2-Substituted thienotetrahydropyridine derivatives: Allosteric ectonucleotidase inhibitors

Laura Schäkel1 | Salahuddin Mirza1 | Markus Pietsch1,2 | Sang-Yong Lee1 | Tim Keuler1 | Katharina Sylvester1 | Julie Pelletier3 | Jean Sévigny3,4 | Thanigaimalai Pillaiyar1 | Vigneshwaran Namasivayam1 | Michael Gütschow1 | Christa E. Müller1

1Pharmaceutical & Medicinal Chemistry, Pharmaceutical Institute, University of Bonn, Bonn, Germany
2Faculty of Medicine and University Hospital Cologne, Institute II of Pharmacology, Centre of Pharmacology, University of Cologne, Cologne, Germany
3Centre de Recherche du CHU de Québec - Université Laval, Québec City, Québec, Canada
4Département de Microbiologie-Infectiologie et d’Immunologie, Faculté de Médecine, Université Laval, Québec City, Québec, Canada

Correspondence
Christa E. Müller, Pharmaceutical & Medicinal Chemistry, Pharmaceutical Institute, An der Immenburg 4, D-53121 Bonn, Germany.
Email: christa.mueller@uni-bonn.de

Present address
Thanigaimalai Pillaiyar, Institute of Pharmacy, Pharmaceutical/Medicinal Chemistry, University of Tübingen, Tübingen, Germany.

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Abstract
The antithrombotic prodrugs ticlopidine and clopidogrel are thienotetrahydro-pyridine derivatives that are metabolized in the liver to produce thiols that irreversibly block adenosine diphosphate (ADP)-activated P2Y12 receptors on thrombocytes. In their native, nonmetabolized form, both drugs were reported to act as inhibitors of ectonucleoside triphosphate diphosphohydrolase-1 (NTPDase1, CD39). CD39 catalyzes the extracellular hydrolysis of nucleoside tri- and diphosphates, mainly adenosine 5'-triphosphate (ATP) and ADP, yielding adenosine monophosphate, which is further hydrolyzed by ecto-5'-nucleotidase (CD73) to produce adenosine. While ATP has proinflammatory effects, adenosine is a potent anti-inflammatory, immunosuppressive agent. Inhibitors of CD39 and CD73 have potential as novel checkpoint inhibitors for the immunotherapy of cancer and infection. In the present study, we investigated 2-substituted thienotetrahydropyridine derivatives, structurally related to ticlopidine, as CD39 inhibitors. Due to their substituent on the 2-position, they will not be metabolically transformed into reactive thiols and can, therefore, be expected to be devoid of P2Y12 receptor-antagonistic activity in vivo. Several of the investigated 2-substituted thienotetrahydropyridine derivatives showed concentration-dependent inhibition of CD39.

The most potent derivative, 32, showed similar CD39-inhibitory potency to ticlopidine, both acting as allosteric inhibitors. Compound 32 showed an improved selectivity profile: While ticlopidine blocked several NTPDase isoenzymes, 32 was characterized as a novel dual inhibitor of CD39 and CD73.

KEYWORDS
CD39, CD73, ecto-5'-nucleotidase, NTPDase1, thienopyridines
1 | INTRODUCTION

Adenosine 5’-triphosphate (ATP) acts as an extracellular signaling molecule by activating nucleotide receptors localized in the cell membrane.[1] The release of ATP into the extracellular compartment is enhanced by inflammation, stress, or cell death and constitutes a danger signal. Extracellular ATP leads to immune cell recruitment and activation, among other effects.[2] The nucleoside adenosine, on the contrary, has opposite effects, including immunosuppressive, anti-inflammatory, and tissue-protective properties by activation of G protein-coupled adenosine receptors.[2,3] BALANCEING the ratio of proinflammatory ATP and anti-inflammatory adenosine is important for the maintenance of physiological functions.[1,4] Imbalances have been reported to contribute to autoimmune diseases or cancers.[5–7] The concentration of extracellular nucleotides and nucleosides is controlled by ectonucleotidases.[8,9]

The family of ectonucleoside triphosphate diphosphohydrolases (NTPDases) dephosphorylates nucleoside triphosphates (NTPs) and diphosphates (NDPs). Eight distinct NTPDases exist, four of which, NTPDases1, -2, -3, and -8, are ectonucleotidases localized in the cell membranes with the active center oriented toward the extracellular domain.[8] The remaining members of the NTPDase family constitute intracellular enzymes. NTPDase1, also termed CD39, is the most prominent NTPDase isozyme. It sequentially dephosphorylates NTPs and NDPs to the respective nucleoside monophosphates (NMPs). In a subsequent step, the NMPs are converted into the corresponding nucleosides, mainly adenosine, by the ecto-5’-nucleotidase (CD73). CD73 is attached to the cell membrane via a glycosylphosphatidylinositol anchor or released into the extracellular medium in its soluble form or via exosomes.[10] As the conversion of ATP into adenosine is mainly controlled by CD39 and CD73, these enzymes are potential targets for the treatment of a variety of diseases including inflammation, infection, and cancer.[11–13] Inhibition of CD39 reduces the concentrations of extracellular adenosine and simultaneously increases those of ATP, ADP, and other nucleotides, which abrogates immune escape mechanisms by cancer cells and tissues overexpressing CD39.[13,14] This effect can be increased by ancillary inhibition of CD73, which prevents hydrolysis of AMP, which can also be formed by alternative mechanisms, and thereby further reduces extracellular adenosine levels.[14]

Crystal structures are available for rat CD39 (e.g., PDB-ID: 3ZX3)[17] and rat NTPDase2 (e.g., PDB-ID: 4BRS and 4CD1).[18,19] However, no crystal structures of human CD39 have been published to date. In contrast, an X-ray crystal structure of human CD73 in complex with AMPCP (PDB-ID: 4H2G)[20] has contributed to the development of nucleotide-based CD73 inhibitors, and a number of further CD73 co-crystal structures with inhibitors have been published since then (e.g., 6Z9D and 6Z9B).[21] Human, rat, and mouse CD39 show high sequence identity (>74%) and similarity (>85%), while the ecto-NTPDase subtypes show much lower sequence identities (35–43%) and similarities (54–60%).

Recently, antibodies blocking the activity of CD39 and CD73 have been developed for the treatment of cancer,[22–25] but might face problems, especially in penetrating solid tumors. Small molecules modulating the enzymes’ activity offer several advantages, for example, the potential for peroral application, favorable pharmacokinetic properties, high penetration into tumor tissues, and possibly into the brain, and low production costs. Only a few small molecule inhibitors of CD39 have been described so far, all of which show limited potency and/or lack of metabolic stability.[26–32] Nucleotide derivatives, analogs of the natural substrates, have been investigated, for example, ARL 67156 (I) and its derivatives (e.g., II).[27,33,34] Nonnucleotide inhibitors comprise polyoxometalates (POMs, e.g., III), anthraquinones, and various other heterocyclic compounds including the clinically approved drugs ticlopidine (IV) and clopidogrel (V), and indole derivatives such as VI.[28–31,35,36] In contrast, CD73 inhibitors with nano- or even picomolar potency have been developed, for example, PSB-12489 (VII), and AB680 (VIII), which is currently being studied in clinical trials for the treatment of cancer.[37–41] Furthermore, potent nonnucleotidic CD73 inhibitors have been published, for example, IX (see Figure 1).[42]

The antithrombotic prodrugs ticlopidine (IV) and clopidogrel (V) are used for the treatment of acute coronary syndromes and the prevention of stroke and heart infarction.[43,44] Their thienotetrahydropyridine structure is oxidatively metabolized in the liver, forming reactive thiol or sulfenic acids. These react with a cysteine residue present in the extracellular domain of the P2Y12 receptor on blood platelets, forming a stable disulfide bond (see Figure 2) and leading to irreversible inhibition of the receptor, which is responsible for the antithrombotic effect.

Thienopyridines have previously been optimized for various pharmacological targets, for example, as inhibitors of MurF enzymes showing antibiotic activity,[47] as inhibitors of Mycobacterium tuberculosis panthothenate synthetase,[48] as inhibitors of glucose-6-phosphatase,[49] and as allosteric enhancers of adenosine A1 receptors.[50]

Native, non-metabolized ticlopidine and clopidogrel had been reported to inhibit the ATP- and ADP-hydrolyzing ectonucleotidase CD39 expressed in human umbilical vein endothelial cells.[50] The authors reported a mixed type of inhibition of human CD39 by ticlopidine and clopidogrel, with apparent K_i values of 14 and 10 μM, respectively, versus ADP as a substrate. The compounds appeared to inhibit ADP hydrolysis by CD39 more strongly than that of ATP, showing 50% inhibition of enzyme activity at 60 μM.[50,51] Selectivity versus several other ectonucleotidases was claimed.[51]

In the search for CD39 inhibitors with drug-like properties, we evaluated ticlopyridine as a potential lead structure, characterized its potency as an inhibitor of CD39 and other ectonucleotidases, and studied its mechanism of inhibition. Subsequently, we identified a closely related class of CD39-inhibitory thienopyridine derivatives, in which the 2-position was blocked to prevent metabolic conversion.

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

A series of thienotetrahydropyridine derivatives, in which the 2-position in the thiophene ring was blocked by substitution, for
example, with an amino, carboxamido, ureido, or thioureido function, were investigated as CD39 inhibitors as they can no longer be metabolized to thiol (or sulfenic acid) derivatives and thus will not act as inhibitors of P2Y12 receptors (see Figure 3).

Thienopyridine derivatives 1-10 (Table 1; see also Scheme S1), that is, alkyl 2-amino-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylates ($R^3 = CO_2R^4$) or 2-amino-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carbonitriles ($R^3 = CN$), were accessible through the Gewald reaction.\[^{[52]}\] In the subsequent step, the amino group was modified, mainly through reaction with isothiocyanates and isocyanates, to generate various thioureido and ureido functionalities, respectively. Such compounds can undergo proton-catalyzed ring-closure reactions to fused 1,3-thiazin-4-ones or 1,3-oxazin-4-ones.\[^{[53]}\] In the course of this study, we have examined the open-chain compounds 11-32, together with their precursors 1-10, as inhibitors of the ectonucleotidase CD39. The synthetic procedures for the test compounds have been published elsewhere.\[^{[54-59]}\] An overview is provided in Scheme S1.

### 2.2 | Biological evaluation

#### 2.2.1 | Assays

Cell membrane preparations of human umbilical cord natively expressing a high level of CD39 were used as an enzyme source for the natively expressed enzyme.\[^{[60]}\] In addition, the most potent inhibitors were additionally tested at recombinantly expressed and purified human soluble CD39, consisting of the catalytic ectodomain of the enzyme and a His-tag for purification. With this artificial protein preparation, no interference is possible, as all other membrane proteins and constituents are removed during the purification process. For selectivity studies, recombinant COS-7-cell membrane preparations expressing the respective human ecto-NTPDase isoenzyme were utilized to eliminate possible interference by other enzymes or isoforms.\[^{[60,61]}\] Human recombinant CD73 was used in its purified soluble form.

The inhibition of enzyme activity was quantified using the following assay systems: (i) capillary electrophoresis coupled to
laser-induced fluorescence (CE-FLF) detection or ultraviolet detection (CE-UV), by which the respective substrate and product are separated according to ionic charge,\(^{[62]}\) (ii) detection of inorganic phosphate released during the enzymatic reaction using the malachite green assay,\(^{[63]}\) and using the radioactively labeled substrate \([3H]AMP\) and measuring the formation of \([3H]\)adenosine by the enzyme CD73.\(^{[64]}\) Data for standard inhibitors of CD39, \(I, IV,\) and \(V\), obtained in the same assays, are provided for comparison (see Table 1).

2.2.2 | Characterization of the lead compound ticlopidine

Initially, we tested ticlopidine and clopidogrel for inhibition of human CD39 in our standard assay based on the detection of phosphate by malachite green\(^{[63]}\) using native human umbilical cord membrane preparations, which contain a very high expression level of CD39 as the only ATP/ADP-hydrolyzing enzyme.\(^{[65]}\) We could confirm that both compounds inhibited CD39 in a concentration-dependent manner (see Figure 4). As described in the literature, both were similarly potent. However, the \(IC_{50}\) values determined in our assay using a purified human CD39 enzyme preparation were about eight-fold higher than those described in the literature, 81.7 \(\mu M\) for ticlopidine and 113 \(\mu M\) for clopidogrel compared to previously reported \(K_{i,app}\) values of 14 and 10 \(\mu M\), respectively, described in the literature.\(^{[30]}\) The somewhat higher values obtained in our laboratory as compared to the literature were not due to the conditions used as different assay systems (malachite green assay, capillary electrophoresis-based assay), increase in the substrate concentration (up to 250 \(\mu M\) of ATP), change of buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] or Tris-HCl), decrease in the pH value (7.4 vs. 6.5), or different protein sources (besides native CD39 we also used recombinantly expressed CD39, and highly purified soluble CD39) confirmed our original data. Our conclusion is that the higher potency reported in the literature is likely due to the enzyme preparation used, which may have contained additional ATP-hydrolyzing enzymes other than CD39. As a reference, we tested the standard CD39 inhibitor ARL 67156 (\(I\)) in our assays, which showed an \(IC_{50}\) value consistent with the data obtained in other laboratories, thus proving the quality of our assays (see Figure 1).

Despite its moderate potency, we selected ticlopidine (\(IV\)) as a lead compound for subsequent studies due to its favorable properties as an approved drug, for example, peroral bioavailability. Ticlopidine was preferred over clopidogrel as it is structurally more simple, nonchiral, shows slightly higher potency, and is less lipophilic, resulting in higher solubility at pH 7.4.

Concentration-dependent inhibition of CD39 by ticlopidine was confirmed for both substrates, ATP and ADP, and no significant difference could be observed in inhibiting the dephosphorylation of both substrates (see Figure 5). This is in contrast to the published results, where higher potency of ticlopidine in inhibiting ADP hydrolysis as compared to ATP hydrolysis had been observed.\(^{[30,51]}\)

The inhibition type of ticlopidine was subsequently determined using recombinant human CD39 expressed in COS-7-cells using cell
| Compound | R¹ | R² | R³ | HEPES-buffer | Malachite green buffer | HEPES-buffer | Tris-buffer |
|----------|----|----|----|--------------|------------------------|--------------|-------------|
| 1        | Me | NH₂ | CO₂Et | 5 ± 1 | -3 ± 4 | -8 ± 10 |
| 2        | Et | NH₂ | CO₂Et | 10 ± 1 | -8 ± 0 | 7 ± 4 |
| 3        | t-BuOCO | NH₂ | CO₂Et | 12 ± 2 | 21 ± 7 | 40 ± 5 |
| 4        | Bn | NH₂ | CO₂Et | 15 ± 2 | 27 ± 2 | 27 ± 4 |
| 5        | Me | NH₂ | CO₂t-Bu | 6 ± 2 | -1 ± 5 | 6 ± 8 |
| 6        | Et | NH₂ | CO₂t-Bu | 7 ± 2 | 0 ± 0 | 13 ± 8 |
| 7        | t-Bu | NH₂ | CO₂t-Bu | 8 ± 2 | 34 ± 0 | 44 ± 2 |
| 8        | Bn | NH₂ | CO₂t-Bu | 5 ± 4 | 55 ± 3 | 61 ± 5 |
| 9        | Bz | NH₂ | CO₂t-Bu | 4 ± 4 | 13 ± 1 | 20 ± 12 |
| 10       | Bn | NH₂ | CN | 9 ± 2 | 51 ± 4 | 55 ± 4 |
| 11       | Bn | CO₂Et | 7 ± 2 | 15 ± 1 | 56 ± 5 |
| 12       | Bn | CO₂Et | 0 ± 3 | 29 ± 9 | 49 ± 4 |
| 13       | Bn | CO₂Et | 0 ± 4 | 43 ± 1 | 29 ± 5 |
| 14       | Bn | CO₂Et | 2 ± 2 | 66 ± 1 | 49 ± 6 |
| 15       | Bn | CO₂Et | 3 ± 2 | 78 ± 4 | 29 ± 6 |

(Continues)
| Compound | R<sup>1</sup> | R<sup>2</sup> | R<sup>3</sup> | CE-LIF assay Inhibition ± SEM (%) at 10 µM | Malachite green assay Inhibition ± SEM (%) at 100 µM |
|----------|--------------|--------------|-------------|------------------------------------------|------------------------------------------|
|          | HEPES-buffer<sup>a</sup> | HEPES-buffer<sup>b</sup> | Tris-buffer<sup>c</sup> | HEPES-buffer<sup>b</sup> | Tris-buffer<sup>c</sup> |
| 16       | Bn CO<sub>2</sub>-t-Bu | -5 ± 1       | 19 ± 6      | 39 ± 2                                   |
| 17       | Bn CO<sub>2</sub>E t <br>3 ± 0 | 26 ± 2 | 29 ± 2 |
| 18       | Bn CO<sub>2</sub>-Bu <br>-6 ± 1 | 9 ± 8 | 21 ± 11 |
| 19       | Bn CN <br>3 ± 3 | 24 ± 3 | 39 ± 4 |
| 20       | Bn CN <br>4 ± 3 | 34 ± 6 | 35 ± 5 |
| 21       | Bn CO<sub>2</sub>-Bu <br>4 ± 2 | 39 ± 3 | 58 ± 5 |
| 22       | Bn CO<sub>2</sub>-Bu <br>8 ± 2 | 35 ± 2 | 75 ± 6 |
| 23       | Bn CO<sub>2</sub>-Bu <br>6 ± 1 | 61 ± 1 | 66 ± 2 |
| Compound | R\(^1\) | R\(^2\) | R\(^3\) | CE-LIF assay Inhibition ± SEM (%) at 10 µM | Malachite green assay Inhibition ± SEM (%) at 100 µM |
|----------|--------|--------|--------|---------------------------------|----------------------------------|
|          | HEPES-Buffer | Tris-Buffer |
| 24       | Bn     | CO\(_2\)-Bu | -1 ± 1 | 16 ± 2                    |
| 25       | Bn     | CO\(_2\)-Bu | 1 ± 1  | 51 ± 3                    |
| 26       | Bn     | CO\(_2\)-Bu | 4 ± 1  | 10 ± 5                    |
| 27       | Bn     | CO\(_2\)-Bu | 2 ± 1  | 57 ± 1                    |
| 28       | Bn     | CO\(_2\)-Bu | -1 ± 5 | 10 ± 1                    |
| 29       | Bn     | CO\(_2\)-Bu | -1 ± 2 | 16 ± 4                    |
| 30       | Bn     | CO\(_2\)-Bu | -2 ± 4 | 21 ± 2                    |
| 31       | Bn     | CO\(_2\)-Bu | -6 ± 1 | 17 ± 5                    |
| 32       | Bn     | CO\(_2\)-Bu | 0 ± 2  | 76 ± 5                    |
| Compound      | CE-LIF assay Inhibition ± SEM (%) at 10 µM | Malachite green assay Inhibition ± SEM (%) at 100 µM |
|---------------|-------------------------------------------|------------------------------------------------------|
|               | HEPES-buffer<sup>a</sup>                  | HEPES-buffer<sup>b</sup>                             |
| ARL-67156 (I) | 99 ± 1                                     | 87 ± 2                                               |
| Ticlopidine (IV) | 17 ± 3                                    | 46 ± 2                                               |
|                |                                           | 48 ± 3                                               |
| Clopidogrel (V) | n.d.                                      | n.d.                                                 |
|                |                                           | 51 ± 3                                               |

Abbreviations: ATP, adenosine 5'-triphosphate; CE-LIF, capillary electrophoresis–laser-induced fluorescence; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; n.d., not determined.

<sup>a</sup>Experimental values determined by the CE-LIF assay using the artificial substrate PSB-017621A (0.5 µM) in HEPES assay buffer.<sup>[62]</sup>

<sup>b</sup>Experimental values determined by the malachite green assay in HEPES assay buffer with the natural substrate ATP (50 µM), (n = 2).

<sup>c</sup>Experimental values determined by the malachite green assay in Tris-HCl buffer with the natural substrate ATP (50 µM), (n = 3).
membrane preparations. To this end, Michaelis–Menten kinetics were measured (see Figure 6). A decrease in $V_{\text{max}}$ with increasing inhibitor concentrations was observed, which indicates an allosteric inhibition type (see Figure 6a). The intersection of lines at the x-axis in the Hanes–Woolf plot supports the characterization of ticlopidine as a noncompetitive inhibitor (see Figure 6b). A $K_i$ value of 127 ± 12 µM for ticlopidine was calculated by nonlinear regression of the values of $V_{\text{max}}$ obtained from Michaelis–Menten plots. This $K_i$ value was consistent with the $IC_{50}$ value of 81.7 µM determined for ticlopidine using a different preparation of human CD39 (see Figure 4).

Thus, we confirmed that ticlopidine acts as an allosteric, non-competitive inhibitor of CD39. As ticlopidine is an approved drug, which is well characterized in terms of its in vivo activities, it appears to be a good starting point in the search for CD39 modulators with improved properties. As ticlopidine and clopidogrel bind to CD39 in their native, nonmetabolized state, but will be oxidized by CYP enzymes in the liver and hydrolyzed to produce reactive thiols that block P2Y12 receptors on platelets (see Figure 2), they are not suitable for drug repurposing as CD39 inhibitors. We, therefore, focused our attention on related thienopyridine derivatives, whose 2-position is blocked by a suitable substituent that prevents oxidative metabolism (Figure 2).

2.2.3 Screening of a thienopyridine library

A library of 32 selected 2-substituted thienotetrahydropyridine derivatives was initially screened for inhibition of human CD39 at a low concentration of 10 µM using a fluorescence-labeled ATP derivative as a substrate and CE-LIF detector to measure product formation [62] (see Table 1). None of the compounds showed any significant CD39 inhibition in this assay. As the thienopyridines are expected to act as allosteric inhibitors as shown for ticlopidine, their activity may be dependent on the substrate used [67,68]. Thus, we switched to the malachite green assay, which allows the use of the natural substrate ATP and quantifies the release of inorganic phosphate generated by the enzymatic dephosphorylation reaction. [63] This time, screening was additionally performed at a higher concentration of 100 µM. The assays were run using two different buffer systems: HEPES or Tris-HCl (see Figure 7 and Table 1). Both buffers yielded, in most cases, consonant screening results (see Figure 7). Six of the compounds showed more than 50% inhibition of CD39 at 100 µM concentration. Compound 32 was the most potent CD39 inhibitor of the present series, showing 76% and 88% inhibition at 100 µM. Therefore, we further characterized its inhibitory potency on CD39, its mechanism of inhibition, and its selectivity versus other ectonucleotidases (see below).

2.2.4 Structure–activity relationships (SARs)

All of the investigated compounds 1–32 had a thieno[2,3-c]tetrahydropyridine core structure that is closely related to that of ticlopidine. Moreover, most of them were benzyl-substituted at the nitrogen atom of the core structure, in analogy to ticlopidine. Only in compounds 1–3, 5–7, and 9 was the benzyl residue replaced. All of the compounds were substituted in the 2- and 3-positions of the thiophene ring. This can be expected to block metabolic conversion into irreversible P2Y12 receptor antagonists as shown for ticlopidine in Figure 2. In the 3-position, a cyano group (10, 19, 20), an ethylcarboxylic acid ester (1–4, 11–15, 17), or a tert-butylcarboxylic acid ester (5–9, 16, 18, 21–32) was present. Position 2 was substituted with an amino group (1–10), a benzamido residue (11), a thiourea group (12–19), a cyclized isothiourea group (20), a urea (21–26, 28–32), or a carbamate (27) function.

R1 position: A small aliphatic methyl or ethyl substituent resulted in inactive compounds (1, 2, 5, and 6), while a larger, more lipophilic isobutyl residue restored some potency (compound 7); however, a benzyl residue was superior (4, 8, 10). Interestingly, a tert-butylxycarbonyl substituent (compound 3) was also well tolerated in that position. We selected the benzyl substituent at N6 for exploring further substituents on the thiophene ring.

R2 position: A substituent in this position was deemed important for protecting the thiophene ring from metabolic oxidation by CYP enzymes in the liver. A simple amino substituent as in compounds 1–10 was initially probed, but might not be ideal as it may also be susceptible to oxidation. It was well tolerated by CD39 (e.g., in compounds 8 and 10). Some larger, substituted thiourea (e.g., 14 and 15) and urea derivatives (e.g., 23 and especially 32) showed similar or, in many cases, even higher inhibitory potency. In contrast, a plain urea function in position 2 led to poor potency (compound 26).

R3 position: The investigated compound library contained three variations in this position: a cyano group, an ethylcarboxylate, or a tert-butylcarboxylate. In the case of the 2-amino-substituted benzylthienotetrahydropyridines, a cyano group led to similar activity as a tert-butylcarboxylate, while ethylcarboxylate derivatives were less potent (compare 4, 8, and 10). However, for derivatives with a large benzothiourea residue in position 2, the difference in potency between the various substituents in the 3-position was smaller (compare 17–19). This showed that the SARs at different positions were interdependent.

2.2.5 Characterization of the new CD39 inhibitor 32

Screening identified N-benzyl-thienotetrahydropyridine derivative 32, bearing a lipophilically substituted urea in position 2 and a tert-butylcarboxylate in the 3-position, as the most potent CD39 inhibitor of the present series. Subsequently, this new CD39 inhibitor was broadly characterized. In silico studies predicted no metabolic conversion of compound 32 into a thiol-reactive metabolite, in contrast to ticlopidine (for details, see the Supporting Information). Concentration-inhibition curves for 32 were constructed in three orthogonal assay systems for membrane-bound and soluble CD39, respectively (see Figure 8 and Table 2). Soluble CD39 constitutes a highly pure recombinant protein consisting only of the extracellular
domain of the enzyme, which harbors the active center, while the transmembrane and intracellular domains have been cut off. The inhibitory potency in all assays and enzyme preparations was similar for 32 as well as ticlopidine. The allosteric binding site for the inhibitors can, therefore, be assumed to be independent of the transmembrane regions of the membrane-bound protein.

2.2.6 | Inhibition type of compound 32 for CD39

The inhibition type of 32 was determined by measuring Michaelis–Menten kinetics as shown in Figure 9. A decrease in $V_{\text{max}}$ with increasing inhibitor concentrations was observed, which indicates an allosteric type of inhibition (see Figure 9a,c). The intersection of lines at the x-axis in the Hanes–Woolf plot supports the characterization of 32 as a noncompetitive inhibitor (see Figure 9b).

2.2.7 | Selectivity studies

As a next step, we investigated the potency of the CD39 inhibitor 32 to inhibit other ectonucleotidases to assess its selectivity, namely, all other ecto-NTPDases (NTPDase2, -3, and -8) and ecto-5’-nucleotidase (CD73). The standard inhibitor ticlopidine and the lead structure 32 were investigated in the same assays for comparison. Initial inhibition screening on the membrane-bound NTPDases was performed at concentrations of 100 and 300 µM (see Figure 10). Ticlopidine tested at a concentration of 300 µM inhibited all investigated ectonucleotidases to a similarly high extent, except for NTPDase8; it can therefore be characterized as a nonselective ectonucleotidase inhibitor at high concentrations, which are, however, clinically irrelevant when used as an antithrombotic drug. The new CD39 inhibitor 32 tested at 100 and 300 µM at ecto-NTPDases showed concentration-dependent inhibition of the ecto-NTPDases, which was, however, lower than the inhibition of CD39, indicating some CD39 selectivity. Only CD73 was also potently inhibited by 32. Concentration–inhibition curves were constructed in the case of over 60% inhibition of enzymatic activity (see Table 2). While compound 32 and ticlopidine showed similar CD39-inhibitory potency for both membrane-bound and soluble CD39, 32 was more selective versus the other NTPDase isoenzymes than ticlopidine. Interestingly, 32 also blocked CD73 with similar potency as CD39 and can therefore be envisaged as a dual CD39/CD73 inhibitor (see Table 2).

3 | CONCLUSIONS

The antithrombotic prodrug ticlopidine in its native, unmetabolized form was confirmed to inhibit the ectonucleotidase CD39, which has been proposed as a novel target for cancer immunotherapy.
Broad characterization of ticlopidine showed that it acted as an allosteric CD39 (NTPDase1) inhibitor, additionally blocking NTPDase2 and NTPDase3, as well as ecto-5′-nucleotidase (CD73). Importantly, we discovered analogs of ticlopidine in which the 2-position is blocked by substitution, thereby preventing oxidative metabolism to reactive thiols (see Figures S2 and S3), a step that is required for the antithrombotic activity of ticlopidine and its derivatives, for example, clopidogrel. The best inhibitor of the present series, compound 32, was characterized as a dual CD39/CD73 inhibitor. Such a profile is desirable to increase the extracellular concentrations of immunostimulatory ATP and to decrease the concentrations of cell proliferation-enhancing, immunosuppressive, and angiogenic adenosine in cancer patients. Future efforts to enhance the ectonucleotidase inhibitory activity of ticlopidine derivatives and analogs, which are devoid of P2Y12 receptor–antagonistic activity, but endowed with drug-like properties, are warranted.

4 | EXPERIMENTAL

4.1 | Chemistry

The investigated compounds were available from previous studies. The synthetic procedures and structural characterizations have already been published as follows, 1–3, 4, 11, 5–9, 15, 16, 18, 21–32, 10, 19, 20, 12, 13, 17, and 14.
4.2 Biological assays

4.2.1 Material

ATP, ammonium heptamolybdate, Brij®L23, calcium chloride, dimethyl sulfoxide (DMSO), magnesium chloride, malachite green, HEPES, polyvinyl alcohol, ticlopidine-HCl, and clopidogrel were obtained from Sigma-Aldrich. Tris(hydroxymethyl)aminomethane was purchased from AppliChem; disodium hydrogenphosphate, lanthanum chloride, sodium chloride, and sulfuric acid were purchased from Carl Roth. N6-[6-(Fluoresceinyl-5′-carboxamido)hexyl]-ATP (PSB-170621A) was obtained from Jena Bioscience. [2,8-3H]AMP (solution in ethanol/water, 1:1, 22.9 Ci/mmol, 1.0 mCi/ml, 849 GBq/mmol) was obtained from Hartmann Analytic, Cellfectin™ II reagent was obtained from Thermo Fisher Scientific, and the baculovirus genomic ProEasy™ vector DNA was obtained from AB vector. Insect-XPRESS™ media (# BE12-730Q; Lonza) and HisPur™ Ni2+-NTA spin columns (# 88226; Thermo Fisher Scientific) were used as protein expression and purification tools.

4.2.2 Enzyme preparation of human CD39

Human umbilical cord membrane preparations were obtained as previously reported with consent and under the approved institutional review board protocol (Comité d’Éthique de la Recherche du CHU de Québec—Université Laval). Stock solutions were aliquoted and stored at −80°C until use in the assay systems.

4.2.3 Expression of human recombinant CD39 (NTPDase1), NTPDase2, NTPDase3, and NTPDase8

NTPDase isoenzymes were recombinantly expressed in COS-7 cells by adding Lipofectamine and an expression vector (pcDNA3) containing the cDNA for the respective ectonucleotidase (human CD39 [GenBank accession no. U87967], human NTPDase2 [NM_203468], human NTPDase3 [AF034840], and human NTPDase8 [AY430414]). Cells were harvested after 40–72 h and membrane preparations were obtained and stored in 95 mM NaCl and 45 mM Tris, pH 7.5, according
TABLE 2  Selectivity of compound 32 and ticlopidine

| Enzyme          | IC₅₀ ± SEM (µM) (or % inhibition at 300 µM) |
|-----------------|--------------------------------------------|
|                 | CD39a                                      |
|                 | 84.8 ± 15.8                                |
|                 | 81.7 ± 5.0                                 |
|                 | CD39b                                      |
|                 | 57.7 ± 10.0                                |
|                 | 72.8 ± 13.9                                |
|                 | Soluble CD39c                              |
|                 | 45.2 ± 4.1                                 |
|                 | 33.3 ± 6.2                                 |
|                 | NTPDase2a                                   |
|                 | ≈ 300 (54% ± 8%)                           |
|                 | 170 ± 24                                   |
|                 | NTPDase3a                                   |
|                 | >300 (37% ± 6%)                            |
|                 | 149 ± 11                                   |
|                 | NTPDase8a                                   |
|                 | >300 (36% ± 4%)                            |
|                 | >300 (28% ± 3%)                            |
|                 | Soluble CD73d                               |
|                 | 45.8 ± 0.9                                 |
|                 | 192 ± 37                                   |

Note: Mean IC₅₀ values are shown in bold.

Abbreviations: AMP, adenosine monophosphate; ATP, adenosine 5′-triphosphate; NTPDase: ectonucleoside triphosphate diphosphohydrolase.

The assays were performed using the malachite green assay with ATP as a substrate (50 µM, Kₘ [CD39] = 17 µM) and recombinant human membrane-bound CD39 (NTPDase1), or with the ATP substrate (100 µM) and recombinant human membrane-bound NTPDase2, -3, or -8 (Kₘ [NTPDase2] = 70 µM; Kₘ [NTPDase3] = 75 µM; Kₘ [NTPDase8] = 46 µM).[69] Initial testing of compounds at 300 µM.

The assay was performed using recombinant CD39 and PSB-017621A as a substrate (0.5 µM); quantification of product formation by capillary electrophoresis with laser-induced fluorescence detection.

The assay was performed with soluble CD39 and ATP as a substrate (150 µM). Detection of product concentration by capillary electrophoresis with UV-detection.

The assay was performed with soluble human CD73 using a radioassay with radioactively labeled AMP as a substrate (5 µM, [2,8-³H]AMP (specific activity 7.4 × 10⁸ Bq/mmol, 20 mCi/mmol)). All data were obtained from three independent experiments (n = 3).

**FIGURE 9**  Inhibition type determination for 32 for CD39. Recombinant human CD39 was used and the malachite green assay was used in the presence of 0, 50, 100, and 150 µM inhibitor 32 with adenosine 5′-triphosphate (ATP) as a substrate (10–100 µM). Data are means ± SEM of three independent experiments. (a) Michaelis–Menten plot; (b) Hanes–Woolf plot where the intersection of lines at the x-axis indicates a noncompetitive inhibition type. The Ki value was calculated for each iteration by nonlinear regression of the Michaelis–Menten plot data applying the following equation: \( V_{max} = V_{max} / (1 + [I]/K_i) \). The mean Ki value was calculated as 321 ± 37 µM. (c) V_max and K_m values of CD39 in the absence or presence of the inhibitor.
to previously published procedures. The protein samples were aliquoted and stored at −80°C.

4.2.4 | Expression and purification of soluble human CD39

Soluble human CD39 was expressed as the recombinant enzyme in Spodoptera frugiperda (sf9) cells using a baculovirus transfection system. The transmembrane domain was excised and the gene of interest (amino acids 38–478) was subcloned into the expression vector pACGP67 B, which induces the release of the expressed protein into the cell culture media as described. The soluble CD39 was collected from the culture media and concentrated using Amicon® Ultra centrifugal filters. The C-terminally His10-tagged protein, which contained a GP67 secretion signal, was purified by HisPurTM Ni2+-NTA spin columns. Further details will be published elsewhere.

4.2.5 | Fluorescence-based capillary electrophoresis assay for CD39

The effects of the test compounds on CD39 activity were initially determined by a previously established capillary electrophoresis (CE)-based assay. Briefly, test compounds were assayed at 10 µM with 40 ng of human umbilical cord membrane preparation natively expressing high levels of CD39, while the concentration–inhibition curves of ticlopidine and compound 32 were determined at 10 different concentrations ranging from 0.1 to 500 µM, and 124 ng of recombinantly expressed CD39. Samples were incubated with 0.5 µM fluorescent ATP derivative PSB-017621A used as an artificial substrate ($K_m = 19.6 \mu M$) suspended in a reaction buffer consisting of 10 mM HEPES, 2 mM CaCl$_2$, and 1 mM MgCl$_2$, pH 7.4, in a final volume of 100 µl. Incubation at 37°C was performed for 4 min, followed by heating at 90°C for 5 min to prevent further enzymatic degradation of the substrate. Before separation and detection of the fluorescantly labeled nucleotides by CE–LIF detection, 10 µl of each sample were diluted with 190 µl of reaction buffer. Measurements were performed using a P/ACE MDQ capillary electrophoresis system (Beckman Instruments) and evaluated with P/ACE MDQ software 32 KARAT obtained from Beckman Coulter (Fullerton). A polyacrylamide-coated capillary [30 cm (10 cm effective length) × 50 µm (id) × 360 µm (od)] was rinsed with background electrolyte (50 mM phosphate buffer (pH 6.5)) for 1 min at 30 psi. Samples were introduced by 30 s of an electrokinetic injection at −6 kV and separated by applying −15 kV of voltage. Fluorescent analytes were detected at 488 nm excitation and 520 nm emission wavelength.

4.2.6 | Malachite green assay for NTPDases

The enzyme activity assays were performed as previously described and adapted in the course of the study to improve the analysis of the investigated compound class. The reaction buffer contained 80 mM Tris-HCl buffer supplemented with 5 mM CaCl$_2$, pH 7.4, or 10 mM HEPES buffer with 2 mM CaCl$_2$ and 1 mM MgCl$_2$, pH 7.4, in a final volume of 100 µl in transparent 96-well half-area plates. All experiments were performed in three independent iterations in triplicate. The final concentration of DMSO was 2% in all experiments. Human umbilical cord membrane preparations containing high levels of CD39 or the respective recombinant COS-7-cell membrane preparations expressing the appropriate NTPDase isozyme (ca. 100 ng of protein depending on the enzyme activity) were preincubated with or without an inhibitor at 37°C with gentle shaking (Eppendorf Thermomixer comfort at 500 rpm) for 5 min. An amount of enzyme was added that allowed 10–20% of substrate conversion. The enzymatic reaction was initiated by the addition of 50 µM ATP (in a few cases, 100 µM ADP was used instead of ATP to compare the effects vs. both substrates; see Figure 5)
for CD39 ($K_m$ [CD39] ATP = 17 µM, ADP = 22 µM) or 100 µM ATP for NTPDases 2, -3, and -8 ($K_m$ [NTPDase2] = 70 µM; $K_m$ [NTPDase3] = 75 µM; $K_m$ [NTPDase8] = 46 µM). The released (inorganic) phosphate was quantified after 20 min at 25°C by measuring the absorption of the malachite green–phosphomolybdate complex at 600 nm. The absorption values were corrected by subtracting the absorption of negative control samples, which were incubated with denatured enzyme (90°C, 15 min), and the phosphate concentration was calculated based on a separate standard curve. The inhibition was calculated by subtracting the corrected absorption of the test sample (T) from the average absorption of positive controls without the inhibitor (B) and divided by B.

\[
\% \text{Inhibition} = \left( \frac{B - T}{B} \right) \times 100.
\]

The inhibitor screening was conducted at a concentration of 100 µM: selectivity studies were performed at 300 µM. The concentration–inhibition curves of ticlopidine, ticlopidine, and 32 were prepared by serial dilution, with the final inhibitor concentrations ranging from 0.1 to 300 µM using 50 ng of membrane preparation of COS-7 cells containing recombinant CD39 or NTPDase3 ($n = 3$). To determine the inhibition type of ticlopidine and 32 for CD39, the initial velocity of the enzyme reaction was measured as a function of the concentration of the substrate ATP (10–100 µM) in the absence and presence of various concentrations of the inhibitor (50, 100, and 150 µM). The results were analyzed and plotted using GraphPad Prism 8 software. Three independent experiments were performed with triplicate observations and presented as mean ± SEM.

### 4.2.7 Capillary electrophoresis assay for soluble CD39

Measurements were performed using a P/ACE MDQ capillary electrophoresis system (Beckman Instruments) with a 20-cm polyacrylamide-coated capillary and evaluated using P/ACE MDQ software 32 KARAT obtained from Beckman Coulter. CD39 activity of the recombinant soluble enzyme was analyzed using a previously reported CE assay with ATP as a substrate. Briefly, the reaction was started after adding 75 ng of the enzyme, diluted in reaction buffer consisting of 10 mM HEPES, 2 mM CaCl2, and 1 mM MgCl2, pH 7.4, to the mixture containing 150 µM substrate either with or without an inhibitor. Samples were incubated at 37°C for 30 min, followed by 10 min of heating at 95°C to terminate the reaction. Samples were introduced by 30 s of an electrokinetic injection at ~6 kV and separated by a ~15 kV voltage application. Full-scale inhibition analysis was performed for a series of inhibitor dilutions (0.005–300 µM) to determine the IC50 values. Three independent experiments were performed with triplicate observations and presented as mean ± SEM.

### 4.2.8 CD73 assay

The inhibitory effects of ticlopidine and compound 32 on human soluble CD73 were assessed using a previously published radiometric assay. Soluble human CD73 (0.09 µg/ml) was incubated with 5.0 µM radioactive substrate [2,8-3H]AMP (specific activity 7.4 × 108 Bq/mmol, 20 mCi/mmol) and the respective test compound for 25 min at 37°C in a shaking water bath. The assay buffer contained 25 mM Tris-HCl, 140 mM NaCl, and 25 mM NaH2PO4 at pH 7.4. Free phosphate and unconverted [2,8-3H]AMP were precipitated upon the addition of 500 µl of cold precipitation buffer (100 mM LaCl3, 100 mM sodium acetate, pH 4.0) during incubation for 30 min on ice. Samples were filtered through GF/B glass fiber filters using a Brandel cell harvester (Brandel), and each reaction vial was washed three times with 400 µl of cold (4°C) demineralized water. Aliquots of the filtrate were mixed with 5 ml of scintillation cocktail (ULTIMA Gold XR9). The product of the enzymatic reaction, radioactive adenosine, present in the filtrate, was subsequently quantified by liquid scintillation counting (TRICARB 2900 TR; Packard/PerkinElmer).

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### CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interests.

### AUTHOR CONTRIBUTIONS

Laura Schäkel, Michael Gütschow, and Christa E. Müller wrote the manuscript with contributions from all coauthors. Markus Pietsch synthesized the compounds and Tim Keuler checked compound identities. Laura Schäkel, Salahuddin Mirza, Sang-Yong Lee, and Katharina Sylvester tested the compounds at ectonucleotidases. Julie Pelletier and Jean Sévigny isolated and purified human CD39. Salahuddin Mirza produced and purified soluble human CD39. Vigneshwaran Namasiyavam performed in silico metabolism studies. Michael Gütschow and Thanigaimalai Pillaiyar contributed to the selection of the compounds. Christa E. Müller designed and supervised the project.

### ORCID

Laura Schäkel  
Markus Pietsch  
Jean Sévigny  
Thanigaimalai Pillaiyar
