Protein kinase inhibitor ceritinib blocks ectonucleotidase CD39 – a promising target for cancer immunotherapy

Laura Schäkel, Salahuddin Mirza, Riekje Winzer, Vittoria Lopez, Riham Idris, Haneen Al-Hroub, Julie Pelletier, Jean Sévigny, Eva Tolosa, Christa E Müller

ABSTRACT

Background An important mechanism, by which cancer cells achieve immune escape, is the release of extracellular adenosine into their microenvironment. Adenosine activates adenosine A2A and A2B receptors on immune cells constituting one of the strongest immunosuppressive mediators. In addition, extracellular adenosine promotes angiogenesis, tumor cell proliferation, and metastasis. Cancer cells upregulate ectonucleotidases, most importantly CD39 and CD73, which catalyze the hydrolysis of extracellular ATP to AMP (CD39) and further to adenosine (CD73). Inhibition of CD39 is thus expected to be an effective strategy for the (immuno)therapy of cancer. However, suitable small molecule inhibitors for CD39 are not available. Our aim was to identify drug-like CD39 inhibitors and evaluate them in vitro.

Methods We pursued a repurposing approach by screening a self-compiled collection of approved, mostly ATP-competitive protein kinase inhibitors, on human CD39. The best hit compound was further characterized and evaluated in various orthogonal assays and enzyme preparations, and on human immune and cancer cells.

Results The tyrosine kinase inhibitor ceritinib, a potent anticancer drug used for the treatment of anaplastic lymphoma kinase (ALK)-positive metastatic non-small cell lung cancer, was found to strongly inhibit CD39 showing selectivity versus other ectonucleotidases. The drug displays a non-competitive, allosteric mechanism of CD39 inhibition exhibiting potency in the low micromolar range, which is independent of substrate (ATP) concentration. We could show that ceritinib inhibits ATP dephosphorylation in peripheral blood mononuclear cells in a dose-dependent manner, resulting in a significant increase in ATP concentrations and preventing adenosine formation from ATP. Importantly, ceritinib (1–10 µM) substantially inhibited ATP hydrolysis in triple negative breast cancer and melanoma cells with high native expression of CD39.

Conclusions CD39 inhibition might contribute to the effects of the powerful anticancer drug ceritinib. Ceritinib is a novel CD39 inhibitor with high metabolic stability and optimized physicochemical properties; according to our knowledge, it is the first brain-permeant CD39 inhibitor. Our discovery will provide the basis (i) to develop more potent and balanced dual CD39/ALK inhibitors, and (ii) to optimize the ceritinib scaffold towards interaction with CD39 to obtain potent and selective drug-like CD39 inhibitors for future in vivo studies.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Ceritinib is an inhibitor of anaplastic lymphoma kinase and an approved anticancer drug.

WHAT THIS STUDY ADDS

⇒ Herein, we demonstrate that ceritinib additionally acts as a non-competitive inhibitor of the ATP-hydrolyzing ectonucleotidase CD39 showing micromolar potency. It therefore results in decreased extracellular concentrations of immunosuppressive, cancer-promoting adenosine.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ In contrast to other investigated kinase inhibitors, ceritinib may have additional immunotherapeutic effects. Moreover, the ceritinib scaffold could be further optimized to obtain more potent CD39 inhibitors for the immunotherapy of cancers.

INTRODUCTION

An important mechanism, by which cancer cells achieve immune escape, is the hypoxia-induced upregulation of ectonucleotidases by hypoxia-inducible factor 1α (HIF-1α) and specific protein 1 (SP-1), resulting in high concentrations of extracellular adenosine in their microenvironment (see figure 1). Adenosine, by activating A2A and A2B receptors on immune cells, constitutes one of the strongest immunosuppressive mediators. The hypoxia–A2A–adenosinergic pathway was demonstrated to impede the recruitment and activity of antitumor T cells in the tumor microenvironment. In previous studies, this immunosuppressive hypoxia–A2A–adenosinergic signaling was successfully blocked by a specific adenosine A2A receptor antagonist, providing the proof of concept that the pharmaceutical reactivation of tumor-reactive T and natural killer cells is feasible. Additional extracellular adenosine promotes cancer cell proliferation, angiogenesis, and metastasis.
In this context, ectonucleotidases have been proposed as novel targets in immunoncology. These membrane proteins catalyze the extracellular dephosphorylation of nucleotides, such as ATP, ADP and AMP, producing adenosine (figure 2). They thereby reduce extracellular concentrations of pro-inflammatory, antiproliferative ATP, leading to an increase in immunosuppressive, cancer-promoting adenosine. This enzymatic cascade constitutes an endogenous feedback loop protecting from ischemia and excessive inflammation, but it can be hijacked by tumors, bacteria, and possibly also by viruses.

Figure 1 Hypoxia-induced purinergic signaling. (A) Tissue hypoxia increases the release of transcription factor SP-1 and hypoxia-inducible factor 1α (HIF-1α), which leads to an increase in the expression and enzymatic capacity of adenosine-producing ectonucleotidases and to an increased expression of adenosine A_{2a} receptors (A_{2a}R), resulting in reduced inflammation. (B) Effects of extracellular adenine nucleotides (ATP, danger-associated molecular pattern molecule) and adenosine via purinergic P2 and adenosine A_{2a} and A_{2b} receptors. ATP is dephosphorylated by a concerted action of ectonucleoside triphosphate diphosphohydrolase 1 (NTPDase1, CD39) and ecto-5'-nucleotidase (CD73) to adenosine. SP-1, specific protein 1; CD, cluster of differentiation.

Figure 2 Generation of adenosine through extracellular hydrolysis of adenine nucleotides by ectonucleotidases. Nucleoside triphosphate diphosphohydrolase-1 (NTPDase1, CD39) and nucleotide pyrophosphatase/phosphodiesterase-1 (CD203a, NPP1, PC-1) as well as their isoenzymes produce AMP, which is hydrolyzed by ecto-5'-nucleotidase (CD73) to yield adenosine. In the course of the reactions, inorganic phosphate (P), or diphosphate (pyrophosphate, PP), is released. As an alternative source for AMP, NAD\(^+\) is converted by the enzyme CD38 to nicotinamide (NAM) and ADP-ribose (ADPR). ADPR can be converted to AMP and ribose-1-phosphate (ribose-1-P) by NPP1 and NPP3. NPPs can also directly transform NAD\(^+\) to AMP by cleaving off nicotinamide mononucleotide (NMM). The ATP analog ARL-67156 is the current standard CD39 inhibitor.
The most abundant ATP-hydrolyzing ectonucleotidase is nucleoside triphosphate diphosphohydrolase-I (NTPDase1, CD39), which converts ATP via ADP to AMP. Further cell membrane-bound ecto-NTPDase isoenzymes include NTPDase2, −3, and −8, while all other NTPDases are located on intracellular organelles. Alternatively, extracellular AMP can be produced by nucleotide pyrophosphatase/phosphodiesterases (NPPs), a promiscuous enzyme family that catalyzes the hydrolysis of various nucleotides. The main NPP isoenzyme, NPP1 (CD203a, PC-1) hydrolyzes ATP, nicotinamide mononucleotide (NAD), ADP-ribose (ADPR) and dinucleoside triphosphates and tetraphosphates. In addition, NAD can be hydrolyzed by CD38 yielding ADPR, which may then be degraded to AMP by NPP1. AMP produced by CD39 and NPPs is further hydrolyzed by ecto-5′-nucleotidase (CD73) yielding adenosine. Adenosine signaling is modulated by additional pathways. For example, ubiquitously expressed alkaline phosphatase and prostate-specific phosphatase are also able to generate adenosine from AMP. Adenosine can be deaminated by adenosine deaminases (ADA1 and ADA2) to inosine, or phosphorylated by adenosine kinase regenerating the nucleotide AMP. The extracellular concentration of adenosine is additionally regulated by equilibrative nucleoside transporters (e.g. ENT1 and ENT2) and by concentrative nucleoside transporters.1,2

CD39, NPP1 and CD73 are upregulated on many types of cancer, providing an immunosuppressive, cancer proliferation-promoting environment. CD73 has even been proposed as a diagnostic marker in metastatic melanoma.24,25

Several anti-hypoxia-adenosinergic drugs are currently in clinical development for the immunotherapy of cancer.26 Besides adenosine A2a receptor antagonists, a small molecule CD73 inhibitor and several antibodies are currently evaluated in clinical trials.27 Inhibition of CD39 might be even more powerful since the resulting increase in ATP and the expected decrease in AMP, the substrate for adenosine generation by CD73, is expected to be synergistic.13,28,29

So far, only moderately potent and/or non-selective and metabolically unstable CD39 inhibitors have been described.30-38 For example, the currently preferred standard CD39 inhibitor, ARL-67156, which has been and is still used in many in vitro and in vivo studies,39-43 displays a K_v value in the micromolar range at human CD39.33,34 This nucleotide analog (figure 2) is a competitive inhibitor showing similarity to the substrate ATP. The ATP cleavage site for dephosphorylation by NTPDases between the β- and γ-phosphate groups is stabilized in ARL-67156 by a dibromomethylene bridge. ARL-67156 was recently shown to be non-selective, also inhibiting CD73 even with higher potency (K_v value of 0.451 μM), and to be metabolically unstable upon incubation with human and mouse liver microsomes; it is therefore not suitable for in vivo studies.35 The optimization of nucleotidic CD39 inhibitors such as ARL-67156 acting as substrate analogs has so far met with limited success.32

In the present study, we pursued a completely new approach to identify novel CD39 inhibitors—the screening of approved ATP-competitive protein kinase inhibitory drugs. Protein kinase inhibitors constitute a growing class of therapeutics for targeted cancer therapy.44-46 Most of the developed and approved inhibitors block the co-substrate binding site for ATP in a competitive manner. Since CD39 harbors a binding site for its substrate ATP, we figured that some of the ATP-competitive protein kinase inhibitors might also inhibit this extracellularly accessible ecto-enzyme. Hit compounds discovered within a library of approved drugs could be an excellent starting point for further optimization due to their already ideal pharmacokinetic and further drug-like properties. Thus, we collected a library of approved therapeutic drugs targeting the ATP binding site of protein kinases, and screened them for CD39 inhibition. This led to the discovery of ceritinib, belonging to a new chemoype of CD39 inhibitors.

METHODS

Assembly of an approved protein kinase inhibitor library

Small molecule protein kinase inhibitors were collected that were approved either by the US Food and Drug Administration (FDA) (http://www.brirm.org/PK/PKIs.htm, version of 12.04.2019), or by the European Medicines Agency and available in Germany (https://www.pharmazeutische-zeitung.de/ausgabe-132018/sortieren-des-grossen-sortiments/). At the time of collection, the library contained a total of 50 drug molecules. All compounds were purchased from Sigma Aldrich (Darmstadt, Germany), with the exception of netarsudil, which was not commercially available (see table 1).

Materials

1, N′-etheno-ATP, etheno-ADP, etheno-AMP, and etheno-adenosine (eATP, eADP, eAMP, and eADO) were purchased from BIOLOG Life Science Institute (Bremen, Germany). Disodium hydrogenphosphate, lanthanum chloride, sodium acetate, sodium chloride and sulfuric acid were obtained from Carl Roth (Karlsruhe, Germany). Disodium hydrogenphosphate, lanthanum chloride, sodium acetate, sodium chloride and sulfuric acid were obtained from Carl Roth (Karlsruhe, Germany). [2,3H]AMP (solution in ethanol/water, 1:1, 22.9Ci/ mmol, 1.0 mCi/ml, 849 GBq/mmol) was purchased from Hartmann Analytic (Braunschweig, Germany). ATP, ammonium heptamolybdate, Brij L23, calcium chloride, dimethyl sulfoxide (DMSO), diprydiamole, magnesium chloride, malachite green, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and polyvinyl alcohol were obtained from Sigma (Steinheim, Germany).

Dulbecco’s Modified Eagle’s Medium (DMEM), fetal calf serum (FCS), penicillin/streptomycin (P/S), and L-glutamine were purchased from Gibco BRL, Gaithersburg, Maryland, USA. RPMI-1640 culture medium was from PAN Biotech GmbH. Cellfectin II reagent (Thermo Fisher Scientific, Massachusetts, USA), baculovirus genomic ProEasy vector DNA (AB vector, California, USA), Insect-XPRESS media (#: BE12-730Q,
Table 1  Collection of protein kinase inhibitors used for CD39 screening

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Continued
Lonza, Switzerland) and HisPur Ni²⁺-NTA spin columns (#: 88226, Thermo Fisher Scientific, Massachusetts, USA) were used as protein expression and purification tools. For capillary electrophoresis, polyacrylamide-coated capillaries were purchased from Chromatographie Service GmbH (Langerwehe, Germany).

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**Table 1 Continued**

**Enzyme preparations**

**Recombinant expression of enzymes**

Human NTPDase-1, −2, −3 and −8 were recombinantly expressed in COS-7 cells (a non-human primate cell line, CV-1 in Origin with SV40 genes), and membrane preparations were obtained according to published protocols.33 34 35 36 37 The human cDNAs for the enzymes NPP1, NPP3, NPP4, NPP5, CD38 and CD73 (Genbank accession no. cDNA sequences NM_006208, NM_005021, NM_021572, NM_014936, NM_001775, and NM_002526, respectively) were obtained from Origene (Rockville, USA). Soluble enzymes were produced as previously reported.32 33 35 37 38 The plasmids contained the catalytic domains of the enzymes to which a 9× histidine tag (Histag) was added at the C-terminus (except for NPP1, which was expressed without tag), in addition to the expression vector pACGP67 A/B. Sf9 insect cells were transfected using Cellfectin II Reagent (Thermo Fisher Scientific, Massachusetts, USA) and ProEasy baculovirus linearized DNA (Cat.#A10S, AB Vector). The expressed protein was released into the supernatant and collected after 48 hours of incubation at 27°C. The enzymes were purified using HisPur Ni²⁺-NTA spin columns according to the manufacturer’s protocol. The protein concentration was determined by the Lowry method.31

**Preparation of umbilical cord membranes natively expressing CD39**

Human umbilical cord membrane preparations were obtained as previously reported.32 In brief, tissue samples were homogenized with a polytron, filtered through a cheesecloth, and centrifuged to remove debris and nuclei. Membrane protein fractions were collected from the pellets obtained after ultracentrifugation. All purification steps were performed at 4°C. Protein stock solutions were aliquoted and stored at −80°C until use in the assays.

**Isolation of human peripheral blood mononuclear cells**

Buffy coats were obtained from the blood bank of the University Medical Center Hamburg-Eppendorf. Peripheral blood mononuclear cells (PBMCs) were isolated by Biocoll (Merck) density gradient centrifugation. Blood was diluted with phosphate-buffered saline (PBS) (Thermo Fisher Scientific) and carefully layered on Biocoll. After centrifugation (25 min, 800 g, RT, the lymphocyte layer was collected and washed twice with cold PBS (650 g, 10 min, 4°C, and 450 g, 5 min, 4°C). The expression of CD39 and CD73 on PBMCs was assessed by flow cytometry.

**Membrane preparations of human cancer cells**

Membrane preparations of human triple-negative breast cancer (TNBC, MDA-MB-231) and melanoma (Ma-Mel-65) cells were prepared as follows. Once cells had reached a confluency of ca. 90%, cells of one 150mm dish were transferred to a confluency of ca. 90%, cells of one 175cm² flask were passed into 20 sterile 150mm dishes and incubated at 37°C, 5% CO₂. After the cells had grown into a monolayer of −90% confluency, the medium was decanted, dishes were rinsed with 5 mL of PBS and frozen at −20°C. Frozen cells were scraped off the dishes with 1 mL of an ice-cold buffer consisting of 25 mM Tris-HCl, 1 mM EDTA, 0.32 M saccharose and 100 µM phenylmethylsulfonyl fluoride (protease inhibitor) at pH 7.4 with a cell scraper. This step was repeated to ensure that all cells were collected from each dish. All cells were transferred to a beaker, and the cell suspension was treated with an Ultraturrax (twice, 30 s each at high speed). To remove nuclei, other
larger organelles, and cell debris, the homogenate was centrifuged at 1000 g for 10 min (4°C). The supernatant was collected and subsequently centrifuged at 48,000 g for 1 hour (4°C). The obtained pellets were resuspended in washing buffer and centrifuged using the same conditions. After two more washing steps, the pellets were resuspended in 0.1 mL per dish of 50 mM Tris-HCl buffer, pH 7.4, and the crude membrane suspensions were stored at −80°C until use. All steps were carried out as fast as possible at a temperature of 4°C to avoid enzyme internalization. Protein concentrations were determined using the Lowry assay. The determined protein concentration was 10.0 µg/µL for TNBC and 9.1 µg/µL for melanoma cell membrane preparations.

Cell culture and monitoring of nucleotide hydrolysis on cancer cells

Human TNBC cells (MDA-MB-231) were grown in DMEM medium supplemented with 10% FCS, and 1% P/S at 37°C and 10% CO₂. Melanoma (MaMel65) cell lines were cultured in RPMI-1640 medium with 10% FCS, 1% P/S, and 1% L-glutamine at 37°C and 5% CO₂. Cells were harvested using trypsin, washed three times using reaction buffer consisting of 10 mM HEPES, pH 7.4, 2 mM CaCl₂, and 1 mM MgCl₂. The hydrolysis of eATP to its product eADO by the concerted action of CD39 and CD73 overexpressed on these cells was monitored. eADO was quantified after precipitation of the nucleotides (eATP, eADP, and eAMP) using LaCl₃ (a detailed assay procedure will be published elsewhere). Briefly, 300,000 cells/reaction (pretreated for 30 min with 20 µM of the nucleoside transport inhibitor dipridamole) were incubated with or without ceritinib (100, 10, or 1 µM) for 1 hour at 37°C. The reaction was terminated by the addition of precipitation buffer consisting of 14.2 mM NaH₂PO₄ and 71.7 mM NaCl/NaOAc under acidic conditions. Then, the samples were centrifuged at 1200 rpm for 10 min. The uncharged eADO remained in the supernatant while the nucleotides were precipitated. Aliquots of 100 µL of supernatant were then transferred to a fresh half area 96-well plate. Relative fluorescence units (at 300/410 nm) were measured by a fluorescence microplate reader (Flexstation, Medical Devices, USA).

Malachite green assay for measuring NTPDase activity (CD39 and NTPDase2, -3 and -8)

Effects of compounds on ATP hydrolysis by CD39, NTPDase2, -3 or -8, and by human cancer cell membrane preparations were evaluated using the malachite green assay. The substrate concentration of ATP was 50 µM for CD39 and the cancer cell membrane preparations, and 100 µM for NTPDase2, -3 or -8. The assay was performed in clear half area plates in a total volume of 50 µL of 10 mM HEPES containing 2 mM CaCl₂ and 1 mM MgCl₂ (pH 7.4) in the presence of a maximal concentration of 2% DMSO. The mixtures of inhibitor and enzyme were preincubated at 37°C for 5 min. Subsequently, the enzymatic dephosphorylation of ATP was started by addition of the substrate. The samples were incubated at 37°C for 15 min, then the reaction was stopped by adding the malachite green detection reagents (20 µL of malachite green solution (0.6 mM) and 30 µL of ammonium molybdate solution (20 mM) in sulfuric acid (1.5 M)). Absorption of the samples at 600 nm was measured using the BMG PHER-Astar FS plate reader (BMG Labtech GmbH, Ortenberg, Germany) after 20 min of incubation at 25°C. The corrected absorption was calculated by subtracting the absorption of the negative control samples, which were incubated with denatured enzyme (90°C, 15 min).

The kinase inhibitors were initially investigated at 10 µM concentration at human CD39, natively expressed in high density in human umbilical cord membranes. The kinase inhibitors were initially investigated at 10 µM concentration at human CD39, natively expressed in high density in human umbilical cord membranes. Further studies were performed using CD39 and the respective NTPDase proteins expressed in COS-7 cell membranes (ca. 100 ng of protein depending on enzymatic activity, adjusted to ensure 10%–20% of substrate conversion). The respective enzyme was incubated with or without 50 µM ceritinib and 50 or 100 µM ATP (Kₐ (CD39)=17 µM; Kₐ (NTPDase2)=70 µM; Kₐ (NTPDase3)=75 µM; Kₐ (NTPDase8)=46 µM). Full concentration-inhibition curves were determined with inhibitor concentrations ranging from 0.1 to 300 µM. For the inhibition type experiments, 57.0 ng of recombinant human CD39 was incubated with 0, 5, 10, 15 and 20 µM of ceritinib and increasing substrate (ATP) concentrations of 10, 25, 50, 100 or 150 µM. All experiments were performed in three independent iterations (n=3), and the obtained data were processed and plotted with GraphPad Prism V.8 software (San Diego, USA).

Capillary electrophoresis assay for monitoring CD39 activity

The effects of ceritinib on CD39 activity were additionally determined by a previously established capillary electrophoresis (CE)-based assay. Briefly, ceritinib in a concentration range of 0.1 to 300 µM was incubated with 100 µM of ATP as substrate (Kₐ (CD39)=17 µM) and recombinant human CD39 preparations suspended in reaction buffer consisting of 10 mM HEPES, 2 mM CaCl₂, and 1 mM MgCl₂ (pH 7.4) in a final volume of 100 µL. Incubation at 37°C was performed for 30 min, followed by heating at 90°C for 10 min in order to prevent further enzymatic degradation of the substrate. The nucleotides were separated using a P/ACE MDQ CE system (Beckman Instruments, Fullerton, California, USA) and evaluated with the P/ACE MDQ software V.32 KARAT obtained from Beckman Coulter (Fullerton, California, USA).

CD73 assay

The effect of ceritinib on soluble human CD73 was determined with a previously established assay. For concentration-inhibition curves of ceritinib, a dilution
series of the compound was incubated at 37°C for 25 min with 0.09 µg/mL of soluble human CD73 and 5.0 µM of the radioactive substrate [2,8-3H]AMP (specific activity 7.4 × 108 Bq/µmol, 20 mCi/µmol) in a shaking water bath. The assay buffer consisted of 25 mM Tris, 140 mM NaCl and 25 mM NaH₂PO₄ at pH 7.4. After incubation, 500 µL of cold precipitation buffer (100 mM LaCl₃, 100 mM sodium acetate, pH 4.0) was added to precipitate free phosphate and unconverted [2,8-3H]AMP for 30 min on ice. The filtrate was collected by filtering the samples through GF/B glass fiber filters using a Brandel cell harvester (M-48, Brandel, Maryland, USA). The reaction vials were washed three times each with 400 µL of cold (4°C) demineralized water. Then, 5 mL of scintillation cocktail (ULTIMA Gold XR) were mixed with aliquots of the filtrate in order to quantify the isolated product of the enzymatic reaction, radioactive adenosine, by liquid scintillation counting (TRICARB 2900 TR, Packard/PerkinElmer).

NPP1 assay
The NPP1 enzymatic activity on the degradation of its substrate AMP was evaluated by analyzing the reaction product ATP by CE. A mixture of 800 ng of NPP1 (crude soluble form expressed in Sf9-insect cells), 50 µM of ceritinib, 2% DMSO and 300 µM of ATP as a substrate in reaction buffer (10 mM N-cyclohexyl-2-aminoethanesulfonic acid (CHES), pH 9.0, 2 mM CaCl₂, 1 mM MgCl₂) were incubated for 30 min at 37°C with gentle shaking. The reaction was terminated by heating at 90°C for 5 min, and afterwards cooled down on ice. The quantitative analysis of the reaction product AMP was performed by CE (AB Sciex, Framingham, USA), according to a published procedure.

NPP3 assay
Inhibition of NPP3 was determined as previously described. Purified soluble NPP3 (95 ng per reaction), expressed in Sf9-insect cells, was incubated with ceritinib at a final concentration of 50 µM, 2% DMSO, and 400 µM of the artificial substrate p-nitrophenyl thymidine 5’-monophosphate (p-Nph-5’-TMP) in a final volume of 100 µL of reaction buffer (50 mM Tris HCl, pH 9.2, 2 mM CaCl₂, 0.1 mM ZnCl₂) for 30 min at 37°C with gentle shaking. The enzyme reaction was terminated by the addition of 20 µL of 1 M aq NaOH solution. The absorption of the formed p-nitrophenolate anion was measured at 400 nm using a BMG PHERAraster FS plate reader (BMG Labtech GmbH, Ortenberg, Germany).

NPP4 assay
The effect on NPP4 activity was tested by employing diade-nosine tetrabiphosphate (AP₄A) as a substrate, which is cleaved to ATP and AMP by the enzyme. A mixture of 1200 ng of NPP4 (soluble form expressed in Sf9-insect cells and purified), 50 µM of ceritinib, 2% DMSO and 300 µM of AP₄A as substrate were incubated for 90 min at 37°C in reaction buffer (10 mM HEPES, 2 mM CaCl₂ and 1 mM MgCl₂ (pH 8.0)) with gentle shaking. The reaction was terminated by heating at 90°C for 5 min, and then cooled down on ice. The quantitative analysis of the reaction product ATP was performed by CE (AB Sciex, Framingham, USA), according to a published procedure.

NPP5 and CD38 assay
Inhibition of the enzymes by ceritinib was measured using the natural substrate NAD⁺. CD38 (5 ng) or NPP5 (250 ng) were mixed with 50 µM ceritinib and 100 µM NAD⁺ in reaction buffer (CD38: 10 mM HEPES, pH 7.4; NPP5: 10 mM CHES (pH 9.0), 2 mM CaCl₂, 1 mM MgCl₂) for 30 min at 37°C. The samples were heated to terminate the reaction (10 min at 95°C), and cooled on ice for 10 min. The reaction products, either ADPR (CD38) or AMP (NPP5), were quantified by CE (AB Sciex, Framingham, USA).

High performance liquid chromatography for measuring AMPase and ATPase activities on PMBCs
To determine the effect of ceritinib on the ATPase and AMPase activity of human PMBCs, 2 × 10⁵ PMBCs were treated with ceritinib for 15 min at 37°C, and then incubated with 1 µM eATP or eAMP for 30 min at 37°C. After the incubation, cells were removed (450 g, 5 min, 4°C) and all samples were passed through 10 kDa size exclusion filters (10,000 g, 10 min, 4°C, Pall Corporation) and stored at −20°C until analysis. The analysis was performed on a reversed-phase high performance liquid chromatography (HPLC) system (Agilent Technologies) with a 250 mm × 4.6 mm C8 Luna column (5 µm particle size, Phenomenex) as stationary phase. The mobile phase consisted of different compositions of HPLC buffer A (20 mM KH₂PO₄, pH 6.0) and B (50% buffer A, 50% methanol), and elution of the nucleotides from the column resulted from an increasing methanol content in the mobile phase (0.0 min (0.0% buffer B), 5.0 min (0.0% buffer B), 27.5 min (100.0% buffer B), 30.0 min (100.0% buffer B), 32.0 min (0.0% buffer B), 43.0 min (0.0% buffer B)). The signals were detected by fluorescence detection (230 nm excitation wavelength, 410 nm emission wavelength). Defined amounts of ethenonucleo-tides were measured as standards for quantification of eATP, eADP, eAMP and eADO.

Statistical analysis of data
All statistical evaluations were performed using GraphPad Prism V8 software (San Diego, USA). One-way or two-way analysis of variance tests were employed to detect the significant differences between groups, and Dunnett’s multiple comparison test was used to compare the respective inhibitor samples to the controls without inhibitor addition. Data were expressed as means±SEM. P values were represented as asterisks; p values of <0.05 were considered statistically significant: 0.01–0.05, significant (*); 0.01–0.001, very significant...
RESULTS

A compound library of protein kinase inhibitors, approved by the US FDA or the European Medicines Agency, was assembled (see table 1). The compounds were initially tested at a concentration of 10 µM for inhibition of human CD39 natively expressed in umbilical cord membranes (figure 3). A single hit compound showed an enzyme inhibition of >50% (corresponding to a hit rate of 2%), namely ceritinib (figure 3). Ceritinib is a potent inhibitor of the anaplastic lymphoma kinase (ALK), a receptor tyrosine kinase. The second most potent CD39 inhibitor was the tyrosine kinase inhibitor drug ponatinib (figure 3) showing a significant inhibition of 33% at 10 µM concentration.

Ceritinib is an FDA-approved drug for the treatment of ALK-positive non-small cell lung cancer. Its IC<sub>50</sub> value against ALK was reported to be in the subnanomolar range, and the inhibitor was proposed to be ATP-competitive based on a crystal structure of a related compound. Ceritinib is administered perorally up to a maximum daily dose of 750 mg and shows some brain permeation.

Maximum plasma levels of the drug were reported to be around 800±205 ng per milliliter corresponding to 1.4±0.4 µM, and the anticancer drug was found to be metabolically highly stable.

In a recent study on patients with brain tumors, ceritinib was found to accumulate in tumor tissue, where an average concentration of 36 µM (2–139 µM) was measured after application of the 10<sup>th</sup> applied dose, while the unbound fraction was low.

Characterization of ceritinib as a CD39 inhibitor

As a next step, we investigated the inhibitory effect of different concentrations of ceritinib on recombinant and umbilical cord membrane-derived CD39 applying two different, orthogonal assays. The malachite green assay allows the quantification of inorganic phosphate released by the CD39-catalyzed hydrolysis of ATP via ADP to AMP. A CE-based assay coupled to ultraviolet (UV) detection (CE-UV) enables the separation and quantification of the nucleotide ATP along with its intermediate and final products ADP and AMP formed by the enzymatic reaction. According to the sensitivities of the different assays, the substrate ATP was employed at a concentration 50 µM in the malachite green assay, while 100 µM of ATP were required for the CE-UV assay. Different human CD39 enzyme preparations were employed, natively expressed CD39 in umbilical cord membrane preparations, and recombinant CD39 expressed in COS-7 cells, respectively. In the malachite green assay on native CD39, an IC<sub>50</sub> value of 11.3 µM was determined for ceritinib. A very similar IC<sub>50</sub> value of 13.7 µM was obtained in the CE-UV assay at the recombinant enzyme (figure 4A). The almost identical IC<sub>50</sub> values despite a twofold difference in substrate concentrations (50 µM and 100 µM) in both assays may hint at a substrate concentration-independent inhibition mechanism (figure 4A). Subsequent studies were performed to elucidate ceritinib's inhibition type on CD39. To this end, K<sub>m</sub> and V<sub>max</sub> values were determined in the absence and in the presence of increasing concentrations of ceritinib. While the inhibitor had no significant effects on the K<sub>m</sub> value of ATP hydrolysis by CD39, the V<sub>max</sub> value showed a progressive decrease with increasing concentrations of the inhibitor (figure 4B–D). This indicates a non-competitive, allosteric inhibition type. A Hanes-Woolf plot was performed that visualizes the non-competitive CD39 inhibition mechanism. A K<sub>v</sub> value of 11.0 µM was calculated from the plotted data which is in excellent agreement with the data obtained in the initial studies using fixed substrate concentrations, where IC<sub>50</sub> values of 11.3 and 13.7 µM were determined for the native and recombinant forms of CD39, respectively.

Next, ceritinib was studied for potential inhibition of other NTPDase isoenzymes related to CD39, and on further relevant human ectonucleotidases. While NTPDases3 and –8 were also inhibited by ceritinib at a concentration of 50 µM, NTPDase2 was not affected. Very low or no inhibition of the ATP-hydrolyzing ectonucleotidase NPP1 and its isoenzymes NPP3, –4, and –5 was observed. Similarly, only moderate inhibition of AMP-hydrolyzing CD73, and of CD38 by ceritinib was detected indicating that the compound is selective for members of the NTPDase family.
Figure 4  Characterization of ceritinib as a CD39 inhibitor. (A) Concentration-dependent inhibition of human CD39 by ceritinib determined with the malachite green (MG) assay (green) on human umbilical cord membrane preparations expressing CD39 (ATP substrate concentration of 50 µM), and determined with the CE-UV assay (black) on recombinant human CD39 (ATP substrate concentration of 100 µM). IC₅₀-values are collected in table 2. (B–D) Determination of the inhibition type of ceritinib at human recombinant CD39 using the MG assay employing 10, 25, 50, 100 and 150 µM of ATP as a substrate, and 0, 5, 10, 15 and 20 µM of the inhibitor. (B) Michaelis-Menten plot for the determination of Vmax and Km values. (C) Hanes-Woolf plot where the intersection of lines on the X-axis indicates a non-competitive inhibition type. (D) Vmax and Km values of CD39 in the presence of the inhibitor ceritinib calculated by GraphPad Prism V.8 from the Michaelis-Menten plot. The Ke value was calculated to be 11.0±0.6 µM by GraphPad Prism V.8 software with non-linear regression of the Michaelis-Menten plot data using the equation Vmax = Vmax/(1+ (I) / Ke). CE, capillary electrophoresis.
the NTPDase family (figure 5). IC₅₀ values were slightly higher at NTPDase3 and NTPDase8 as compared with CD39 (NTPDase1) (figure 5). All results are collected in table 1.

Effects of ceritinib on the ATPase and AMPase activity of PBMCs

The effect of ceritinib was further investigated on primary human PBMCs. In the donors used for this experiment, the percentage of CD39-expressing PBMCs ranged between 7% and 16% and the percentage of CD73-expressing PBMCs between 11% and 14%. The cells were incubated with the fluorescent CD39 substrate 1,N⁶-eATP or the fluorescent CD73 substrate 1,N⁶-eAMP in the absence and presence of ceritinib. Fluorescent substrates and products were separated by reversed-phase HPLC coupled to fluorescence detection (excitation: 230 nm, 410 nm emission wavelength), and subsequent quantitative analysis of nucleotides was performed (figure 6A and B). Increasing concentrations of ceritinib resulted in an accumulation of the substrate eATP, while its hydrolysis product eAMP was concomitantly decreased, and its formation was completely blocked at a ceritinib concentration of 50 µM and higher (figure 6A).

Ceritinib had only a minor effect on the hydrolysis of eAMP by PBMCs (figure 6B), which is in agreement with our findings on recombinant CD73, which is only weakly inhibited by ceritinib (figure 5A and table 2).

Effect of ceritinib on the ATPase activity of cancer cell membrane preparations

Hypoxia is a hallmark of the tumor microenvironment, and HIF-1α induces an overexpression of CD39 and CD73, which contributes to immune escape and a cancer growth-promoting microenvironment. Therefore, we next investigated the effect of ceritinib on ATP hydrolysis by human cancer cell lines, that is, melanoma cells (MaMel65) and TNBC (MDA-MB-231) cells. This TNBC cell line is known to express both CD39 and CD73. As expected, ATPase activity of the cancer cell membranes decreased significantly with increasing concentrations of ceritinib (figure 6C). The potency of ceritinib for inhibiting the ATPase activity of both TNBC and melanoma cells was similar to that of the recombinantly expressed CD39, indicating that CD39 is in fact the major enzyme responsible for ATP hydrolysis in these cell lines.

Effects of ceritinib on the ATPase activity of live cancer cells

Finally, we investigated the inhibitory effects of ceritinib on live cancer cells employing the same human cell lines, TNBC and melanoma cells. Ceritinib displayed a concentration-dependent inhibition of the hydrolysis of eATP added to the cells, which was significant at 10 µM (figure 6D). It should be noted, however, that ceritinib, being a potent protein kinase inhibitor, can be cytotoxic at higher concentrations, especially upon extended exposure.

DISCUSSION

The screening of protein kinase inhibitors proved to be a successful strategy for the identification of structurally novel CD39 inhibitors; the most potent hit compound was the approved anticancer drug ceritinib. The compound was found to display selectivity for NTPDases within the family of ectonucleotidases, preferably inhibiting NTPDase3 (B) and NTPDase8 (C) by ceritinib determined by the malachite green assay applying an ATP substrate concentration of 100 µM. For calculated IC₅₀ values see table 1. NPP, nucleotide pyrophosphatase/phosphodiesterase; NTPDase, nucleoside triphosphate diphosphohydrolase.
catalytic domain of the enzyme. Membrane-localized receptors, for example, nucleotide P2Y receptors, have been reported to harbor a so-called meta-binding site which is responsible for the first contact with the physiological ligand, subsequently guiding it to the orthosteric site. Similarly, nucleotide-metabolizing (bacterial) enzymes were described to be allosterically modulated by their substrates, that is, they harbor a second nucleotide binding site. We hypothesize that ceritinib binds to such an allosteric ATP/nucleotide-interacting site in the extracellular domain of CD39 and thereby blocks ATP hydrolysis. The NTPDase1 (CD39) isoenzymes NTPDase3 and NTPDase8, but not NTPDase2, appear to harbor the analogous allosteric binding site since ceritinib inhibited them as well at slightly higher concentrations (about twofold, see figure 5 and table 2). However, ceritinib was highly selective versus other ectonucleotidase families, including NPPs, CD73, and CD38 (see table 2).

The CD39-inhibitory effects, along with its selectivity versus CD73, were not only observed in recombinant enzyme preparations, but also on native immune as well as cancer cells (figure 6). Ceritinib was proven to inhibit the ability of human cancer cell membrane preparations and PBMCs to dephosphorylate ATP/eATP. Significant effects were observed at TNBC cell membranes already at a concentration of 1 µM leading to reduced ATP degradation (figure 6C). Blood plasma levels of up to 1.4 µM had been determined in patients treated with ceritinib, and local concentrations at the cell membrane, where the enzyme is localized, may be even higher. Ceritinib
of some other protein kinases, including the insulin-like growth factor 1 receptor, the insulin receptor, and the serine/threonine protein kinase STK22D (TSSK1). The compound had been found to display growth inhibition and cellular toxicity of tumor cells, which may be dependent on its strong inhibition of ALK and other kinases; ceritinib’s cytotoxic mechanism of action may, in fact, be due to its polypharmacology, to which CD39 inhibition might additionally contribute.

Nevertheless, our findings are valuable, for example, as a basis for further drug development. In fact, the structure of ceritinib is an excellent starting point for optimization of its CD39-inhibitory potency. Since the structure-activity relationships of ceritinib derivatives and analogs as ALK inhibitors have been extensively studied, it appears to be feasible to reduce or abolish ALK inhibition, and at the same time to increase its CD39-inhibitory potency to obtain potent, selective CD39 inhibitors. Another option would be to design balanced dual ALK/CD39 inhibitors, further increasing CD39-inhibitory activity without compromising ALK inhibition. Such dual ALK/CD39 inhibitors could further improve therapeutic efficacy by combining a targeted anticancer approach (ALK inhibition) with enhanced immunotherapeutic anticancer activity.

Ceritinib is not only the most potent allosteric CD39 inhibitor described to date, moreover, it is the first brain-permeable CD39 inhibitor. Thus, it could be a useful tool compound for further preclinical studies targeting CD39.

Currently, there are only few commercially available CD39 inhibitors that have drug-like physicochemical properties and high metabolic stability. Therefore, ceritinib could be directly considered for further in vitro and in vivo studies targeting CD39 (along with the related NTPDase3 and –8).

Future studies on the structure-activity relationships of ceritinib to optimize its potency at CD39 and to reduce ALK inhibition are warranted.

### Table 2 Inhibitory potencies of ceritinib on human ectonucleotidases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC₅₀ ± SEM (µM) or % inhibition at 50µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human umbilical cord membrane preparations with high CD39 expression</td>
<td>11.3±0.8 (Kᵢ=11.2±0.6)†</td>
</tr>
<tr>
<td>Recombinant human CD39</td>
<td>13.7±0.6 (Kᵢ=11.0±0.6)†</td>
</tr>
<tr>
<td>NTPDase2</td>
<td>&gt;&gt;50 (–8)</td>
</tr>
<tr>
<td>NTPDase3</td>
<td>22.7±4.0</td>
</tr>
<tr>
<td>NTPDase8</td>
<td>20.3±1.5</td>
</tr>
<tr>
<td>Soluble CD73</td>
<td>&gt;50 (33)</td>
</tr>
<tr>
<td>Membrane-bound CD73</td>
<td>&gt;50 (30)</td>
</tr>
<tr>
<td>NPP1</td>
<td>&gt;50 (8)</td>
</tr>
<tr>
<td>NPP3</td>
<td>&gt;50 (1)</td>
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<td>NPP4</td>
<td>&gt;50 (15)</td>
</tr>
<tr>
<td>NPP5</td>
<td>&gt;50 (1)</td>
</tr>
<tr>
<td>CD38</td>
<td>&gt;50 (19)</td>
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</tbody>
</table>

*In all cases, three independent experiments were performed. Details on the assays can be found in the Methods Section.†Determined by non-linear regression of the Michaelis-Menten plot data (see figure 4).

**NPP, nucleotide pyrophosphatase/phosphodiesterase; NTPDase, nucleoside triphosphate diphosphohydrolase.**

can penetrate cell membranes, the blood-brain barrier, and tumor tissues very well. At a pH of 7.4, it is neutral (uncharged) and therefore quite lipophilic. The piperidine nitrogen (see figure 3B) has weakly basic properties (pkᵢ close to 10), which means that it will not be protonated, even in the acidic tumor environment (pH 6.8), remain lipophilic, and keep its excellent ability for tissue permeation. Ceritinib was found to exhibit comparable pharmacokinetics in mice as in humans. In vivo, the drug was found to cross the blood brain barrier and accumulate in brain tumor tissue. In fact, ceritinib appears to accumulate at cell membranes and in tumor tissues, where average concentrations of 36nmol/g (36µM) have been observed even in brain tumors under steady-state conditions. The concentration of the drug in some patients reached levels of above 100µM in tumor tissues, a concentration at which we observed very high or even nearly complete inhibition of CD39 (figures 4A and 6C–D) and of ATP hydrolysis on immune and cancer cells (figure 6). Thus, it is conceivable that ceritinib inhibits CD39 activity – at least in part – in cancer patients treated with the drug. CD39-inhibitory activity might contribute to the potent anticancer effects of ceritinib seen in patients by intercepting hypoxia-induced adenosine signaling leading to a reactivation of T cells. In contrast, two other investigated ALK inhibitors, alectinib and crizotinib, did not inhibit CD39 (see compounds 4 and 13 in table 1 and figure 3).

Ceritinib is, of course, not an ideal CD39 inhibitor due to its very high ALK-inhibitory potency and its inhibition of some other protein kinases, including the insulin-like growth factor 1 receptor, the insulin receptor, and the serine/threonine protein kinase STK22D (TSSK1). The compound had been found to display growth inhibition and cellular toxicity of tumor cells, which may be dependent on its strong inhibition of ALK and other kinases; ceritinib’s cytotoxic mechanism of action may, in fact, be due to its polypharmacology, to which CD39 inhibition might additionally contribute.

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