



Cladribine as a Potential Object of Nucleoside Transporter-Based Drug Interactions

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Abstract

Cladribine is a nucleoside analog that is phosphorylated in its target cells (B and T-lymphocytes) to its active triphosphate form (2-chlorodeoxyadenosine triphosphate). Cladribine tablets 10 mg (Mavenclad[®]), administered for up to 10 days per year in 2 consecutive years (3.5-mg/kg cumulative dose over 2 years), are used to treat patients with relapsing multiple sclerosis. Cladribine has been shown to be a substrate of various nucleoside transporters (NTs). Intestinal absorption and distribution of cladribine throughout the body appear to be essentially mediated by equilibrative NTs (ENTs) and concentrative NTs (CNTs), specifically by ENT1, ENT2, ENT4, CNT2 (low affinity), and CNT3. Other efficient transporters of cladribine are the ABC efflux transporters, specifically breast cancer resistance protein, which likely modulates the oral absorption and renal excretion of cladribine. A key transporter for the intracellular uptake of cladribine into B and T-lymphocytes is ENT1 with ancillary contributions of ENT2 and CNT2. Transporter-based drug interactions affecting absorption and target cellular uptake of a prodrug such as cladribine are likely to reduce systemic bioavailability and target cell exposure, thereby possibly hampering clinical efficacy. In order to manage optimized therapy, i.e., to ensure uncompromised target cell uptake to preserve the full therapeutic potential of cladribine, it is important that clinicians are aware of the existence of NT-inhibiting medicinal products, various lifestyle drugs, and food components. This article reviews the existing knowledge on inhibitors of NT, which may alter cladribine absorption, distribution, and uptake into target cells, thereby summarizing the existing knowledge on optimized methods of administration and concomitant drugs that should be avoided during cladribine treatment.

1 Introduction

Multiple sclerosis (MS) is a chronic and debilitating autoimmune disorder of the central nervous system, affecting about 2.8 million people worldwide [1]. B and T-lymphocytes are believed to play a major role in the pathophysiology of MS [2]. Cladribine tablets 10 mg [3] were shown to have significant efficacy in the treatment of relapsing MS in placebo-controlled phase III trials [2, 4, 5]. A cumulative dose of

3.5 mg/kg body weight (consisting of two annual courses each comprising two treatment weeks; at the start of the first month and at the start of the second month of each year) has been approved in more than 80 countries including across Europe and the USA for the treatment of adults with certain types of relapsing MS [3, 6].

Recent immunophenotyping data demonstrated that effective doses of oral cladribine induced a 20–30% reduction of CD8⁺ T-cells and a 40–45% reduction of CD4⁺ T-cells during the first 12 months after initiation of treatment. This was accompanied by a comparable reduction in memory T-cells. The most prominent effect of cladribine, however, was a marked (80–85%) and long-lasting reduction in CD19⁺ B-cells [7, 8], suggesting that cladribine primarily confers a substantial B-cell reduction characterized by slow re-population kinetics, along with a long-term memory B-cell reduction [9]. Based on this mechanism, cladribine was characterized to act as an immune reconstitution therapy, which means the induction of immune resetting processes resulting in long-term efficacy.

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Key Points

Cladribine tablets are used to treat patients with relapsing multiple sclerosis.

In this article, the authors consider how interactions with nucleoside transporters may affect the clinical efficacy of cladribine tablets, provide a summary on optimized approaches to administration, and identify which concomitant drugs should be avoided.

Cladribine is a chlorinated analog of deoxyadenosine. The chlorine substitution for hydrogen in its purine ring renders cladribine largely resistant to deamination by adenosine deaminase, thereby prolonging its intracellular residence time with accumulation in the target cell cytoplasm [10]. Cladribine is a prodrug that is phosphorylated intracellularly to its active form, 2-chlorodeoxyadenosine triphosphate (2-Cd-ATP). Formation of 2-Cd-ATP is a sequential three-step process: first, cladribine is phosphorylated to 2-chlorodeoxyadenosine monophosphate by nuclear/cytosolic enzyme deoxycytidine kinase (DCK) and mitochondrial deoxyguanosine kinase. Thereafter, 2-chlorodeoxyadenosine monophosphate is further phosphorylated by nucleoside monophosphate kinase to 2-Cd-ADP, and nucleoside diphosphate kinase to 2-Cd-ATP. In most cells, 2-chlorodeoxyadenosine monophosphate is promptly dephosphorylated by 5'-nucleotidase (5'-NT) and, consequently, accumulation of 2-Cd-ATP depends on the intracellular ratio of DCK and 5'-NT enzymes [11]. Cells with a high endogenous DCK/5'-NT activity ratio (as is the case in B and T-lymphocytes) accumulate deoxynucleotides to toxic concentrations, resulting in cell death. By this mechanism, cladribine exerts a selective mode of action on B and T-lymphocytes [12, 13]. Thus, constitutive variations in the expression levels and activities of DCK and 5'-NT between immune cell subtypes explain differences in immune cell sensitivity to cladribine.

Recently, an important relationship was shown between DCK activity and the effect of cladribine on B and T-cells, depending on their activation status. Accordingly, cladribine induced apoptosis in stimulated T and B-cells and unstimulated B-cells [10], which may well explain the overall more pronounced activity on B-lymphocytes. Moreover, cells of the innate immune system are generally less affected than cells of the adaptive immune system [13, 14]. The preferential reduction in lymphocyte subpopulations, followed by the pattern of lymphocyte recovery (termed immune reconstitution), is believed to 'reset' the immune system to a less autoreactive state [15].

As a nucleoside analog, cladribine is a hydrophilic compound that relies on transporter-mediated cellular uptake as a prerequisite for its downstream metabolic activation, intracellular function, and clinical effectiveness. Numerous studies have shown that the distribution of cladribine across biological membranes is facilitated by a number of uptake and efflux transporters. The role of different ABC, solute carrier (SLC), and nucleoside transporter (NT) proteins in transmembrane permeability of cladribine to facilitate absorption, distribution throughout the body, intracellular uptake into target immune cells, and renal excretion of the compound has been recently reviewed [16].

Among the key ABC (ATP-binding cassette) efflux transporters, only breast cancer resistance protein (BCRP) has been shown to be an efficient transporter of cladribine, while P-glycoprotein (P-gp) does not transport cladribine well. Breast cancer resistance protein modulates the oral absorption of cladribine, while its renal excretion appears to be most likely driven by BCRP, equilibrative NT1 (ENT1), and P-gp [11].

Nucleoside transport in humans is mediated by members of two unrelated protein families, the SLC28 family of cation-linked concentrative NTs (CNTs) and the SLC29 family of energy-independent, equilibrative NTs (ENTs). These families contain three (CNT1, CNT2, and CNT3) and four (ENT1, ENT2, ENT3, and ENT4) members, respectively [17, 18]. Together, they play key roles in nucleoside and nucleobase uptake for salvage pathways of nucleotide synthesis. Moreover, they facilitate cellular uptake of several nucleoside and nucleobase drugs.

Transporter-mediated intestinal absorption, distribution throughout the body, and intracellular uptake of cladribine appear to be essentially mediated by ENTs and CNTs, specifically by ENT1, ENT2, and ENT4, and CNT2 (low affinity) and CNT3, while intestinal efflux of cladribine is modulated by BCRP, and renal excretion of cladribine appears to be most likely driven by BCRP, ENT1, and P-gp. The latter may play a role in the renal excretion of cladribine despite its poor cladribine transport efficiency in view of the high renal abundance of P-gp [19, 20]. There is no evidence that SLC uptake transporters, such as OATPs, OATs, and OCTs, are involved in the transport of cladribine [11].

Nucleoside transporter-based drug interactions affecting absorption and target cellular uptake of a prodrug such as cladribine are unlikely to be relevant in terms of safety, i.e., they are not expected to give rise to clinically notable adverse events. However, they are likely to reduce systemic bioavailability and target cell exposure, thereby possibly hampering clinical efficacy. Alteration of clinical efficacy by drug interactions is clinically hardly notable because decreased efficacy is not necessarily associated with notable adverse events; therefore, this type of interaction has been denoted "silent interactions" [16]. In order to manage

optimized therapy, i.e., to preserve the full therapeutic potential of cladribine treatment in clinical practice, it is of critical importance that clinicians are aware of the existence of such “silent interactions” based on inhibition of NTs. This article therefore reviews the potential of certain food components such as dietary purines, various lifestyle drugs, and marketed medicinal products for the alteration of cladribine absorption, distribution, and uptake into target cells by inhibition of NT, thereby summarizing the existing knowledge on optimized methods of administration and concomitant drugs that should be avoided during cladribine treatment.

2 Methodology

This work aims to review food components, lifestyle drugs, and marketed prescription drugs reported to act as inhibitors of ENTs (ENT1, ENT2, and ENT4), and CNTs (CNT2 and CNT3). The NT selection of interest was based on published preclinical and clinical evidence, indicating that cladribine is a substrate of these proteins, and thus may become object of NT-based food/drug interactions in the presence of NT inhibitors. The information on the NT substrate characteristics of cladribine, and the role, function, as well as tissue and immune cell abundance of the NT of interest was part of a comprehensive cladribine transporter review recently published by our group [11]. In addition, a systematic review of published literature on NT inhibitors was undertaken using Medline In-Process (PubMed) and limited to English-language publications. As target cell uptake and efflux are of particular relevance to the disposition (and efficacy) of cladribine, search terms included the keywords “cladribine/2-CdA”, “ENT and CNT inhibitors”, “adenosine”, “adenosine re-uptake inhibitors”, and specific white blood cell subsets, with a particular emphasis on B and T-lymphocytes. Apart from the primary PubMed search that was conducted in May 2019, repeated secondary searches have been conducted on an ongoing basis during the preparation of this article, i.e., the authors continued to screen databases for upcoming relevant publications at regular intervals. Listed references of identified pertinent publications were also systematically reviewed to identify additional relevant publications that were not identified by the primary and secondary database searches.

In the absence of published *in vivo* drug–drug interaction (DDI) studies for specific NT inhibitors, the assessment of a potential *in vivo* DDI risk was based on available *in vitro* data of identified NT inhibitors describing transporter inhibition such as the half-maximal inhibitory concentrations (IC_{50}) and/or the inhibition constants (K_i). These *in vitro* data were applied to calculate regulatory thresholds for *in vivo* DDI risk according to current DDI regulatory guidelines [European Medicines Agency (EMA) and US Food and Drug Administration (FDA)] in the context of their available

in vivo exposure data, with a particular emphasis on the non-protein-bound free fraction (i.e., fraction unbound = f_u) of the compounds and their reported maximum *in vivo* exposure achieved at steady state after administration of the highest recommended clinical doses. As no NT-specific cut-off values for potential *in vivo* relevance are currently provided in regulatory guidelines (EMA/FDA) or by the International Transporter Consortium, i.e., commonly accepted cut-off values are only available for unidirectional intestinal, hepatic, renal, and blood–brain barrier uptake and efflux transporters, and bidirectional renal proton antiporters (i.e., MATEs), but not for bidirectional equilibrative (i.e., passive) transporters such as ENTs, and different transporters may have different cut-off values, we decided to apply the most conservative approach recommended in the EMA guideline for renal transporters, which is the use of inhibitor concentrations of 50-fold unbound maximum concentration ($C_{max,u}$) divided by IC_{50} or K_i , which results in a cut-off value of 0.02. This means that NT-inhibiting activities yielding a cut-off ≥ 0.02 were considered possibly clinically relevant for orally administered drugs. In addition to the generally applicable regulatory thresholds for anticipated *in vivo* DDI risks of various non-NTs, it was recently proposed specifically for the assessment of clinical significance of ENT1 inhibition as assessed by specific adverse events known to be associated with elevated adenosine levels to define a high degree of ENT1 inhibition *in vivo* when $C_{max,u}$ covers a concentration of $\geq 4 \times IC_{50}$ as determined by cellular adenosine uptake assays or $\geq 13.3 \times K_i$ as determined by [3H] NBTI (nitrobenzylthioinosine) binding assays [21].

3 Background and Role of NTs and Their Inhibition

Inhibition of ENTs and CNTs may affect intestinal absorption of nucleoside derivatives such as cladribine, as well as their distribution into endothelial cells, red and white blood cells, uptake and excretion by the liver and kidney, and permeation of the blood–brain barrier, thereby impeding access to the central nervous system [18, 22]. To date, there are few clinical DDI studies available showcasing the effects of potent ENT/CNT inhibitors on the pharmacokinetics and distribution of sensitive ENT/CNT substrates. However, there are many studies linking NT expression in cells of various hematological malignancies (e.g., CLL, CML) and solid tumors (e.g., pancreatic cancer) to intracellular accumulation and clinical response to nucleosidic anti-cancer drugs. Therefore, NTs could be considered as predictive biomarkers of drug targets and indicators of responsiveness to those treatments [22].

For the assessment of a potential ENT/CNT-based risk for drug interactions, the first step is to review the currently

available evidence, as to which marketed products have been identified and characterized by *in vitro* testing as potent ENT/CNT inhibitors. The second step is an attempt to translate these *in vitro* findings to potential *in vivo* relevance by looking for accepted biomarkers of NT inhibition. As adenosine represents the main endogenous substrate of NTs, which are essential for adenosine re-uptake, extracellular adenosine levels can be elevated as a result of NT inhibition *in vivo*, although other mechanisms (e.g., inhibition of adenosine metabolism) can also result in elevated plasma adenosine levels [23]. Among the NT most relevant for adenosine re-uptake, ENT1 has been identified as a major player. Nevertheless, other transporters such as CNT2 and CNT3 also contribute to purinergic modulation based on their high affinity for adenosine and concentrative capacity, and depending on their tissue or cellular abundance [22, 24]. However, most of the currently available marketed medicinal products that have been reported to inhibit NT display almost exclusively high potencies for inhibition of ENTs, primarily ENT1 and to some extent also ENT2 (Table 1). An exception to this rule is the tyrosine kinase inhibitor (TKI) imatinib, which has been shown to be a potent and fairly selective CNT2 inhibitor (IC_{50} CNT2 = 2.3 $\mu\text{mol/L}$) [25] (Table 1). Apart from imatinib, there are few potent or specific pharmacological CNT inhibitors among marketed products. Thus, the few products that have been reported to inhibit CNTs at relatively low $\mu\text{mol/L}$ concentrations are rather to be considered as pan ENT/CNT inhibitors that usually inhibit ENT1 with much higher potency than ENT2 and CNTs. Therefore, the present review primarily focuses on marketed ENT1 inhibitors.

Some pharmacokinetic considerations are relevant for the potential *in vivo* relevance of *in vitro* NT inhibition by identified perpetrator drugs. These include $C_{\text{max,u}}$ achieved with recommended doses, possible accumulation of plasma concentrations upon repeat dosing, and plasma protein binding, as only the free fraction of a perpetrator drug actually can confer transporter inhibition. Other factors include the transporter affinities and dissociation kinetics of putative perpetrator drugs and their mechanism of inhibition, i.e., competitive, non-competitive, or irreversible inhibition. In a recent study, it was shown that currently available ENT1 perpetrator drugs display relatively fast dissociation kinetics, and yield only short-to-moderate residence times between 1 and 44 minutes at the transporter protein [26]. The underlying mechanism of ENT1 inhibition is predominantly competitive with few exceptions of non-competitive inhibition [27]. To the best of our knowledge, there are no irreversible NT inhibitors among currently marketed drugs. The lack of irreversible ENT inhibitors along with the short residence times at the transporter protein allows, in principle, clinical management of such interactions by relatively simple means, namely, by time separation of the intake of the ENT

substrate and perpetrator drugs, under careful consideration of the inhibitor's terminal plasma half-life.

Because of the paucity of *in vivo* drug interaction studies with potent ENT inhibitors, reliable quantitative assessments that may build the scientific basis for judgments on potential clinical relevance are currently not possible. However, reasonable mechanistic considerations on potential drug interaction risks, based on the criteria detailed above, along with transporter expression pattern and their established roles in the transport of nucleosides and nucleosidic drugs, are considered valuable information to prevent possibly significant drug interactions in the clinical use of ENT/CNT substrates such as cladribine. In this context, it is noteworthy that both alterations of the bioavailability of cladribine and its distribution into target cells (i.e., lymphocytes) by potent ENT inhibition most likely represent a "silent interaction" that cannot be detected by clinical adverse events but may result in altered or sub-optimal clinical efficacy. It is therefore important to pay attention to the avoidance of co-administration of known potent ENT inhibitors shortly before and during the few days of cladribine treatment. In addition to DDI, the potential NT-inhibitory effects of food components such as dietary purines, alcohol, and lifestyle drugs such as cannabinoids are also considered below.

3.1 Food Effects

Based on existing knowledge on the ENT/CNT substrate characteristics of cladribine, the intestinal uptake of cladribine can, in principle, be facilitated by intestinal apical uptake transporters such as concentrative NTs CNT2 [17, 28] and CNT3 [17, 29, 30], while efflux from the intestinal enterocytes into the mesenteric blood is, in principle, facilitated by the basolateral located ENTs [24, 31, 32]. Among the ENT family, ENT1 is likely to be the key transporter mediating nucleoside efflux into the mesenteric blood as its expression and activity are higher than that of co-localized ENT2 [17]. CNT2 has been suggested as the jejunal NT of purine nucleosides [33], and CNT3 transports both pyrimidine and purine nucleosides [17].

For the antiviral nucleoside analog ribavirin, which displays similar ENT1/2 and CNT2/3 substrate characteristics as cladribine [22], a study in healthy volunteers examined the effects of the dietary purine content of meals on the pharmacokinetics of orally administered ribavirin. Participants were administered a single 600-mg oral dose of ribavirin either after a high-purine meal (purine content 192.1 mg) or a low-purine meal (purine content 7.56 mg). Maximum concentration and area under the concentration–time curve from zero to 14 h values of ribavirin following a low-purine meal were 136% (90% confidence interval 120–155) and 139% (90% confidence interval 120–159) higher than corresponding data obtained after the high-purine meal, indicating a

Table 1 Summary of NT inhibitors

| Compound class | Class representative(s) with NT inhibitor characteristics | Supposed mechanism | In vitro inhibition | References ^a | C _{max} (dose) | Protein binding (%) | $(C_{\max} \times f_u)/K_i$ or $(C_{\max} \times f_u)/IC_{50}$ |
|----------------|---|---|---|--|--|---------------------|--|
| Food | Purine-rich meals | Competitive inhibition of intestinal CNT2 and CNT3 | n.d. | Li et al. [34] | | | n.d. |
| Alcohol | Alcohol | Selective ENT1 inhibition In vitro ENT1 inhibition shown at lower ethanol exposures that are known to occur in vivo by consumption of one to two drinks per day Down-regulation of ENT1 expression (long-term effect) | 2 mM reduced gemcitabine cytotoxicity in the human bladder cancer cell line HBT2 | Ramadan et al. [45] | | | n.d. |
| Cannabinoids | Cannabidiol | Competitive, selective ENT1 inhibition | IC ₅₀ : 124 nmol/L for uptake inhibition of [³ H]adenosine uptake into EOC-20 microglia IC ₅₀ : 190 nmol/L for uptake inhibition of [³ H]adenosine into RAW264.7 macrophages Ki: 120 nmol/L for adenosine uptake inhibition Ki: 237 ± 94 nmol/L for competitive inhibition of [³ H]NBMPR binding to ENT1 in EOC-20 cells | Carrier et al. [48]; Millar et al. [53] | 3.39 ng/mL (10-mg oro-mucosal spray buccal) 93.3 ng/mL (1.5 mg vaporized) | ≥ 94# | ENT1: < 0.02 ENT1: 0.08 |

Table 1 (continued)

| Compound class | Class representative(s) with NT inhibitor characteristics | Supposed mechanism | In vitro inhibition | References ^a | C _{max} (dose) | Protein binding (%) | (C _{max} × f ₀)/K _i or (C _{max} × f ₀)/IC ₅₀ |
|--|--|---|--|---|---|--|--|
| | Tetrahydrocannabinol (delta-9- tetrahydrocannabinol; plant-derived phytocannabinoid) | Competitive selective ENT1 inhibition | IC ₅₀ : ~270 nmol/L for uptake inhibition of [³ H]adenosine into EOC-20 microglia IC ₅₀ : 334 nmol/L for uptake inhibition of [³ H]adenosine into RAW264.7 macrophages Maximal inhibition of adenosine transport ~60% in microglia and macrophages | Carrier et al. [48] | 7.88 ng/mL [#] (10 mg p.os.) 116.2 ng/mL (8 mg vaporized) | 97 [#] | ENT1: <0.02 ENT1: 0.07 |
| Adenosine re-uptake Inhibitors | Dipyridamole | Dual ENT1 and ENT2 inhibition; much higher potency for ENT1 inhibition | Ki (ENT1): 48 nmol/L Ki (ENT2): 6.2 μmol/L IC ₅₀ (ENT1): 15.1 nmol/L | Dresse et al. [85] Visser et al. [58] Wang et al. [86] Lenz and Wilson [87] Vlachodimou et al. [26] | 1680 ng/mL [#] (75 mg) | 96.5–98.1 [#] | ENT1: 2.43 ENT2: 0.02 |
| | Dilazep | Fairly selective ENT1 inhibition; ENT2 inhibition unlikely to be relevant in vivo | Ki (ENT1): 19 nmol/L Ki (ENT2): 134 μmol/L | Visser et al. [58] Sambhi et al. [88] | 25.6 ng/mL (100 mg t.i.d at steady state) | n.a. | n.d. |
| | Cilostazol | | IC ₅₀ (ENT1): ~10 μmol/L IC ₅₀ (ENT2): ~10 μmol/L | Liu et al. [71] Pletal [®] product label [70] | 1532.7 ng/mL [#] (200 mg) | 95–98 [#] | ENT1: 0.02 ENT2: 0.02 |
| P2Y ₁₂ receptor antagonists | Ticagrelor | Specific ENT1 inhibition | IC ₅₀ (ENT1): 260 nmol/L Ki (ENT1): 41 nmol/L for [³ H] NBMPR displacement | Armstrong et al. [62] | 1490.7 ng/mL [#] (180 mg; fasted) | >99% [89] | ENT1: 0.11 (IC ₅₀) |
| | Metabolites AR-C124910XX (AR-C133913XX) | | IC ₅₀ (ENT1): 2.2 μmol/L IC ₅₀ (ENT1): 3.5 μmol/L | | 410.9 ng/mL [#] (180 mg of Ticagrelor) | >99% [89] (AR-C124910XX) 40–57 % (AR-C133913XX) | ENT1: <0.02 (AR-C124910XX) |

Table 1 (continued)

| Compound class | Class representative(s) with NT inhibitor characteristics | Supposed mechanism | In vitro inhibition | References ^a | C _{max} (dose) | Protein binding (%) | (C _{max} × f _b)/K _i or (C _{max} × f _b)/IC ₅₀ |
|--|---|---|---|---|---|---------------------|--|
| Dihydropyridine calcium channel blockers | Nimodipine | Fairly selective non-competitive ENT1 inhibition; ENT2 inhibition unlikely to be relevant in vivo | IC ₅₀ (ENT1): 61 nmol/L IC ₅₀ (ENT2): 13.1 μmol/L | Kirsten et al. [76] Blardi et al. [77] Li et al. [27] Nimotop [®] product label [74] Li et al. [78] | 131.6 ng/mL [#] (90 mg) | >95 [#] | ENT1: 0.26 ENT2: < 0.02 |
| | | Dual non-competitive ENT1/ENT2 inhibition; ENT1 inhibition unlikely to be relevant in vivo | IC ₅₀ (ENT1): 13.7 μmol/L IC ₅₀ (ENT2): 1.3 μmol/L | Kirsten et al. [76] Li et al. [27] Eschenhagen et al. [90] Boswell-Casteel et al. [18] | 56.44 ng/mL [#] (90 mg) | 92–98 [#] | ENT1: < 0.02 ENT2: < 0.02 |
| | Nifedipine | Fairly selective non-competitive ENT1 inhibition; unlikely to be relevant in vivo | IC ₅₀ (ENT1): 8.7 μmol/L | Li et al. [27] Dow and Graham [91] | 70.5 ng/mL (40 mg t.i.d.) | ≥ 95 [#] | ENT1: < 0.02 |
| | Nitrendipine | Dual non-competitive ENT1/ENT2 inhibition; ENT1/2 inhibition unlikely to be relevant in vivo | IC ₅₀ (ENT1) = 10.1 μmol/L IC ₅₀ (ENT2) = 3.7 μmol/L | Kirch et al. [92] Lasseter et al. [93] Kann et al. [94] Goa and Sorkin [95] Kirsten et al. [76] Li et al. [27] | 7.53 ng/mL [#] (20 mg) 41.6 ng/mL (20 mg/d over 1 week) 8.60 ng/mL (20 mg/d over 1 week) | 98 [#] | ENT1: < 0.02 ENT2: < 0.02 |
| | Felodipine | Fairly selective non-competitive ENT1 inhibition | IC ₅₀ (ENT1): 12.4 μmol/L | Li et al. [27] | 36.7 nM [#] (10 mg) | ≥ 99 [#] | ENT1: < 0.02 |

Table 1 (continued)

| Compound class | Class representative(s) with NT inhibitor characteristics | Supposed mechanism | In vitro inhibition | References ^a | C _{max} (dose) | Protein binding (%) | (C _{max} × f _b)/K _i or (C _{max} × f _b)/IC ₅₀ |
|---------------------------------------|---|--|---|--|---|---------------------|--|
| Non-steroidal anti-inflammatory drugs | Piroxicam | Dual competitive ENT1/ENT2 inhibition, with larger inhibitory effects on ENT1 ENT1/2 inhibition unlikely to be relevant in vivo | ENT1: 30% inhibition of adenosine uptake at 100 μmol/L | Perini and Suarez-Kurtz [96] Li et al. [79] | 4250 ng/mL [#] (40 mg) | 99 [#] | n.d. |
| | Sulindac | Dual competitive ENT1/ENT2 inhibition, with larger inhibitory effects on ENT1 ENT1/2 inhibition unlikely to be relevant in vivo | Inhibition of [³ H] adenosine uptake by 13–18% | Li et al. [79] | 4110–6460 ng/mL [#] (150 mg) | ≥ 93.1 [#] | n.d. |
| | Sulindac sulphide (active metabolite of sulindac) | Dual competitive ENT1/ENT2 inhibition, with larger inhibitory effects on ENT1; ENT2 inhibition unlikely to be relevant in vivo | IC ₅₀ (ENT1): 33.77 μmol/L for inhibition of adenosine uptake IC ₅₀ (ENT1): 24.19 μmol/L for displacement of [³ H] NBMPR ENT2: 35% inhibition of adenosine uptake at 300 μmol/L | Li et al. [79] Li et al. [78] | 1970–2480 ng/mL [#] (150 mg of Sulindac) | 97.9 [#] | ENT1: < 0.02 |
| Benzodiazepines | Midazolam | Presumably selective ENT1 inhibition | Ki: 6 μmol/L for displacement of [³ H] NBMPR | Seubert et al. [81] Link et al. [97] | 169.5 ng/mL* [98] (7.5 mg; iv) | 96–98* [98] | ENT1: < 0.02 |

Table 1 (continued)

| Compound class | Class representative(s) with NT inhibitor characteristics | Supposed mechanism | In vitro inhibition | References ^a | C _{max} (dose) | Protein binding (%) | (C _{max} × f _u)/K _i or (C _{max} × f _u)/IC ₅₀ |
|------------------------------------|---|--|---|--|--|-------------------------------|--|
| BCR-ABL tyrosine kinase inhibitors | Nilotinib | Selective ENT1 inhibition | IC ₅₀ (ENT1): 0.7 μmol/L | Damaraju et al. [83] Tian et al. [99] | 1360 ng/mL (300 mg twice daily) | Approximately 98 [#] | ENT1: 0.07 |
| | Ponatinib | Selective ENT1 inhibition | IC ₅₀ (ENT1): 9.0 μmol/L | Damaraju et al. [83] | 77 ng/mL* [100] (45 mg) | >99 [#] | ENT1: < 0.02 |
| | Bosutinib | Dual ENT1/2 inhibition, unlikely to be relevant in vivo Down-regulation of ENT1 membrane expression | IC ₅₀ (ENT1): 13 μmol/L IC ₅₀ (ENT2): 24 μmol/L | Damaraju et al. [83] | 200 ng/mL* [101] (500 mg with food) | 94* [101] | ENT1: < 0.02 ENT2: < 0.02 |
| | Dasatinib | Dual ENT1/2 inhibition, unlikely to be relevant in vivo | IC ₅₀ (ENT1): 60 μmol/L IC ₅₀ (ENT2): 84 μmol/L | Damaraju et al. [83] Christopher et al. [102] | 247.7 ng/mL (180 mg) | 96* [103] | ENT1: < 0.02 ENT2: < 0.02 |
| | Imatinib | Fairly selective CNT2 inhibition | IC ₅₀ (CNT2): 2.3 μmol/L IC ₅₀ (CNT3): 62 μmol/L IC ₅₀ (ENT1): 110 μmol/L | Damaraju et al. [83] Eschenhagen et al. [90] | 3701.8 ng/mL* [104] (800 mg; steady state) | 95* [104] | CNT2: 0.16 CNT3: < 0.02 ENT1: < 0.02 |
| Immunosuppressants | Cyclosporin A | Presumably selective ENT1 inhibition | ENT-mediated uptake inhibition of adenosine increased adenosine plasma levels in kidney transplant patients >2-fold | Guieu et al. [84] | | | n.d. |

Resources of human PK and PB data: unless otherwise stated the PK and PB data were taken from current drug labels or European Assessment Reports (indicated by * next to the data) or from the drug database PharmaPendium[®] (indicated by # next to the data); where ranges are given for PB, always the lowest PB value, i.e., the highest f_u was used for the calculation of cut-off values μM micromolar (10⁻⁶ mol/L), C_{max} maximum concentration, CNT concentrative nucleoside transporter, ENT equilibrative nucleoside transporter, f_u fraction unbound, I/I total plasma concentration of the interacting drug at steady state (when data were available), IC₅₀ half-maximal inhibitory concentrations, iv intravenously, Ki inhibition constant, n.a. not available, n.d. not determined, nM nanomolar (10⁻⁹ mol/L), NT nucleoside transporter, PB protein binding, PK pharmacokinetic, *l.i.d.* three times daily

^aListed are references for in vitro data and in vivo human PK data

reduced maximum exposure and extent of absorption of the ENT1/2 and CNT2/3 substrate ribavirin, probably owing to competition of dietary nucleosides with ribavirin for intestinal CNT and ENT transporters [34].

In a cladribine food-interaction study employing a typical FDA-recommended high-fat, high-calorie breakfast [35], the rate of cladribine absorption was delayed in the fed state (median time to maximum concentration 1.5 h; range 1–3 h) as compared with the fasted state (median time to maximum concentration 0.5 h; range 0.5–1.5 h), and the geometric mean maximum concentration (C_{\max}) was reduced by 29%. In contrast, total cladribine exposure (geometric mean area under the concentration–time curve from zero to infinity) was only marginally affected with 72.8 ng·h/mL for the fed state vs 75.7 ng·h/mL for the fasted state [35]. Based on this, it was concluded that cladribine tablets can be administered without regard to food [3, 6]. It should be noted, however, that the high-fat, high-calorie test meal employed in the study only contained relatively low amounts of dietary purines of approximately 24 mg, which is about eight-fold less than the high-purine meal employed in the ribavirin study. It remains therefore unknown whether purine-rich meals [including certain fish and seafood (such as anchovies, haddock, herring, mussels, sardines, scallops, and trout) and some meats (such as bacon, turkey, veal, venison, and organ meats like liver)] may have the potential to alter the bioavailability of cladribine tablets.

3.2 Effects of Alcohol

Clinicians should be aware of the possible effects that alcohol intake could have on the effectiveness of cladribine, particularly as problem drinking has been reported in patients with MS [36]. A number of studies have identified ethanol as a pharmacological inhibitor of ENT1-mediated adenosine uptake, resulting in increased extracellular adenosine concentrations in experimental *in vitro* studies using human lymphocytes [37], primary cultures of rat hepatocytes [38], human placental cells [39], human bronchial cells [40], S49 mouse lymphoma cells and rodent neuronal-glia cells (NG108-15) [41–44], and in the cardiomyocyte cell line, HL-1 [45]. Ethanol is known to selectively inhibit ENT1, but not ENT2 (Table 1).

Ramadan and co-workers showed partial ENT1 inhibition at lower ethanol exposures that are known to occur *in vivo* by an average consumption of one to two drinks per day (which is equivalent to peak blood alcohol levels of ~ 10 mmol/L ethanol) while about 25-mmol/L ethanol blood concentrations are commonly achieved in social surroundings [45]. This suggests that ethanol-mediated ENT1 inhibition is likely to be relevant *in vivo*. The same group also showed that gemcitabine cytotoxicity is reduced in the presence of 2-mmol/L ethanol concentrations in the human bladder

cancer cell line, HBT2. The authors proposed a direct partial inhibition of ENT1 upon acute ethanol exposure and a long-term down-regulation of ENT1 expression, resulting in less ENT1 protein at the plasma membrane upon chronic alcohol exposure [45].

Ethanol-mediated ENT1 inhibition may alter intestinal absorption of cladribine as well as ENT1-mediated uptake into lymphocytes and tubular secretion in the kidney. A reduced bioavailability together with an altered uptake into lymphocytes may theoretically have potential to result in an attenuated efficacy of cladribine treatment, while safety appears less of a concern. Therefore, it appears advisable that patients refrain from alcohol consumption before taking cladribine tablets.

3.3 Tetrahydrocannabinol and Cannabidiol

Two recent high-quality systematic reviews concluded that the only strong evidence for medical marijuana in neurological disorders was for reducing the symptoms of patient-reported spasticity and central pain in MS and that the only complementary and alternative medicine intervention in MS with strong supportive evidence was cannabinoids [46]. A UK survey conducted in 2014 in patients with MS revealed that one in five patients used cannabis products to alleviate their symptoms, especially muscle spasms or stiffness (spasticity) and pain [47].

The *Cannabis sativa* components tetrahydrocannabinol (THC) and cannabidiol (CBD) were reported to inhibit adenosine transport with IC_{50} values in the nanomolar range by acting as competitive inhibitors at the equilibrative transporter ENT1 [48]. In particular, CBD and, less potently, THC decreased uptake of [3 H]adenosine to a similar extent as [3 H]thymidine in both murine microglia and RAW264.7 macrophages. Binding studies confirmed that CBD binds to ENT1 with a $K_i < 250$ nmol/L, and the K_i for CBD to inhibit adenosine transport was 120 nmol/L [48] (Table 1).

Marketed medicinal products containing the synthetic THC derivative dronabinol (delta-9-tetrahydrocannabinol) are available in USA and Canada, and include capsules for oral use [49] and an oral solution [50]. Although both are not indicated for the symptomatic treatment of MS symptoms, some off-label use may be assumed. Mean plasma C_{\max} achieved with the highest approved Marinol[®] capsule strength, i.e., 10 mg in the fasted condition, are reported with 7.88 ± 4.54 ng/mL [49], corresponding to about 250 nmol/L. Maximum concentration values achieved with the highest recommended dose (4.2 mg/m² body surface area) of the oral solution product are reported with about 3.3 ng/mL distinctly lower [50]. Considering these exposure data together with the delta-9-THC plasma protein binding of 97%, the maximum unbound C_{\max} values are unlikely to

confer a relevant ENT1 inhibition in vivo, i.e., ($C_{\max} \times f_u / IC_{50}$ is < 0.02 ; see Table 1).

In addition to the available oral THC mono products, there is a fixed combination medicinal product available in 19 European countries and Switzerland (European Union since 2010, CH since 2013), which is specifically indicated for symptom improvement in adult patients with moderate-to-severe spasticity due to MS. This product contains THC and the non-psychoactive cannabinoid-component CBD, and is presented as an oromucosal spray (USAN: nabiximols; tradename Sativex[®]; one puff delivers a 100- μ L spray containing 2.7 mg of THC and 2.5 mg of CBD) [51]. The highest recommended dose consists of 12 sprays per day separated into five sprays in the morning and seven sprays in the evening. The latter corresponds to maximum recommended total single oromucosal doses of 18.9 mg and 17.5 mg of THC and CBD, respectively. The inter-subject variability of THC and CBD pharmacokinetic parameters is high. Following a single Sativex[®] administration of four sprays under fasted conditions, the mean maximum plasma THC levels (C_{\max}) ranged between 0.97 and 9.34 ng/mL, while the observed C_{\max} for CBD ranged between 0.24 and 2.57 ng/mL, i.e., about ten-fold for both THC and CBD. After 9 consecutive days of dosing C_{\max} ranged from 0.92 to 6.37 ng/mL for THC and from 0.34 to 3.39 ng/mL for CBD, respectively, indicating an absence of accumulation of THC and CBD plasma concentrations upon repeated dosing [51]. This means also for CBD that with repeat administration of recommended doses of Sativex[®], the achieved maximum unbound C_{\max} values are unlikely to confer a relevant ENT1 inhibition in vivo, i.e., $C_{\max} \times f_u / IC_{50}$ is < 0.02 ; see Table 1.

In addition to approved and well-standardized, THC-containing and CBD-containing pharmaceutical products, medical cannabis is also provided in the form of herbal products derived from dried cannabis flowers (medical cannabis or medical marijuana). In this context, it is worthy to note that plasma concentrations achieved with THC/CBD products for oral or oromucosal administration, THC, and CBD plasma concentrations are much lower compared with concentrations achieved following inhalation (e.g., smoking or vaporisation) of cannabinoids at similar doses. For instance, a dose of 8 mg of vaporised THC extract, administered by inhalation, resulted in a mean C_{\max} of more than 116.2 ng/mL (corresponding to 0.37 μ mol/L) within minutes of administration, which exceeds mean C_{\max} values upon oral administration of 10 mg delta-9-THC by about 15-fold [52]. This yields a cut-off value for $C_{\max} \times f_u / IC_{50}$ of 0.07, thereby indicating a possible relevant ENT1 inhibition in vivo (Table 1). The same applies to CBD maximum exposure concentrations observed after vaporisation with a mean C_{\max} value of 76.3 ng/mL (range 2.3–339 ng/mL) achieved with just a CBD 1.5-mg single dose or smoking of cannabis cigarettes containing CBD 1.5 mg, which resulted

in a mean C_{\max} value of 93.3 ng/mL (range 0.65–350 ng/mL) in frequent cannabis smokers [53, 54]. This translates to an approximately 27-fold maximum CBD exposure concentration upon smoking of cannabis cigarettes as compared to the CBD steady-state exposure achieved with recommended doses of the oral mucosal spray (Sativex[®]). This translates to a cut-off value of 0.09 when $C_{\max} \times f_u / IC_{50}$ is considered, and 0.08 when $C_{\max} \times f_u / K_i$ is considered. Both outcomes point to a possible relevant ENT1 inhibition in vivo (Table 1). Taken together, systemic THC/CBD exposure concentrations achieved with inhalation/smoking are deemed sufficiently high to predict that in vivo inhibition of ENT1 by both compounds is likely.

The terminal half-life of THC is reported to be 25–36 h upon oral administration [49], while the half-life of CBD was reported between 1.4 and 10.9 h after an oromucosal spray, 2–5 days after long-term oral administration and 31 h after smoking [53]. In addition, THC and CBD may be retained in the adipose tissue for as long as 4 weeks from which they are slowly re-distributed at sub-therapeutic concentrations into the systemic circulation [51]. Thus, it is recommended that patients with MS refrain from the use of cannabinoid products, in particular from inhaled administration, for at least 1 week before and during treatment with cladribine tablets in order to optimize ENT1-mediated absorption of cladribine and uptake into lymphocytes.

3.4 Adenosine Re-Uptake Inhibitors

Adenosine re-uptake inhibitors such as dipyridamole [55] and dilazep [56] are old drugs developed in the 1950s and 1960s. They were designed to retard the cellular uptake of extracellular adenosine into endothelial cells and red blood cells, thereby potentiating the receptor-mediated physiological effects of adenosine, by potent inhibition of ENTs, in particular ENT1. They were deemed to be clinically useful because of their cerebral and coronary vasodilating action, and inhibition of platelet aggregation through enhancement of adenosine effects.

Dipyridamole was introduced for the treatment of ischemic heart disease in 1959. Its undesired and harmful coronary “steal” phenomenon was observed a few years later [57]. Although its clinical use in cardiac indications such as coronary artery disease and myocardial infarction has been considered obsolete in the scientific community for decades, the drug is still marketed for these indications in some countries (e.g., Japan and India). Dipyridamole is also marketed in the European Union and the USA as an adjunct to oral anti-coagulation in combination with acetylsalicylic acid or coumarin anticoagulants in patients with prosthesis-related thromboembolic events, e.g., in the prevention of postoperative thromboembolic complications of cardiac valve replacement [55].

Dipyridamole and dilazep are known to inhibit ENT1 at nanomolar concentrations, with K_i values of 48 nmol/L and 19 nmol/L, respectively [58, 59]. Considering the dipyridamole exposure data together with the lowest reported plasma protein binding of 96.5%, the maximum unbound C_{\max} values are likely to confer a relevant ENT1 inhibition in vivo, i.e., $C_{\max} \times f_u/K_i$ is calculated with 2.43, which is by far the highest cut-off value for all identified ENT1 inhibitors (Table 1), which is consistent with the established evidence that dipyridamole represents the most potent prototypic ENT1 inhibitor known to date. Dipyridamole also inhibits ENT2 at low micromolar concentrations ($K_i = 6.2 \mu\text{mol/L}$), which however, is unlikely to exert clinical relevance (Table 1).

Unfortunately, no protein binding data are available in the public domain for dilazep, which prevents calculation of ENT1 and ENT2 inhibition cut-off values. However, when the reported K_i values for ENT1 inhibition in the lower nanomolar range are compared with the K_i values for ENT2 inhibition in the higher micromolar range (Table 1), it can be inferred that dilazep most probably acts as a specific ENT1 inhibitor in vivo. As both dipyridamole and dilazep products are available as oral formulations, they are in principle to be considered perpetrators of the intestinal transporter-mediated absorption of cladribine as well as ENT1-mediated uptake into lymphocytes and red blood cells, and tubular secretion of cladribine in the kidney. However, because of the required 3-h time separation of cladribine dosing from other orally administered drug products, as per the cladribine label, intestinal ENT-mediated absorption interactions are not expected to play a significant role in clinical practice. As the mean (dominant) plasma half-lives of dipyridamole and dilazep are short (about 3 h for both compounds), it is recommended that patients suspend intake of dipyridamole or dilazep at least 1 day before starting treatment with cladribine tablets. If withdrawal is not possible, selection of alternative products with no, or minimal, ENT1 transporter-inhibiting properties should be considered.

3.5 Ticagrelor

Ticagrelor is an oral, direct-acting, selective, and reversibly binding P2Y₁₂ receptor antagonist that prevents ADP-mediated P2Y₁₂-dependent platelet activation and aggregation to inhibit the prothrombotic effects of ADP [60]. In addition to the inhibition of platelet activation and aggregation via P2Y₁₂ receptor antagonism, part of the antiplatelet effect of ticagrelor is due to a drug-induced increase in extracellular adenosine levels via ENT1-mediated adenosine re-uptake inhibition, resulting in adenosine-mediated platelet inhibition via the A_{2A} receptor. Thus, ticagrelor has a unique dual mode of action, with a primary effect mediated by P2Y₁₂

antagonism complemented by adenosine re-uptake inhibition as a secondary mechanism [61].

Ticagrelor inhibited adenosine uptake in MDCK cells expressing ENT1, with an IC₅₀ of 260 nmol/L (Table 1), which is approximately 35-fold less than that of dipyridamole (IC₅₀ = 7.4 nmol/L), a known prototypic inhibitor of ENT1 [62]. These findings are in line with those from another study in human breast carcinoma cells, where the potency of ticagrelor with respect to inhibition of adenosine uptake was approximately ten-fold less than that of dipyridamole [63]. The major ticagrelor metabolites AR-C124910XX and AR-C133913XX also inhibited adenosine uptake with IC₅₀ values of 2.2 $\mu\text{mol/L}$ and 3.5 $\mu\text{mol/L}$, respectively (Table 1).

The authors further demonstrated that ticagrelor can displace the potent inhibitor [³H]NBTI (nitrobenzylthioinosine) from ENT1 in MDCK cells, confirming that ticagrelor binds with high affinity ($K_i = 41 \text{ nmol/L}$) directly to ENT1 to inhibit adenosine uptake [62] (Table 1). In this cell system, the K_i of ticagrelor was about 15-fold less than that of dipyridamole ($K_i = 2.6 \text{ nmol/L}$). In turn, no significant inhibitory activity was observed in MDCK cells expressing ENT2 or CNT2/3, characterizing ticagrelor as a specific ENT1 inhibitor.

Both ticagrelor and the active metabolite AR-C124910XX are highly bound to human plasma protein (> 99.8%), i.e., the unbound fraction is ~0.2% [64]. However, despite this high protein binding, the unbound ticagrelor C_{\max}/IC_{50} for ENT1 inhibition is calculated with 0.078 (Table 1), thereby exceeding the EMA guideline criteria for the assessment of systemic transporter inhibition of potential clinical relevance (cut-off of ≥ 0.02 applied). The predicted in vivo relevance of ticagrelor-mediated ENT1 inhibition was confirmed by clinical studies in healthy subjects (180-mg single dose) and in patients with non-ST-elevation acute coronary syndrome undergoing percutaneous coronary intervention (90 mg twice daily [bid] over 15 days), both showing that ticagrelor enhanced adenosine-mediated coronary blood flow velocity via an adenosine-mediated mechanism [65, 66]. Finally, a study investigating the impact of ticagrelor on adenosine plasma concentrations in patients with acute coronary syndrome (180-mg loading dose followed by 90 mg bid) compared to a clopidogrel treatment (600-mg loading dose followed by 75 mg bid), showed more than twice as high adenosine plasma concentrations in patients receiving ticagrelor than patients receiving clopidogrel [1.5 μM (interquartile range: 0.98–1.7 μM) vs 0.68 μM (interquartile range: 0.49–0.78 μM ; $p < 0.01$)] [67]. These results confirm that the concentration of ticagrelor attained after oral administration with the recommended clinical dose regimen is sufficient to inhibit cellular uptake of adenosine in vivo, thereby increasing its plasma concentration. These findings are also supported by the adverse event profile of ticagrelor,

which includes dyspnea and bradyarrhythmias, symptoms that are attributed to elevated adenosine levels due to ENT1 inhibition [21, 68, 69].

Taken together, it is concluded that ticagrelor inhibits cellular adenosine uptake selectively via ENT1 inhibition at concentrations of clinical relevance, suggesting that ticagrelor treatment should be suspended for at least 3 days (the mean terminal disposition half-life of ticagrelor ranges between 7.7 and 13.1 h) before initiation of treatment with cladribine tablets, and should only be resumed 1 day after the last dose of cladribine. If withdrawal is not possible, selection of an alternative antiplatelet product with no, or minimal, ENT1 transporter-inhibiting properties should be considered.

3.6 Cilostazol

Cilostazol (Pletal[®]) is a dual inhibitor of phosphodiesterase III and adenosine re-uptake with antiplatelet activity that is indicated for the improvement of the maximal and pain-free walking distances in patients with intermittent claudication [70]. Cilostazol inhibits adenosine uptake into muscle, endothelial cells, red blood cells, and platelets with an IC₅₀ of ~5–10 μmol/L [71, 72] (Table 1). As with other adenosine re-uptake inhibitors, clinically relevant concentrations of cilostazol (3–5 μmol/L) have been shown to increase rabbit cardiac interstitial and circulatory adenosine levels [72]. It is noteworthy that the 3,4-dehydro metabolite of cilostazol (OPC-13015), one of two major metabolites, is about five times more potent than cilostazol as a phosphodiesterase III inhibitor and three times more potent as an adenosine re-uptake inhibitor [71], and its plasma concentration (as measured by area under the concentration–time curve) is about 41% of the cilostazol parent drug [70]. It was later shown that the adenosine uptake inhibition by cilostazol was actually due to direct inhibition of ENT1 [21]. Thus, it is reasonable to assume that the total ENT1-inhibiting potency in vivo might be the result of a combined effect of the cilostazol parent drug and its major 3,4-dehydro metabolite OPC-13015. Additionally, the adverse event profile of cilostazol includes dyspnea and bronchospasm in patients with pre-existing respiratory diseases, symptoms that are attributed to elevated adenosine levels due to ENT1 inhibition [21]. This suggests that the inhibition of ENT1 by recommended doses of cilostazol (i.e., 100 mg bid) is clinically relevant. It is important to note that the calculated cut-off values of cilostazol of 0.02 for both ENT1 and ENT2 inhibition (Table 1) correctly point to the potential in vivo relevance of cilostazol-mediated dual ENT1/2 inhibition. Therefore, patients should be advised to suspend cilostazol treatment for at least 3 days (the mean terminal disposition half-life of cilostazol is about 10.5 h) before initiation of treatment with cladribine tablets, resuming 1 day after the

last dose of cladribine. If withdrawal is not possible, selection of an alternative product with no, or minimal, ENT1 transporter-inhibiting properties should be considered.

3.7 Dihydropyridine CCBs

Calcium channel blockers (CCBs) are among the five major drug classes recommended for the treatment of hypertension [73], and have similar effectiveness as other major drug classes on blood pressure, major cardiovascular events, and mortality outcomes. This is a heterogeneous class of agents including dihydropyridines (DHP-CCBs) and nondihydropyridines (e.g., verapamil and diltiazem). The two subclasses do not show substantial differences in effectiveness [73].

Nimodipine (Nimotop[®]) is a DHP-CBB that is not indicated for the treatment of hypertension but approved for the prevention of ischemic neurological deficits and cerebral vasospasm after aneurysmal subarachnoid hemorrhage [74]. The substance is known to inhibit adenosine transport in the brain, heart, and red blood cells with IC₅₀ values in the nanomolar range [27, 75, 76]. A pharmacokinetic study in patients with cerebral ischemia has shown that intravenous (bolus of 0.03 mg/kg) and oral (30, 60, and 90 mg) administration of nimodipine increases the plasma level of adenosine [77]. It has therefore been suggested that the inhibition of adenosine uptake is a relevant mechanism for the clinical effects of nimodipine. Other DHP-CBB such as nitrendipine, nifedipine, and nicardipine inhibit adenosine transport in vitro to different extent [18, 78].

Li et al. investigated the mechanism by which dihydropyridines inhibit adenosine uptake [27]. Among the dihydropyridines tested, nimodipine was identified as a very potent inhibitor of ENT1 (IC₅₀ = 60 ± 31 nmol/L), whereas nicardipine, nitrendipine, felodipine, and nifedipine exhibited at least 100-fold less potent inhibitory activity (nicardipine IC₅₀ = 8.7 ± 8.2 μmol/L; nitrendipine IC₅₀ = 10.1 ± 1.3 μmol/L; felodipine IC₅₀ = 12.4 ± 3.1 μmol/L; nifedipine IC₅₀ = 13.7 ± 3.2 μmol/L) (Table 1). Nifedipine, nitrendipine, and nimodipine also inhibited ENT2 with IC₅₀ values in the lower micromolar range (nifedipine IC₅₀ = 1.3 ± 0.1 μmol/L; nitrendipine IC₅₀ = 3.7 ± 1.5 μmol/L; nimodipine IC₅₀ = 13.1 ± 4.9 μmol/L) (Table 1), while nicardipine and felodipine did not exert significant effects on ENT2 [27].

Kinetic studies suggested that DHP-CCBs act as non-competitive inhibitors of ENT1 and ENT2. In this respect, DHP-CCBs are distinct from most other nucleoside transport inhibitors, which are usually competitive inhibitors. The interactions of DHP-CCBs with ENT1 and ENT2 are reversible [27].

When EMA DDI guideline criteria for the assessment of systemic transporter inhibition of potential clinical relevance are considered, only nimodipine is expected to act

as a clinically relevant inhibitor of ENT1 in vivo, with a cut-off value of 0.26 when $C_{\max} \times f_u/IC_{50}$ for a 90-mg oral dose is considered. In turn, all other DHP-CCBs with reported in vitro ENT-inhibiting properties are unlikely to exert these effects in vivo (Table 1). The dominant half-life for nimodipine ranges between 1.1 and 1.7 h. Accordingly, co-administration of nimodipine does not need to be washed out before initiation of cladribine treatment but should be avoided (if possible) during the 4–5 days of treatment with cladribine tablets. If withdrawal is not possible, selection of an alternative product with no, or minimal, ENT1 transporter-inhibiting properties should be considered. All other DHP-CCBs are unlikely to exert clinically relevant inhibition of ENT1 or ENT2 when used at recommended doses.

3.8 Nonsteroidal Anti-Inflammatory Drugs

Li and co-workers investigated the effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on adenosine uptake in human aortic smooth muscle cells. Aspirin, ibuprofen, and naproxen had no effect on adenosine uptake, while etodolac, indomethacin, ketoprofen, mefenamic acid, and sulindac inhibited adenosine uptake by 13–18%, and piroxicam inhibited adenosine uptake by 30% [79] (Table 1). However, sulindac sulphide (the active metabolite of sulindac) completely inhibited adenosine uptake with an IC_{50} of 40 $\mu\text{mol/L}$ (Table 1). The action of sulindac sulphide on adenosine uptake is due to the direct blockade of NTs rather than to the inhibition of COX. Kinetic data of adenosine uptake indicated that the inhibition of adenosine transport by sulindac sulphide is competitive, and results also showed that the inhibitory effect on ENT1 was more potent (IC_{50} of 33 $\mu\text{mol/L}$) than that on ENT2 (35% inhibition of adenosine uptake at 300 $\mu\text{mol/L}$) (Table 1). The peak blood concentration of sulindac sulphide in patients who take sulindac (150 mg bid) is about 20 $\mu\text{mol/L}$, and the authors of the study estimated that such a concentration can weakly (but significantly) inhibit ENT1-mediated adenosine uptake [79]. However, when the most conservative EMA cut-off criterion for potential in vivo relevance of transporter inhibition is applied (i.e., $C_{\max,u} \times f_u/IC_{50}$ is ≥ 0.02), it does not appear that sulindac sulphide may be able to confer a clinically relevant ENT1 inhibition in vivo (Table 1). Taken together, NSAIDs, as a class, are not regarded as clinically relevant NT inhibitors in vivo.

3.9 Benzodiazepines

Benzodiazepines are widely used for the symptomatic treatment of a variety of MS symptoms including spasticity (stiffness), tremor dizziness/vertigo, and anxiety [80]. There are data to indicate that benzodiazepines show affinity for ENT1. Competition binding assays of five structurally

distinct benzodiazepines (midazolam, alprazolam, flurazepam, diazepam and chlordiazepoxide) in membranes from porcine striatum and guinea pig ventricles showed that all benzodiazepines completely displaced the ENT1-specific inhibitor [^3H]NBMPR in a concentration-dependent manner [81]. Midazolam was the most potent ENT1 inhibitor with a K_i value of 6 $\mu\text{mol/L}$ (Table 1). The K_i values of all other benzodiazepines tested ranged between 39.8 and 53.7 $\mu\text{mol/L}$ when the guinea pig ventricle data are considered. The authors concluded that benzodiazepines are weak ENT1 inhibitors at concentrations achieved with clinically recommended doses to facilitate their primary mode of action, i.e., GABAergic transmission in vivo. The affinity of benzodiazepines for ENT1 was shown to be about 1000-fold lower than that of the prototypical high-affinity ENT1 inhibitor NBMPR or dipyrindamole ($K_i = 1.9$ nmol/L in human erythrocytes). Apart from that, benzodiazepines are highly bound to plasma proteins, resulting in much lower free concentrations of the drugs in vivo. Finally, midazolam, the most potent ENT1-inhibiting benzodiazepine identified, has a short plasma disposition half-life and is primarily used as an intravenous formulation in perioperative or diagnostics settings (e.g., endoscopic procedures) for anesthesia and sedation, although tablet formulations are also available in some countries for the short-term treatment of insomnia. However, when the most conservative EMA cut-off criterion for potential in vivo relevance of transporter inhibition is applied (i.e., $C_{\max,u} \times f_u/IC_{50}$ is ≥ 0.02), it does not appear that recommended intravenous or oral doses of midazolam may be suspected to confer a clinically relevant ENT1 inhibition in vivo (Table 1). Taken together, benzodiazepines as a class are not considered to act as clinically relevant ENT1 inhibitors in vivo, and thus must not be suspended before or during treatment with cladribine tablets.

3.9.1 BCR-ABL TKIs

BCR-ABL TKIs are indicated for the treatment of Philadelphia chromosome-positive chronic myelogenous leukemia and acute lymphoblastic leukemia. The leukemic cells of both hematological malignancies carry a constitutively active BCR-ABL oncogene (BCR: breakpoint cluster region; ABL: Abelson murine leukemia), which is the therapeutic target of inhibition by BCR-ABL TKIs [82].

Damaraju and colleagues examined interactions of the BCR-ABL TKIs bosutinib, dasatinib, imatinib, nilotinib, and ponatinib with recombinant human NTs (ENT1 and ENT2; CNT1, CNT2, and CNT3) produced in yeast *Saccharomyces cerevisiae*. Nilotinib inhibited ENT1-mediated uridine transport most potently (IC_{50} 0.7 $\mu\text{mol/L}$) followed by ponatinib > bosutinib > dasatinib > imatinib. Imatinib inhibited CNT2 with an IC_{50} of 2.3 $\mu\text{mol/L}$ (Table 1). Ponatinib inhibited all five NTs with the greatest effect seen for ENT1 (IC_{50}

9 $\mu\text{mol/L}$) [83]. Tyrosine kinase inhibitors inhibited [^3H] uridine uptake in a competitive manner.

In cultured human CEM lymphoblastoid cells, which possess a single human NT type (ENT1), accumulation of [^3H] cytarabine, [^3H] cladribine, or [^3H] fludarabine was reduced by each of the five TKIs, and also caused a reduction in cell surface expression of the ENT1 protein. The authors concluded that, at concentrations achieved in human plasma, ponatinib and bosutinib may exert their effects on ENT1 by down-regulation of the membrane abundance of ENT1 over prolonged exposure times, whereas imatinib and dasatinib may have less effect on ENT1 activity and levels [83]. When the most conservative EMA cut-off criterion for potential *in vivo* relevance of transporter inhibition is applied (i.e., $C_{\text{max,u}} \times f_u / \text{IC}_{50} \geq 0.02$), only nilotinib exceeded this threshold for possibly relevant ENT1 inhibition *in vivo* as well as imatinib for CNT2 inhibition (Table 1). However, as all BCR-ABL TKIs are associated with severe myelosuppressive side effects such as grade 3–4 neutropenia and thrombocytopenia, concurrent treatment with cladribine tablets in patients with MS is contraindicated as per prescribing information [3, 6].

3.10 Immunosuppressants

Long-term administration of the immunosuppressive drug cyclosporin A (CsA) was shown in a clinical study in kidney transplant recipients to block ENT-mediated uptake of adenosine to increase its plasma levels. Adenosine plasma levels in CsA-treated kidney transplant recipients were significantly higher (mean 0.76 $\mu\text{mol/L}$) than in the kidney transplant recipient CsA-untreated control group (mean 0.31 $\mu\text{mol/L}$). The authors further showed that elevated adenosine plasma levels correlated with CsA blood levels, and that adenosine plasma level increases were due to ENT-mediated adenosine uptake inhibition by CsA [23, 84]. As the initiation of treatment with cladribine tablets is contraindicated in immunocompromised patients, including patients currently receiving immunosuppressive or myelosuppressive therapy, no practical implications of the CsA-mediated ENT1 inhibition are expected for cladribine-treated patients with MS.

4 Discussion and Conclusions

Cladribine has been shown to be a substrate of various NTs. Intestinal absorption and distribution of cladribine throughout the body appear to be exclusively mediated by ENTs and CNTs, specifically by ENT1, ENT2 and ENT4, and CNT2 (low affinity) and CNT3. It is important to note that, owing to the required 3-hour time separation of cladribine dosing from other orally administered drug products, as per the cladribine tablets label, intestinal NT-mediated absorption

interactions are not expected to play a significant role in clinical practice. This is because currently available ENT1 inhibitors act in a reversible, competitive, or (rarely) non-competitive manner, and display relatively fast dissociation kinetics with only short-to-moderate residence times between 1 and 44 min at the transporter protein [25, 26]. To the best of our knowledge, there are no irreversible NT inhibitors among currently marketed drugs.

Therefore, this review focusses particularly on NT-based distribution interactions into the primary target cells of cladribine (i.e., lymphocytes), which may go along with a reduced efficacy. The key transporter for the intracellular uptake of cladribine into B and T-lymphocytes is ENT1, with ancillary contributions of ENT2 and CNT2. Thereby, ENT1 and ENT2 work in a bidirectional manner along a transmembrane concentration gradient, i.e., they are also capable of facilitating cladribine efflux from lymphocytes, when plasma concentrations fall below intracellular concentrations.

As the renal clearance of cladribine involves active tubular secretion, and accounts for almost 50% of the total cladribine clearance (22.2 L/h for renal clearance and 23.4 L/h for non-renal clearance), renal transporter inhibition needs in principle also to be considered [16]. All five NTs are expressed in the kidney. It is likely that ENT1 and ENT2 facilitate renal uptake of cladribine through the basolateral membrane (i.e., blood side), while ENT1 may also be involved in the apical urinary excretion of cladribine [11]. Thus, inhibition of renal ENT1/2 may have the potential to alter the renal clearance of cladribine, thereby possibly resulting in higher systemic exposure. Unfortunately, no studies are available to support this mechanistic hypothesis and to quantify possible effects of renal ENT1/2 inhibition on renal cladribine clearance and systemic cladribine exposure. However, effects of ENT1/2 inhibition on the renal clearance of cladribine are expected to be modest as ENT1/2-mediated tubular secretion of cladribine only represents an ancillary renal clearance mechanism on top of glomerular filtration, and may be possibly compensated by other apical renal efflux transporters that have been shown to transport cladribine more (e.g., BCRP) or less (e.g., P-gp) efficiently. Even if systemic cladribine exposure would be increased by renal ENT1/2 inhibition, it is expected that this would not give rise to safety concerns, as cladribine is an inactive prodrug, and at the time of renal ENT1/2 inhibition, ENT1/2 inhibition is also expected to be present at lymphocyte membranes, which means that an increased target-cell uptake and activation of the prodrug cladribine appears unlikely in the presence of a clinically relevant systemic ENT1/2 inhibition despite some systemic cladribine exposure increase.

Taken together, based on our review, the issue of the potential clinical relevance of NT inhibition regarding the

safety or efficacy of cladribine can be summarized as follows. The vast majority of NT-inhibiting lifestyle drugs and approved medical products act as specific or at least fairly specific ENT1 inhibitors (Table 1). There are hardly any CNT inhibitors among marketed products, with the exception of the CNT2 inhibitor imatinib (see Table 1), which is contra-indicated in cladribine-treated patients. Hence, the entire story of clinically relevant NT-based DDIs of cladribine is essentially about ENT1 inhibition, which is predominantly of concern in terms of altered cladribine uptake into lymphocytes, thereby hampering clinical efficacy, resulting in a “silent interaction”, which can hardly be detected clinically. This efficacy concern is based on the observation that ENT1 efficiently transports cladribine in cultured human leukemic cells with a K_m of 23 μM and overexpression of ENT1 leads to a significant increase in the cytotoxicity of cladribine (10 μM) compared with control cells [31]. In turn, it can be reasonably inferred that ENT1-mediated uptake-inhibition of cladribine goes along with reduced lymphocyte toxicity, thereby altering the clinical efficacy of cladribine. In order to manage cladribine therapy optimally, i.e., to ensure uncompromised target cell uptake to preserve the full therapeutic potential of cladribine, it is of importance that clinicians are aware of the existence of NT-inhibiting medicinal products, various lifestyle drugs, and food components such as dietary purines, which should be avoided during cladribine treatment.

In turn, there is no conceivable mechanism to suggest that NT inhibition may confer any safety risk, as cladribine is an inactive prodrug, requiring target-cell uptake and metabolic activation to exert its pharmacological action. Therefore, it can be reasonably inferred that increased systemic exposure concentrations of the prodrug cladribine, which cannot be excluded to occur because of renal NT inhibition, are unlikely to confer clinically significant adverse reactions. This consideration is supported by the established adverse event profile of cladribine, showing that clinically significant adverse events (e.g., lymphopenia, other white blood cell alterations, and infections and infestations) are altogether closely related to the primary pharmacology of cladribine, which renders the hypothetical possibility of any off-target toxicities mediated by the prodrug cladribine unlikely.

Although the strength of the available evidence on the in vivo relevance of NT inhibition varies, and predictions on the net effects in terms of potential cladribine distribution and exposure alterations are difficult to make, it appears prudent to avoid concomitant treatments and lifestyle drugs with in vitro evidence of a high degree of ENT1/2 inhibition, i.e., for which significant inhibiting activities were shown (i.e., $C_{\max} \times f_u/K_i$ or $\text{IC}_{50} \geq 0.02$) or for which clinically relevant ENT1 inhibition is suggested by typical adenosine-related adverse effects (e.g., dyspnea, bronchospasm, and bradyarrhythmias) as is the case for ticagrelor and cilostazol

or the clinical relevance of ENT1 inhibition is evidenced by increased adenosine plasma levels (e.g., ticagrelor and CsA).

Cardiovascular drugs with significant ENT-inhibiting potential include legacy products such as dipyridamole, dilazep, and cilostazol, which are no longer widely used but are still available in a few countries. These should not be co-administered with cladribine tablets.

Only a few prescription drugs with relevant NT-inhibiting properties have been identified.

The DHP-CBB nimodipine, which is indicated for the prevention of ischemic neurological deficits and cerebral vasospasm after aneurysmal subarachnoid hemorrhage, is another very potent and fairly selective ENT1 inhibitor in vitro, which has been shown to increase plasma adenosine levels upon oral single dosing [77], and it does exceed the regulatory cut-off threshold for possible in vivo relevance (i.e., $C_{\max} \times f_u/K_i$ or $\text{IC}_{50} \geq 0.02$), and thus is expected to confer a clinically meaningful ENT1 inhibition. Nimodipine should therefore not be co-administered with cladribine tablets. All other DHP-CBBs are unlikely to confer significant ENT1/2 inhibition in vivo.

The P2Y₁₂ receptor antagonist ticagrelor and its major metabolites are potent specific ENT1 inhibitors. Despite their high protein binding, significant ENT1 inhibition has been shown to increase systemic adenosine levels and to elicit prototypical adenosine-related adverse events such as dyspnea and bradyarrhythmias. Therefore, ticagrelor treatment should be suspended for at least 3 days (the mean terminal disposition half-life of ticagrelor ranges between 7.7 and 13.1 h) before initiation of treatment with cladribine tablets, and should only be resumed 1 day after the last dose of cladribine. Alternatively, patients can be switched to other anti-platelet drug products.

Among the class of NSAIDs, only sulindac has been shown to elicit dual competitive ENT1/2 inhibition to a degree that may be relevant in vivo, mainly owing to the effects of its major active metabolite sulindac sulphide. However, even sulindac sulphide does not exceed the regulatory cut-off threshold for possible in vivo relevance (Table 1), and thus is not expected to confer a clinically meaningful ENT1 inhibition. Thus, NSAIDs reported to act as dual competitive ENT1/2 in vitro are not regarded as clinically relevant NT inhibitors, and must not be avoided before or during cladribine treatment.

Some benzodiazepines have also been shown to inhibit ENT1 in vitro, the most potent being midazolam with a K_i of 6 $\mu\text{mol/L}$, which however does not exceed the regulatory cut-off threshold for possible in vivo relevance (Table 1), and thus is not expected to confer a clinically meaningful ENT1 inhibition. Among the class of TKI inhibitors, nilotinib and ponatinib have been shown to be potent ENT1 inhibitors with potential in vivo relevance, while imatinib was shown to be a fairly selective and potent CNT2 inhibitor.

Among the class of immunosuppressants, CsA was shown to elevate plasma adenosine levels upon long-term administration in kidney transplant patients by about two-fold, presumably by selective ENT1 inhibition. However, as the initiation of treatment with cladribine tablets is contraindicated in immunocompromised patients, including patients currently receiving immunosuppressive or myelosuppressive oncological therapies, no practical implications of the TKI-mediated or CsA-mediated ENT1 inhibition are expected for cladribine-treated patients with MS.

Frequently used lifestyle drugs such as alcohol and cannabis, and in particular inhaled or smoked THC/CBD-containing products, should be avoided before and during treatment with cladribine tablets because of their potent ENT1-inhibiting properties. In addition to ENT1 inhibition, long-term use of alcohol has been shown to result in down-regulation of ENT1 expression. For inhaled/smoked THC/CBD-containing products, it is recommended that patients with MS refrain from the use for at least 1 week before and during treatment with cladribine tablets in order to optimize ENT1-mediated uptake of cladribine into lymphocytes. In turn, oral or oral-mucosal medical THC/CBD products are unlikely to confer a clinically relevant ENT1 inhibition *in vivo* (Table 1).

Last but not least, although direct evidence is lacking, cladribine tablets should not be taken together with purine-rich meals, as dietary purines have been shown to compete for CNT2/3-mediated intestinal uptake of nucleosidic drugs as shown for ribavirin. However, it has been shown that cladribine tablets can be taken with high-fat high-calorie meals with a low purine content (e.g., the FDA-recommended standard high-fat breakfast).

A principle limitation of our review regarding the assessment of the possible *in vivo* relevance based on *in vitro* ENT inhibitor data together with human pharmacokinetic and protein binding data is the lack of specific regulatory (EMA/FDA/PMDA) or scientific (e.g., International Transporter Consortium) guidance for bidirectional equilibrative transporters. We therefore applied to our assessments the most conservative cut-off criterion provided in the EMA DDI guideline, which is the use of inhibitor concentrations of 50-fold $C_{\max,u}$ divided by IC_{50} or K_i , which results in a cut-off value of 0.02. Although this cut-off value is conservative, it is acknowledged that it is arbitrary and was not specifically developed for ENTs. However, our review showed that all ENT1-inhibiting substances for which it was shown that they are capable of increasing plasma adenosine concentrations to a significant extent exceed the chosen cut-off value of 0.02, which is a first confirmation of the practical applicability and usefulness of this cut-off for the assessment of possible *in vivo* relevance of *in vitro* identified ENT inhibitors. Although this review aimed to optimize the clinical efficacy of cladribine treatment by identification of potent

NT inhibitors that should be avoided during treatment with cladribine tablets, the results of this review are considered to some extent generalizable for other treatments with nucleosidic compounds, which may have similar or comparable NT substrate characteristics.

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Declarations

Conflict of Interest RH served as an external clinical pharmacology expert advisor for various aspects in the clinical development of cladribine, and received financial support for research, consulting, and training services from Merck Healthcare KGaA, Darmstadt, Germany. PK served as an external pharmacology expert advisor for *in vitro* transporter studies. He is a co-founder of Habilitas Kft, which received financial support for generation of an Internal Transporter Report from Merck Healthcare KGaA Darmstadt, Germany. He was also an employee of Solvo Biotechnology, Budapest, Hungary, outside this advisory work. ASK, MF, and AB are employees of Merck Healthcare KGaA Darmstadt, Germany. AG was an employee of Ares Trading SA, Eysins, Switzerland, an affiliate of Merck KGaA at the time of the study, and is currently a consultant to Merck Healthcare KGaA, Darmstadt, Germany. AM was at the time of preparation of this manuscript, an employee of the Merck Institute of Pharmacometrics, Lausanne, Switzerland, an affiliate of Merck KGaA.

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Availability of Data and Material Any requests for data by qualified scientific and medical researchers for legitimate research purposes will be subject to Merck KGaA's Data Sharing Policy. All requests should be submitted in writing to Merck KGaA's data sharing portal: <https://www.merckgroup.com/en/research/our-approach-to-research-and-development/healthcare/clinical-trials/commitment-responsible-data-sharing.html>. When Merck KGaA has a co-research, co-development, or co-marketing or co-promotion agreement, or when the product has been out-licensed, the responsibility for disclosure might be dependent on the agreement between parties. Under these circumstances, Merck KGaA will endeavor to gain agreement to share data in response to requests.

Code Availability Not applicable.

Author Contributions Not applicable.

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