 50% (0–2.0 minutes) and to 90% (2.0–2.2 minutes). From minute 2.2, initial conditions were applied (2.2–3.0 minutes).

Finally, all analytes and internal standard compounds were detected by multiple-reaction monitoring using ionization in positive mode with the following parameters: curtain gas: 35 psi, ion spray voltage 3500 V, temperature 500 °C; ion source gas 1: 30 psi; ion source gas 2: 70 psi. Compound concentrations were determined with Analyst 1.6.3 (AB SCIEX) by calculating the peak area ratio of analyte and internal standard.

**LysoTracker Displacement Assay and Immunofluorescence**

A549 cells (MET wild type) were purchased from ATCC (Manassas, VA) and grown in Dulbecco’s modified Eagle medium with 10% (v/v) FBS in 10% CO₂ at 37 °C. For all assays, 50 ng/mL Gibco (Life Technologies, Carlsbad, CA) penicillin/streptomycin was added. A549 tumor cells were seeded after using a cell strainer with 7500 cells per well in 100 μl medium in clear-bottom, black 96-well plates (part number: 655090, Greiner Bio-One) and incubated overnight at 10% CO₂ and 37 °C in medium without phenol red. Treatment with compounds dissolved in DMSO was conducted using a Tecan D300 dispenser. Chloroquine diphosphate salt (part number: C6628, Sigma Aldrich) 50 μM was used as a control for 0% lysosomal staining, and 0.5% DMSO vehicle for 100% lysosomal staining. Thirty minutes after compound treatment, 25 nM LysoTracker Red DND-99 (Life Technologies Europe, Netherlands) was added manually (10 μl pre diluted). After shaking for 5 seconds, the plates were incubated for another 30 minutes at 37°C and 10% CO₂ then washed once with PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. After two washing steps with PBS, Hoechst (Molecular Probes, 1:10,000) was added. Imaging was performed using an ImageXpress Micro Confocal system (Molecular Devices, San Jose, CA).

For washout conditions, 10,000 A549 tumor cells per well were seeded in 100 μl medium in 96-well polypropylene v-bottom plates (part number: 651261, Greiner Bio-One) and treated and washed as described above. Afterward, cells were resuspended in fresh medium and transferred to a black 96-well plate with a clear bottom. In parallel, cells from control plates (no washing steps in the polypropylene plate) were transferred to black 96-well plates with a clear bottom. Control and washout plates were incubated at 37°C and 10% CO₂ for 3 hours. After incubation, chloroquine was added, with LysoTracker Red added 30 minutes later, and plates were processed as described above for the standard conditions.

For lysosomal-associated membrane protein 1 (LAMP1) staining, fixed cells were permeabilized with 0.1% Triton X-100 for 10 minutes and washed once with PBS and blocked with 1% goat serum (part number: B15-035, PAA Laboratories), 0.1% BSA (part number: K11-013, PAA Laboratories), and 0.1% sodium azide (the Healthcare Business of Merck KGaA, Darmstadt, Germany) for 1 hour. LAMP1 primary antibody (part number: 9091, D2D11 XP Rabbit mAb, Cell Signaling Technology) was diluted 1:100 in PBS containing 0.1% BSA and incubated overnight at 4°C. After washing three times the next day, the secondary goat anti-rabbit Alexa Fluor 488 antibody (part number: A-11070, batch number: 2018208, Thermo Fisher Scientific, IL) was diluted 1:2000 in PBS containing 0.1% BSA and incubated for 75 minutes. Cells were washed three times, and wells were filled up to 100 μl; PBS and 0.05% sodium azide were added for long-term storage. Imaging was performed using a 20x objective and the ImageXpressULTRA High Throughput Confocal Imaging System and the associated software MetaXpress, version 6.6.1.42 (both from Molecular Devices). Images from LysoTracker and LAMP1 staining were overlayed with Fiji 1.53c (Schindelin et al., 2012).

**Statistical Analysis**

All experimental results are expressed as mean ± S.D. or mean ± S.E.M. of three or more independent experiments; details are specified in the figure legends. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, CA). The statistical significance of differences between controls and washout conditions for IC₉₀ values and target engagement was determined using multiple unpaired Student t tests. We corrected for multiple comparisons using the Holm-Sidak method and considered a P value of less than 0.05 to be significant.

**Results**

**Cell Viability and pMET Persistence after Washout.** After a 14-hour washout, tepotinib displayed persistent pMET inhibitory effects in A549 cells stimulated with HGF (Schadt et al., 2010; Bladt et al., 2013). In the present study, the human lung cancer cell line EBC-1 was used to evaluate cell viability and pMET status and to assess tepotinib’s cell persistence in addition to that of the MET inhibitors capmatinib, savolitinib, and crizotinib. EBC-1 was selected as a model cell line because EBC-1 cells harbor MET amplification, express high levels of MET, and have high basal MET phosphorylation, and their viability is sensitive to MET inhibition (Lutterbach et al., 2007). Additionally, EBC-1 cells allowed a robust assay readout in washout settings and polypropylene plates, which was not the case for other tested cell lines (data not shown). Figure 1, A and B illustrates the potency shifts for tepotinib and capmatinib after washout; dose-response curves for crizotinib and savolitinib are available in Supplemental Fig. 1, A and B. Figure 1C summarizes cell viability (IC₉₀) after treatment and IC₉₀ values after washout. Tepotinib, capmatinib, and savolitinib showed similar potencies in the viability assay in steady-state conditions (IC₉₀ 1–2 nM), and crizotinib showed a lower potency (11 nM), consistent with previously reported data (Baltschukat et al., 2019). Applying washout conditions, more precisely 1-hour compound treatment followed by three washing steps and after 72-hour viability assessment, mean IC₉₀ values increased for all inhibitors but differed in their extent. The IC₉₀ value of tepotinib and crizotinib shifted 14- and 10-fold, respectively, whereas the IC₉₀ for capmatinib and savolitinib shifted by factors of ≥300. We also investigated the more direct effect of these four compounds on MET phosphorylation (Y1234/1235; Fig. 1, D–F and Supplemental Fig. 1, C and D), which paralleled the effect observed for viability. The pMET IC₉₀ values for tepotinib and crizotinib shifted by a factor of 5, and shifts observed for capmatinib and savolitinib were again more extreme, with factors >150. To ensure that the washout effects were specific to the compound and cell interaction, we excluded plastic binding of the inhibitors (Palmgren et al., 2006) by using polypropylene plates and cell-free controls (Supplemental Fig. 2).

**Effect of Drug-Target Complex Duration on the Differences Between the MET Inhibitors.** Tepotinib has a long residence time of >1000 minutes (Willemse-Seegers et al., 2017), which could contribute to persistent effects in a washout setup. We analyzed residence times and kₑff for all
four MET inhibitors (Fig. 2A); crizotinib had the fastest $k_{off}$, and tepotinib had the slowest. Figure 2B shows best estimates of residence times from $k_{off}$ values averaged over all timepoints with inhibitor occupancies higher than 10%; the residence time of tepotinib was estimated at 20 hours, followed by capmatinib (14 hours), savolitinib (10 hours), and crizotinib (4 hours).
Target engagement kinetics were analyzed in a cellular system using a MET NanoBRET assay (Fig. 2C) (Robers et al., 2015). In nonwashout conditions, target engagement potencies ranged from 9–28 nM, with crizotinib less potent than the other inhibitors. We tried to recapitulate the washout conditions of the EBC-1 viability and pMET assays but had to adjust the protocol to HEK293T cells, a standard model cell line for mechanistic NanoBRET assays. Keeping the compound incubation time constant at 1 hour and applying three washing steps in polypropylene plates, cells had to be transferred to cell culture plates, and different incubation times were addressed. After 2 hours, we saw initial splits in potencies, which increased at 4 hours (Supplemental Fig. 3). Extended incubation did not further increase potency shifts, but this could be due to technical limitations of the assay setup at later timepoints (data not shown). Figure 2C illustrates the potency shift for tepotinib and shift factors for all MET inhibitors 4 hours after washout. Additional replicates can be found in Supplemental Fig. 4. Despite having the shortest residence time, the mean IC50 value for crizotinib shifted by a factor of 16, which is comparable to the shift of tepotinib (factor 19, longest residence time). The difference in ranking order in the persistence of effects in the cellular NanoBRET assay after washout and protein-based surface plasmon resonance assessment indicates that parameters other than on-target residence time contribute to the persistent cellular effects of tepotinib and crizotinib described above (Fig. 1).

**Sustained Effects after Washout and Intracellular Compound Concentrations.** Intracellular concentrations of MET inhibitors from EBC-1 cell lysates and corresponding extracellular compound concentrations in the incubation medium (after 1-hour treatment, before washout) were investigated as additional parameters influencing their sustained cellular effects. Figure 3A shows the measured compound concentration after cell lysis with 100 μl acetonitrile normalized to 10,000 cells per well. Figure 3B shows the detailed compound recovery from cell lysates and incubation medium for the 5 μM treatment. The partition coefficient reflects the ratio between the intracellular drug amount and the remaining compound in the incubation medium after treatment. It was calculated by building the arithmetic mean of the partition coefficient from all incubation concentrations for each MET inhibitor. A partition coefficient of 1.75 ± 0.61 was determined for crizotinib, 0.68 ± 0.39 for tepotinib, 0.003 ± 0.002 for capmatinib, and 0.001 ± 0.0007 for savolitinib. At 5 μM, tepotinib and crizotinib showed >50% intracellular compound concentrations, indicating a cellular accumulation of the compounds, which could be cell organelle or membrane-binding associated.

**MET Inhibitor Differences in Physicochemical Profile.** Based on initial structural considerations, we hypothesized that physicochemical differences might explain differences in intracellular compound concentrations. To further substantiate this working hypothesis, the major physicochemical compound descriptors were calculated (hydrogen bond donators/acceptors, topological polar surface area) or measured [lipophilicity (logP), pKa1, pKa2] (Table 1). The physicochemical profiles of all tested MET inhibitors were compliant with the “rule of 5” criteria (Benet et al., 2016), characterizing pharmaceutically relevant physicochemical property ranges. Despite the molecular weight of the MET inhibitors covering a considerable range (345–493 g/mol) and excepting the slightly increased number of hydrogen bond donators of crizotinib, the numbers of hydrogen bond acceptors and donators, as well as the calculated topological polar surface area, were comparable. However, logP and the acid dissociation constant (pKa1) separate the four compounds into two categories: lipophilic weak bases (tepotinib and crizotinib) and neutral molecules (capmatinib and savolitinib).
Translation of Tepotinib and Crizotinib Physicochemical Profiles into Lysosomal Compound Reservoirs. Lipophilic weak bases are known to accumulate in acidic cell compartments such as lysosomes, so we assessed lysosomal retention using two approaches: effects of cotreatments with chloroquine and an indirect fluorescence-based assay (Schmitt et al., 2019). Cotreatment with a lysosomotropic agent (e.g., chloroquine) should compete with lipophilic weak bases (tepotinib and crizotinib) for storage capacities of acidic cell compartments and, therefore, reduce the cellular compound concentration and activity in washout settings. We applied selected MET inhibitor concentrations within their dose-response range and combined this treatment with either DMSO control or chloroquine at 10 or 50 μM. For tepotinib, Fig. 4A shows reduced inhibition of MET phosphorylation with increasing concentrations of chloroquine cotreatments, indicating a competition of tepotinib and chloroquine for acidic compound reservoirs. We observed similar effects for crizotinib, but capmatinib and savolitinib activities were not affected by cotreatments with chloroquine (Supplemental Fig. 5). A similar competition of lysosomal storage capacities should occur between compounds and the fluorescence probe LysoTracker. Lysosomal retention is expected to result in dye displacement and reduction of the fluorescence signal. We selected A549 for quantification analysis based on assay robustness, but similar effects were detected in the EBC-1 cell line (Supplemental Fig. 6A). Additionally, LAMP1 lysosome immunofluorescence staining did not detect differences between DMSO controls and tepotinib-treated samples (Supplemental Fig. 6B), and colocalization of LAMP1 and LysoTracker (DMSO control) confirmed the staining of the desired subcellular compartment. Figure 4B shows reduced LysoTracker fluorescence with tepotinib, detected by confocal imaging. In Fig. 4C, this effect is quantified using image analysis. Chloroquine was used as a reference compound, and the IC_{50} value determined by Schmitt et al. (2019) using rat hepatocytes matches our IC_{50} value of 2.0 μM in A549 cells (Supplemental Fig. 6C). Tepotinib and crizotinib displaced LysoTracker lysosome staining dye dependently, with IC_{50} values of 1.8 μM and 0.4 μM, respectively. Capmatinib and savolitinib did not affect the fluorescent probe signals. Lastly, we investigated the reversibility of the lysosomal retention of tepotinib. Tepotinib was incubated for 1 hour, and cells were washed and then transferred from polypropylene plates to imaging-compatible plates and left for 4 hours to attach. At 12.5 μM tepotinib (Fig. 4D), the LysoTracker signal was displaced completely in nonwashed references, whereas in washout conditions, a LysoTracker fluorescence signal was visible, indicative of a decrease in tepotinib concentration in the lysosomal compartment. The change in the displacement of the LysoTracker dye after washout indicates reversibility of tepotinib lysosomal retention, rendering the lysosome a compound reservoir, not a trap.

Discussion

Prominent differences existed between the MET inhibitors in washout experiments, altering the potency ranking of the tested compounds. We observed a link between the compounds’ physicochemical properties (high pKa and logP) and persistence in washout conditions. We suggest that compound distribution within cells is highly dynamic and influenced by multiple factors (Fig. 5). Besides lysosomal retention and residence time, transport across cell membranes and the amount of unbound intracellular compound could contribute to persistency.

Duration of the Drug-Target Complex and its Effect on Target Inhibition in Open Systems. Drug-target complex stability is determined by its dissociation rate constant. Long residence time and a rapid rebinding driven by the high proximal drug concentration would extend the overall duration of target occupancy (Copeland, 2016). The recovery rate of MET activity after washout has been shown to increase with decreasing MET receptor residence time for a series of MET tyrosine kinase inhibitors (Farrell et al., 2017). In the present study, Fig. 5A illustrates the impact of a stable drug-target complex on overall cellular compound distribution; a slow k_{off} rate enables persistent target inhibition despite extracellular compound depletion by metabolism or excretion in vivo (or washing steps in vitro). Our analysis of MET inhibitor/target residence times by surface plasmon resonance (Fig. 2, A and B) allowed us to rank dissociation rates of the four MET inhibitors, starting with the slowest k_{off}: tepotinib, capmatinib, savolitinib, and crizotinib. This ranking was not
| Compound     | Structure | MW [g/mol] | HAc [N] | HDon [N] | TPSA [Å²] | logP  | pKa1  | pKa2  |
|--------------|-----------|------------|---------|----------|-----------|-------|-------|-------|
| Tepotinib    | ![Tepotinib Structure](image1.png) | 492.58     | 8       | 0        | 95        | 3.99  | 9.25  | —     |
| Crizotinib   | ![Crizotinib Structure](image2.png) | 450.34     | 6       | 3        | 78        | 3.87  | 9.21  | 5.29  |
| Capmatinib   | ![Capmatinib Structure](image3.png) | 412.43     | 7       | 1        | 85        | 2.36  | 4.57  | 2.18  |
| Savolitinib  | ![Savolitinib Structure](image4.png) | 345.37     | 9       | 0        | 92        | 1.28  | 5.80  | —     |

HAc, hydrogen bond acceptors; HDon, hydrogen bond donators; MW, molecular weight; TPSA, topological polar surface area.
reflected in the potency shifts in NanoBRET washout data nor pMET or cell viability, where potency shifts (lowest first) were ranked: tepotinib/crizotinib, capmatinib, and savolitinib. Our explanation for the discrepancy between protein and cellular target engagement is that additional cellular effects, such as lysosomal retention, mask the effect of residence time. Additional (later) timepoints for the NanoBRET assay would strengthen our hypothesis but were not feasible using the assay setup and are subject to future investigations. Looking at the two nonlysosomotropic agents, savolitinib and capmatinib, the potency shift ranking matches the residence time ranking, and the better washout performance of capmatinib could be explained by its longer residence time. More molecules with different residence times and similar physico-chemical properties could be analyzed further.

**Contribution of Lysosomal Retention to Sustained Target Inhibition.** Intracellular acidic/lipophilic cell compartments, including lysosomes, are known to be accessible by certain compounds (Miao et al., 2013). The mechanism of lysosomal retention of compounds has been studied since it was described by de Duve et al. (1974). Lysosomes in normal cells have a significantly lower pH (approximately 4 to 5) than the surrounding cytosol, and this acidic subcellular compartment enables lysosomotropic behavior, which is well described for lipophilic weak bases. Intralysosomal concentration/capacity depends on the pKa of the compound and the permeability coefficient z of its neutral and protonated forms (Duvvuri et al., 2004, 2005). The bidirectional passive permeation of the lysosomal membrane also depends on the compound's logP. Furthermore, lysosomal retention depends on secondary factors, including compound size, intramolecular dipole, active transport, and other mechanisms affecting general biodistribution. Focusing on the measured pKa and lipophilicity descriptors of the four MET inhibitors, the compounds were separated into lipophilic weak bases (tepotinib and crizotinib) and neutral molecules (capmatinib and savolitinib). We have shown that tepotinib and crizotinib are lysosomotropic and capmatinib and savolitinib are nonlysosomotropic agents. Crizotinib's lysosomotropicism has been described earlier (Honeywell et al., 2018; Van Der Steen et al., 2020).

Such a lysosomal compound reservoir could contribute to persistent effects of a compound. To our knowledge, there is only one washout study published, that shows that the CDK4/6 inhibitor palbociclib is reversibly stored in lysosomes, linking the lysosomal compound reservoir to long-term activity of the inhibitor (Llanos et al., 2019). We started to show the reversibility of lysosomal retention experimentally by reduced LysoTracker displacement after washout (Fig. 4D). Two limitations to our dataset could be addressed by expanding cell lines and compounds to confirm the benefits of lysosomal retention and by expanding kinetic analyses to confirm the reversibility of lysosomal retention.

As depicted in Fig. 5, kinetics are important in the reversibility of lysosomal retention; as the noncharged molecule is membrane permeable, the equilibrium will be restored after extracellular compound removal by washing steps. Cytosolic compound could cross the cell membrane, noncharged compound could cross the lysosomal membrane, and within the lysosome, deprotonation of compounds will restore the equilibrium. In summary, the degree of lysosomal retention, membrane transport, and resulting kinetics depend on individual compound physicochemical characteristics, and our data indicate benefits for the sustained target inhibition of MET by lysosomotropic agents such as crizotinib and tepotinib.

Lysosomal trapping, or sequestration, is associated with a separate set of effects and implications, including reduced effective cellular concentrations or resistance mechanisms (Gong et al., 2003; Duvvuri et al., 2006; Ndolo et al., 2010; de Klerk et al., 2018; Englinger et al., 2018; Halaby, 2019). However, most of these aspects have been studied in steady-state experimental settings, where lysosomal retention generally reduces cytoplasmic concentration and prevents pharmacologic activity at the target site. This change in a compound's unbound intracellular concentration contributes to asymmetry between the extracellular and intracellular drug concentration: the “potency drop-off” (Hann and Simpson, 2014; Trunkle et al., 2020). Lysosomal retention may also be associated with negative phenomena such as phospholipidosis (Lowe et al., 2012; Muehlbacher et al., 2012). However, in an open system such as an organism, a large cellular drug depot near the pharmacologic target could be an advantage, assuming an intact equilibrium between compartments. Once the cytosolic drug concentration decreases, a back diffusion from the lysosomal compartment into the cytosol restores compound availability (Kazmi et al., 2013). These reservoirs could maintain high levels of target engagement in dynamic systems to support $C_{min}$-driven pharmacology.

Our findings indicate that physicochemical differences are closely linked to intracellular accumulation of MET inhibitors during washout. Basic compounds often show a high volume of distribution (Peters, 2011) and high partition into tissue. The tissue distribution also depends on the tissue type and its relative content of lysosomes (Schmitt et al., 2021). Johne et al. (2020) demonstrated in a mass balance study that tepotinib, a lipophilic weak base, distributes preferably into tissue and was found in higher concentrations in xenograft tumors compared with plasma concentration (Bladt et al., 2013). In the context of cancer, tumor cells tend to have a higher intracellular pH value compared with normal differentiating cells, but there is increased acidification in the tumor cell environment (White et al., 2017), and lipophilic weak bases distribute preferably to the reduced pH value of the tumor tissue. The reversed pH gradient in tumor tissues is extensively described in the literature (Webb et al., 2011). Metabolism could additionally affect biodistribution in multiple ways. We considered efflux pumps such as P-glycoprotein (P-gp;ABCB1). However, P-gp should have minimal or no effect on our results because all four MET inhibitors are substrates of P-gp (Cortot et al., 2022). Additionally, the experiments in this study are based on the lung cancer cell lines EBC-1 and A549 with low P-gp expression [Cancer Dependency Map Portal (RRID:SCR_017655)]. Therefore, the contribution of lysosomes to sustained target engagement is a reasonable assumption. Further studies are needed to investigate if the lysosomotropic compounds tepotinib or crizotinib induce lysosomal swelling, lysosomal leakage, and, potentially, a lysosome-mediated cell death and how our observations translate to in vivo and/or clinical settings.

**Importance of Sustained Effects to Induce the Desired Phenotype by MET Inhibition.** As lysosomal retention is compound- and context-dependent, and consequently able to modulate desired and undesired pharmacologic effects, these correlations offer the opportunity to optimize pharmacokinetic and pharmacodynamic characteristics. Depending on the target,
persistent inhibition may be critical to induce a phenotypic effect. The MET oncogene is such a target. An in vitro study with the MET inhibitor JNJ-605 describes a rebound effect with increased MET phosphorylation 15 minutes after washout. Despite being transient, this rebound was enough to resume tumor growth, confirming that short-lived pMET inhibition is ineffective (Pupo et al., 2016). Second, data for different doses and durations of crizotinib treatment in GTL16 and U87-MG mouse models suggest that near-complete inhibition of MET phosphorylation (>90% inhibition) for the duration of administration is necessary to maximize therapeutic benefit (Zou et al., 2007), and customized regimens of different inhibitors (including tepotinib) may be required to achieve this goal (Srivastava et al., 2018). Third, pharmacokinetic-pharmacodynamic modeling for tepotinib and savolitinib have been based around the need for prolonged, high levels of MET phosphorylation for clinical activity and identified once- and twice-daily dosing, respectively, as optimal (Jones et al., 2021; Xiong et al., 2021). In the present

**Fig. 4.** Physicochemical profiles of tepotinib and crizotinib translate into lysosomal compound reservoirs. EBC-1 cells were cotreated with tepotinib and chloroquine for 1 hour, before media were replaced three times to remove the compound from the supernatant, and MET phosphorylation was measured after 24 hours. (A) Activity of cotreatments compared with tepotinib monotreatment. Mean ± S.E.M.; N = 3; two-way ANOVA; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. LysoTracker staining of A549 cells (green) and nuclear counterstain using DAPI (blue). Imaging was performed using the high-content imager Micro Confocal (Molecular Devices) and a 20x objective. Scale bars, 50 μM. (B) A549 cells treated for 1 hour with 0.1 μM and 10 μM tepotinib and 0.5% DMSO (control). (C) Images quantified counting vesicles using MetaXpress software. Two-point normalization using 0.5% DMSO (100%) and 50 μM chloroquine (0%). (D) Treatment with 12.5 μM tepotinib control and washout. DAPI: 4',6-diamidino-2-phenylindole; ns, not significant.
study, we confirm that persistent target inhibition results in a stronger phenotypic viability effect as our washout effects on pMET and viability correlate well (Fig. 1). We also show that persistent effects in our in vitro setting depend on the molecular physicochemical properties and could be engineered to favor lysosomal compound reservoirs.

In summary, in vitro washout conditions changed the ranking of four MET inhibitors, tepotinib, capmatinib, savolitinib, and crizotinib, regarding cellular potency. A lysosomal compound reservoir was shown to be beneficial. Further studies are needed to understand if additional factors affect cellular activity in open systems and/or to address translation to in vivo settings.

Any requests for data by qualified scientific and medical researchers for legitimate research purposes will be subject to the Healthcare Business of Merck KGaA, Darmstadt, Germany, data sharing policy. All requests should be submitted in writing to the healthcare business of Merck KGaA, Darmstadt, Germany, data sharing portal (https://www.emdgroup.com/en/research/our-approach-to-research-and-development/healthcare/clinical-trials/commitment-responsible-data-sharing.html).

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Authorship Contributions

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Supplementary Information

Sustained cellular effects of MET inhibitors like tepotinib are driven by prolonged target engagement and lysosomal retention

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Supplementary Figure S1

EBC-1 cells were treated with selected MET inhibitors for 1 hour, before media were replaced three times to remove the compound from the supernatant, and 72-hour viability was measured. Dose–response curve for crizotinib (A) and savolitinib (B) show activity after washout compared to non-washed control treatment. MET phosphorylation 24 hours after washout for crizotinib (C) and savolitinib (D) was measured with 0.5 µM tepotinib as inhibitor control and DMSO as vehicle. DMSO: dimethyl sulfoxide; MET: mesenchymal-epithelial transition factor; NSCLC: non-small cell lung cancer; wt: wild type.
EBC-1 cells were treated with selected MET inhibitors for 1 hour, before media were replaced three times to remove the compound from the supernatant, and 72-hour viability was measured. For the unspecific binding control ("plastic binding control"), plates without cells were processed like washout samples. Cells were added after the washing steps to check the effect of residual compound in a plate. MET: mesenchymal-epithelial transition factor; NSCLC: non-small cell lung cancer; wt: wild type.
Cellular target engagement of MET was determined by NanoBRET assay. Cells were treated for 1 hour with the respective MET inhibitors. For washout samples, IC$_{50}$ determination was performed after 2 and 4 hours of incubation following washout: media were replaced three times to remove the compound from the supernatant.
DMSO and tracer were used as 100% occupancy control. Shift factors were calculated using the ratio of washout and control IC$_{50}$ values. DMSO: dimethyl sulfoxide; MET: mesenchymal-epithelial transition factor.
Cellular target engagement of MET was determined by NanoBRET assay. Cells were treated for 1 hour with the respective MET inhibitors. For washout samples, IC\textsubscript{50} determination was performed after 4 hours of incubation following washout: media were replaced three times to remove the compound from the supernatant. DMSO and tracer were used as 100% occupancy control. Mean IC\textsubscript{50} and standard deviation are based on 3–6 independent experiments and analyzed by multiple unpaired Student t-tests. **P < 0.01. DMSO: dimethyl sulfoxide; MET: mesenchymal-epithelial transition factor.
EBC-1 cells were co-treated with selected MET inhibitors and chloroquine for 1 hour, before media were replaced three times to remove the compound from the supernatant, and MET phosphorylation was measured after 24 hours. pMET levels after co-treatments compared to mono-treatments for crizotinib (A), capmatinib (B), and savolitinib (C). Mean ± SEM; N = 3; two-way ANOVA; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ANOVA: analysis of variance; pMET: phosphorylated mesenchymal-epithelial transition factor; SEM: standard error of the mean.
Supplementary Figure S6

LysoTracker staining (green) and nuclear counterstain using DAPI (blue). Imaging was performed using the high content imager ImageXpressULTRA (Molecular Devices) and a 20x objective. Scale bars indicate 50 µm. (A) EBC-1 cells treated for 1 hour with 10 µM tepotinib and 0.5% DMSO (control). (B) A549 cells treated for 1 hour with 10 µM tepotinib and 0.5% DMSO (control) and immunofluorescent co-staining of lysosomes using LAMP1 (red). (C) A549 cells treated for 1 hour with 50 µM chloroquine and images quantified counting vesicles using MetaXpress software (version 6.6.1.42). Two-point normalization using 0.5% DMSO (100%) and 50 µM chloroquine (0%). DAPI: 4’,6-diamidino-2-phenylindole; DMSO: dimethyl sulfoxide; LAMP1: lysosomal-associated membrane protein 1.