

Narciclasine – an Amaryllidaceae Alkaloid with Potent Antitumor and Anti-Inflammatory Properties

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Key words

- Amaryllidaceae
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Abstract

▼ The isocarbostryril alkaloid narciclasine, also known as lycoricidinol, was discovered in *Narcissus* species (Amaryllidaceae) in 1967. A few years later, the 60S subunit of ribosomes, and thus protein biosynthesis, were shown to be directly targeted by narciclasine. Due to its selective and highly potent cytotoxic action on cancer cells, narciclasine was intensively investigated as an antitumor compound both *in vitro* and *in vivo*. However, narciclasine did not show a strong pharmacological activity in animal tumor models. During the last decade, new fascinating actions, mechanisms, and targets of narciclasine have emerged. This review intends to present a brief but comprehensive overview of these novel insights. Beneficial therapeutical actions have been reported particularly in brain tumor models. The translation elongation factor eEF1A, which does

not only participate in protein biosynthesis but also in the regulation of the actin cytoskeleton, was discovered as new direct target. Moreover, narciclasine was found to trigger actin stress fiber formation via the activation of the small GTPase RhoA. Progress has also been made regarding the pharmacokinetic characterization of the alkaloid. The synthesis of a great number of narciclasine derivatives led to a substantial understanding of its pharmacophore and of the structure-activity relationships. However, an optimized compound did not result from these efforts. Most importantly, a new field of indication has emerged: Narciclasine was proven to exert profound anti-inflammatory actions *in vivo*. Taken together, there has been a strong advance in the preclinical knowledge about the alkaloid. Nevertheless, narciclasine has not been tested in human clinical trials up to now.

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Amaryllidaceae Alkaloids

▼ The natural compound narciclasine was named after the plant genus *Narcissus* (daffodil), which belongs to the Amaryllidaceae (amaryllis) family. The medical use of different *Narcissus* species dates back to ancient times. Famous physicians of this period, such as Hippocrates of Kos (4th century BC) or Pedanius Dioscorides (1st century AD), recommended narcissus oil as a treatment against cancer, in particular uterine tumors [1]. This tradition was perpetuated during medieval times, e.g. in France by Henri de Mondeville (14th century AD) [1]. The pharmacologically most interesting secondary metabolites present in plants of the Amaryllidaceae family are alkaloids. Their scientific evaluation started in 1877 with the isolation of lycorine (● Fig. 1) [2], the prototypical and most widely spread representative of the Amaryllidaceae alkaloids. Its chemical

structure was published in 1956 [3]. The Amaryllidaceae family consists of 75 genera with about 1600 species [4], from which approximately 500 different alkaloids have been identified up to now. Based on the underlying skeleton, these compounds can be classified into at least nine different groups. Some authors even expanded this classification to 18 groups [5–7]. The most important prototypes of the nine classes are norbelladine, lycorine, homolycorine, haemanthamine, tazettine, montanine, galanthamine, crinine, and narciclasine (● Fig. 1) [7–10]. The Amaryllidaceae alkaloids have been found to exert a huge variety of pharmacological properties, such as antiproliferative, antitumor/cytotoxic, acetylcholinesterase inhibitory, analgesic, hypotensive, antibacterial, and antifungal activities [11]. The intensive research of the last decades, consisting of isolation, structure elucidation, analysis of structure-activity relationship, total synthesis, and pharmaco-

logical characterization, has shed light on their tremendous potential. In fact, in 2000/2001, the Amaryllidaceae alkaloid galanthamine (● Fig. 1), which was discovered in *Galanthus woronowii* (Caucasian snowdrop), was approved for the treatment of mild to moderate forms of Alzheimer's disease in Europe and in the USA.

The Discovery of Narciclasine

Narciclasine (● Fig. 1) was first isolated in 1967 from bulbs of several *Narcissus* species within a research program looking for antigrowth factors [12]. One year later, a chemical structure was proposed [13], but turned out to be incorrect. It was revised in 1970 [14,15] and the stereochemistry was fully elucidated by X-ray analysis in 1972 [16]. Narciclasine bears the substructures of isoquinoline, phenanthridine, and isocarbostryl. Due to the amide structure, it is not a basic alkaloid. Interestingly, the Amaryllidaceae alkaloid lycoricidinol, which was first isolated in 1968 as plant-growth regulator from the bulbs of *Lycoris radiata* (red spider lily) [17], turned out to be identical to narciclasine [18]. Thus, narciclasine was discovered independently by two different groups at almost the same time. In the following years, the compound as well as congeners thereof (e.g. narciprimine, 7-deoxynarciclasine, *trans*-dihydrnarciclasine, or 4-*O*- β -D-glucosyl-narciclasine) were also found in a variety of other Amaryllidaceae genera (e.g. *Haemanthus*, *Galanthus*, *Hymenocallis*, or *Leucojum*) [8,19–23]. The amount of narciclasine in these plants was reported to vary from 1.5 mg/kg (fresh bulbs) to 200 mg/kg [8,19]. An intriguing discovery was made in 2005: narciclasine was found in the Texas grasshopper *Brachystola magna* [24]. As this animal does not prefer species of the Amaryllidaceae family as food, undiscovered plant sources of narciclasine might still exist. Surprisingly, despite the intensive research on narciclasine, the first total synthesis was not developed until 1997 [25]. Up to now, six different total syntheses of narciclasine and one of the enantiomeric molecule have been published [26,27]. The number of steps necessary to build up the compound ranges between 11 and 22.

The Biosynthesis of Narciclasine

Narciclasine originates from *O*-methylnorbelladine, the central precursor of all Amaryllidaceae alkaloids. The biosynthesis of this precursor was intensively investigated in the late 1950s and early 1960s by tracer experiments with radiolabeled precursors and intermediates [28]: *O*-methylnorbelladine derives from the aromatic amino acids phenylalanine and tyrosine. Phenylalanine is transformed to protocatechuic aldehyde via *trans*-cinnamic acid, *p*-coumaric acid, and caffeic acid. Protocatechuic aldehyde reacts with tyramine, the decarboxylated form of tyrosine, yielding an imine (Schiff's base) that is reduced and methylated to *O*-methylnorbelladine. The subsequent biosynthetic route to narciclasine was studied in the early 1970s [8,19,29–32]: It was suggested to start with the cyclization of *O*-methylnorbelladine by a *para-para* phenol coupling reaction (● Fig. 2). The resulting compound is eventually converted into narciclasine via the intermediate 11-hydroxyvittatine and by an elimination of two carbon atoms (● Fig. 2). Although considerable progress has been made in the last years in the field of metabolic engineering for the production of plant isoquinoline alkaloids [33], the knowledge about the bio-

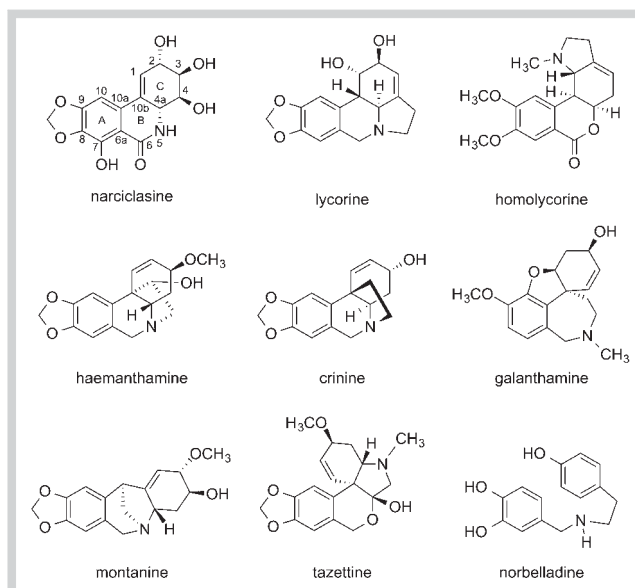


Fig. 1 Chemical structures of narciclasine and eight other prototypes of Amaryllidaceae alkaloids.

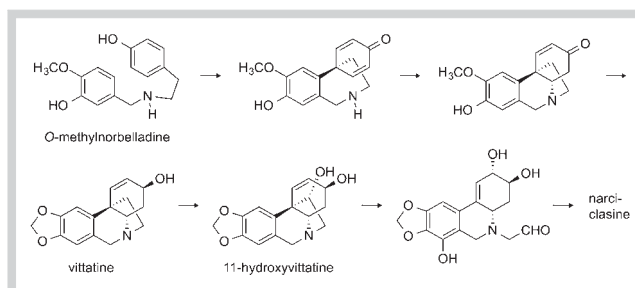


Fig. 2 Proposed biosynthetic route from *O*-methylnorbelladine to narciclasine.

synthesis of narciclasine has not expanded significantly since the 1970s. Neither the involved enzymes nor their respective genes have been characterized. This knowledge, however, would be strongly needed for a rational biotechnological approach, in particular to solve the supply issue, by producing narciclasine in an efficient way in plants or in microorganisms, e.g. by heterologous expression.

Preclinical Knowledge About Narciclasine

The subsequently presented knowledge about the pharmacological actions, the underlying mechanisms, and the target of narciclasine are graphically summarized in the ● Fig. 3.

Anti-tumor activity

Narciclasine inhibits cell growth by blocking protein biosynthesis. The initial publication describing the isolation of narciclasine also contains the first evaluation of its bioactivity: The compound showed a strong mitosis-blocking activity, since it effectively inhibited the growth of wheat grain radicles (0.05–0.5 μ g/mL) as well as of murine sarcoma cells *in vivo* (oral and subcutaneous

application) [12]. The first study proposing a mechanistic explanation of these actions was published in 1975: Carrasco et al. [34] reported that the alkaloid inhibits protein synthesis in rabbit reticulocytes as well as in a yeast-derived cell-free system by blocking peptide bond formation at the ribosome. These results were corroborated and largely expanded by further detailed studies published between 1975 and 1978 by the group of Vazquez [35–39]: Narciclasine blocked protein synthesis with an IC_{50} of 70 nM and inhibited HeLa (human cervix carcinoma) cell growth with a comparable IC_{50} of 100 nM. RNA synthesis was not affected, whereas DNA synthesis was slightly diminished, but only at much higher concentrations. The mechanistic basis of this effect has not been clarified, however, a direct interaction of narciclasine with the DNA (complex formation) was excluded [40]. The binding site of narciclasine was located in the 60S subunit of the ribosome and found to overlap with that of the known peptidyl transferase inhibitors anisomycin and trichodermin. Two decades later, narciclasine was utilized as a chemical probe to study the fine structure of the ribosomal peptidyl transferase center [41]. In 2014, X-ray crystallography was applied to decipher the exact binding mode of 16 different ribosome inhibitors, among them narciclasine, at the atomic level in yeast ribosomes [42]. This study confirmed that narciclasine inhibits the step of peptide bond formation during elongation by binding to the 60S tRNA A-site.

Mechanisms beyond ribosome inhibition. In the early 1990s, the group of G. Pettit reported about the broad cytotoxic activities of narciclasine against a variety of cancer cells after the compound was tested in the NCI panel of 60 human tumor cell lines [43]. The mean IC_{50} of narciclasine in this screening was 15.5 nM. Interestingly, melanoma cell lines were the most sensitive tumor cells. Besides the *in vitro* screening approach, the NCI also performed a number of tests on tumor models in mice [44]. However, the overall efficacy of narciclasine was only modest and associated with a considerable toxicity. Surprisingly, the mechanisms underlying the narciclasine-induced death of cancer cells was not investigated until 2007: Dumont et al. [45] showed that narciclasine was cytotoxic to all investigated cancer cell lines (IC_{50} : 30 nM), whereas much higher concentrations were needed to interfere with the viability of fibroblasts (IC_{50} : 7.5 μ M). Ingrassia et al. [46] confirmed this selectivity towards cancer cells (mean IC_{50} : 38 nM) and reported that endothelial cells (HUVECs) are more sensitive than fibroblasts, since narciclasine inhibited the proliferation of endothelial cells with an IC_{50} of approx. 90 nM. This points towards a possible antiangiogenic action of the alkaloid. In fact, unpublished data from our own lab confirm this hypothesis.

Narciclasine used at the high concentration of 1 μ M was proven to cause apoptotic cancer cell death via activation of the Fas and death receptor 4 (DR4) death-inducing complex (DISC) and the subsequent recruitment of caspase-8 [45]. Interestingly, whether downstream effector caspases (e.g. caspase-3) are activated directly or via mitochondria depends on the cell type: In the prostate cancer cell line PC-3, narciclasine directly activated effector caspases via the Fas/DR4-triggered assembly of DISC and the activation of caspase-8. In the breast cancer cell line MCF-7, however, the activation of effector caspases depended on the processing of Bid, the release of cytochrome c from mitochondria and the subsequent formation of the apoptosome [45]. In human promyelocytic HL-60 cells and in human oral cavity squamous carcinoma HSC-2 cells, also low concentrations of narciclasine (18 and 50 nM, re-

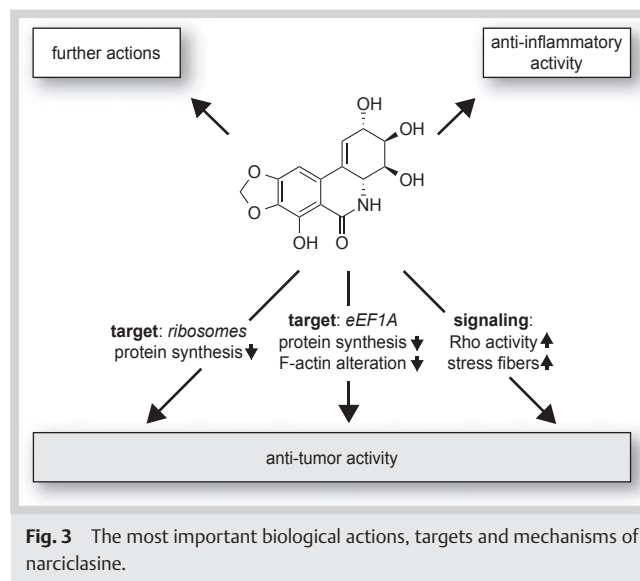


Fig. 3 The most important biological actions, targets and mechanisms of narciclasine.

spectively) have been proven to induce apoptosis (cell shrinkage, DNA fragmentation, caspase-3 activation) [47].

In contrast, narciclasine used at the concentration of 100 nM was also found to impair tumor cell growth without inducing apoptosis: The alkaloid blocked the proliferation and migration of glioblastoma cells *in vitro*, but did not trigger apoptotic cell death [48]. The authors of the study did not claim the inhibition of translation as mechanistic basis of the antitumor effect, but provided evidence that narciclasine activates the small GTPase RhoA [48]. Of note, RhoA was not suggested or investigated as a direct target of narciclasine. The activation of RhoA eventually led to the formation of F-actin stress fibers *via* the Rho kinase/LIM kinase/cofilin pathway. The increased generation of stress fibers was speculated to be the basis for the inhibition of cytokinesis and thus mitosis/proliferation, as well as for the decreased migratory capacity of glioblastoma cells. In the same study, the authors also tested narciclasine *in vivo* in a murine orthotopic model of human glioblastoma tumors. In contrast to the above mentioned disappointing *in vivo* results gathered by the NCI [44], narciclasine (1 mg/kg, orally or via tail vein injections) was able to considerably act against the glioblastoma tumor and to significantly increase the survival of the glioblastoma-bearing mice [48].

Van Goietsenoven et al. [49] studied narciclasine in another hard-to-treat brain tumor. They implanted human brain metastatic and apoptosis-resistant VM-48 melanoma cells into the brains of immunodeficient mice. Animals that were treated with narciclasine (1 mg/kg, orally) showed a significant therapeutic benefit that was even slightly stronger than that of the established chemotherapeutic agent temozolomide. The authors investigated the action of narciclasine on these melanoma cells in detail and discovered a new direct target of the alkaloid, the eukaryotic translation elongation factor eEF1A [49]. Binding of narciclasine to eEF1A was predicted by molecular docking analysis and proven in a cell-free system with recombinant human eEF1A as well as in a cellular assay using two melanoma cell lines. eEF1A is a very interesting protein: On the one hand, it delivers aminoacyl-tRNAs to the empty A-site of ribosomes, on the other hand, it binds to actin and participates in the organization of the actin cytoskeleton [50]. Thus, it regulates the morphology, cytokinesis, and migration of cells. Surprisingly, although migratory processes

in cancer cells are inhibited by narciclasine [48], a collagen invasion assay revealed that the invasive capacity of human cervix carcinoma cells (HeLa) was not influenced by narciclasine at a concentration of 50 nM [51]. This might be due to the fact that cancer cell invasion does not only consist of a migratory component. Another important factor is the interaction of the tumor cell with the extracellular matrix.

Anti-inflammatory actions

The first report on its anti-inflammatory properties was published in 1999: Mikami et al. [52] demonstrated that narciclasine effectively prevents paw swelling in a rat arthritis model. They also showed that narciclasine suppresses the production of TNF- α in LPS-activated murine macrophages. The authors ascribed this effect to the protein synthesis-blocking action of narciclasine [53]. In 2011, the alkaloid was reported to inhibit LPS-triggered NO production in a murine macrophage cell line (RAW264) as well as the generation of TNF- α in a human monocytic cell line (THP-1) [54]. One year later, Lubahn et al. [55] provided evidence that narcistatin, a water-soluble cyclic phosphate prodrug of narciclasine (see below), was able to reduce inflammation (by approx. 70%) and joint destruction (by approx. 50%) in rat adjuvant-induced arthritis after disease onset. Moreover, narcistatin also decreased the production of pro-inflammatory cytokines in different types of leukocytes. In 2015, our group characterized the anti-inflammatory effect of a narciclasine-containing extract of *Haemanthus coccineus* in two murine models of inflammation (dermal edema formation by arachidonic acid or croton oil and kidney injury caused by unilateral ureteral obstruction) [56]. The extract blocked the pro-inflammatory activation of leukocytes and endothelial cells. Interestingly, we could not detect any action on the activation cascade of the most prominent pro-inflammatory transcription factor NF κ B, but found a strong inhibition on the NF κ B-dependent gene transcription in endothelial cell. We are currently performing further experiments in order to fully understand the anti-inflammatory potential and underlying mechanisms of the alkaloid.

Further pharmacological activities

In 1992, data on the antiviral actions of narciclasine were published. The compound was found to be active *in vitro* against three flaviviruses (Japanese encephalitis, yellow fever, and dengue fever), but not against the human immunodeficiency virus 1 (HIV-1) and the vaccinia virus. However, the activity was very weak and the concentrations needed were too close to those inducing cytotoxicity in uninfected cells [57].

More recently, in 2012, the influence of narciclasine on the circadian clock of cells was investigated. The alkaloid reversibly extended the circadian period. This effect was not caused by the inhibition of protein translation, but by an altered transcription of the core clock gene *Bmal1* [58].

Interestingly, in 2015, Kim et al. [59] investigated the action of narciclasine in the context of the Alzheimer's disease. The alkaloid decreased the production of amyloid beta ($A\beta$) by attenuating amyloid precursor levels *in vitro*. In a murine Alzheimer model, a narciclasine-containing extract from the plant *Lycoris chejuensis* reduced the levels of $A\beta$ and plaques, and showed beneficial effects on cognitive functions.

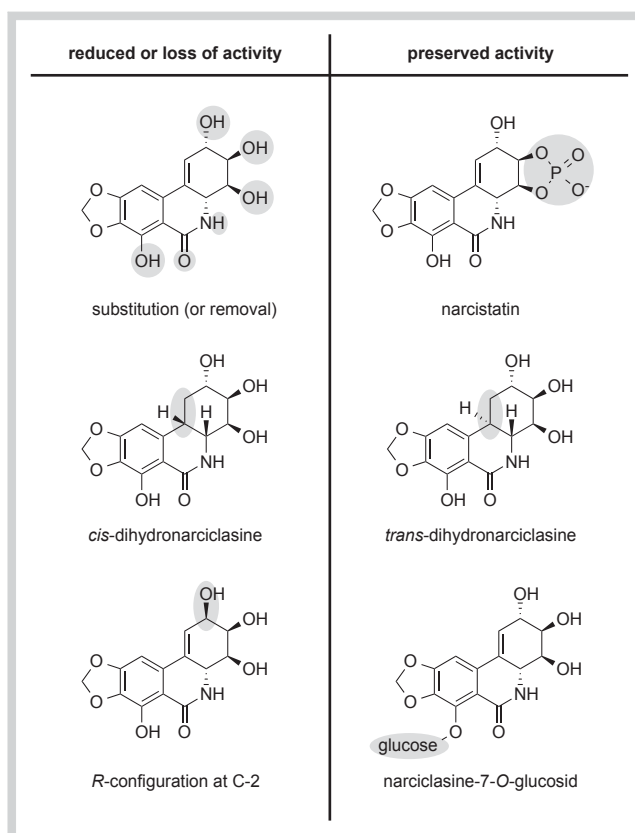


Fig. 4 Summary of the structural modifications of narciclasine and their impact on the biological activity.

Narciclasine as a Lead Compound

By analyzing the action of several derivatives, first insights into the structure-activity relationship of narciclasine were already established in the 1970s. The tri-hydroxylated ring C seems to be a critical part of the molecule (● Fig. 4): Hydrophobic substituents decreased the inhibitory activity on peptide bond formation and reduced the cytotoxicity against cancer cells [60, 61]. A loss of each of the three hydroxyl groups also attenuated the biological activity [62–64]. Interestingly, after reduction of the double bond between C-1 and C-10b (● Fig. 4), the resulting *cis*-dihydronarciclasine showed a weaker activity, whereas the *trans*-derivative was as active as narciclasine [51]. Pettit et al. [65] analyzed the biological activity of 7-deoxynarciclasine and 7-deoxy-*trans*-dihydronarciclasine (● Fig. 4). Both modifications diminished the cytotoxic activity against tumor cells. A huge number of narciclasine derivatives were synthesized by the group of R. Kiss [46]: Narciclasine was modified by the systematic addition of different substituents to each of the hydroxyl groups (C-2/3/4), to the phenolic OH group (C-7) and to the N atom of the lactam. In addition, the O atom of the lactam function was exchanged with a C atom (● Fig. 4). Unfortunately, most of these modifications led to a weaker antiproliferative activity. Also the *S*-configuration at C-2 (● Fig. 4) was shown to be of importance for the actions of the alkaloid [46]. Only narciclasine glucosylated at the phenolic group at C-7 (● Fig. 4) exhibited an *in vivo* activity comparable to that of the parent compound, but showed a slightly increased bioavailability. Taken together, the medicinal

chemistry approach to optimize the biological activity of narciclasine did not generate a largely improved compound.

Nevertheless, an important physicochemical property of narciclasine, its poor water solubility, could be improved: The group of G. Pettit converted narciclasine into the water soluble (4 mg/mL) prodrug narcistatin (● Fig. 4), a cyclic phosphate bridging the two hydroxyl groups at C-3 and C-4. Narcistatin, which is readily hydrolyzed to narciclasine by unspecific esterases, showed the same pharmacological potency as the parent compound [66].

The group of R. Kiss also evaluated both the pharmacokinetic properties and the adverse effects of narciclasine. In mice, the oral administration of 10 mg/kg resulted in a peak concentration of 300 ng/mL. The oral bioavailability was calculated as 32%, the terminal elimination half-life after iv application was 66 min [46]. Of note, the alkaloid was suggested to be able to cross the blood brain barrier, since brain tumors in mice were successfully treated [48,49]. Using liver microsomes from different species (rat, mouse, dog, human), narciclasine was found to be metabolically quite stable: After 45 min, only the rodent species evoked a slight decline in the concentration of narciclasine of less than 20% [46]. However, the observed actions in the brain are not necessarily caused by the compound itself. Its physicochemical properties are not favorable for the permeation of the blood brain barrier. Thus, unknown metabolites of narciclasine could be responsible for these actions. Unfortunately, this interesting issue has not been investigated so far. McNulty et al. [67] reported an inhibitory activity of the alkaloid against the human cytochrome P450 isoenzyme CYP3A4, which points towards an unfavorable interaction profile with a great number of drugs that serve as a substrates of this enzyme.

The side effects of narciclasine were studied in rats: The compound was administered by oral gavage for five consecutive days (Monday to Friday) for 3 weeks at doses of 0, 1, 3, 10, and 25 mg/kg/day. The no observed adverse effect level (NOAEL) was 1 mg/kg/day. Higher doses (3 and 10 mg/kg/day) caused piloerection, diarrhea, lethargy, stomach abnormalities and alterations in red and white blood cell parameters. The highest dose was too toxic and caused death in most of the animals within three days [46].

Summary

Taken together, narciclasine shows strong cytotoxic activity (nanomolar range) against a variety of tumor cells *in vitro*. The *in vivo* efficacy was very good in brain tumor models, whereas the action was not very pronounced in other types of cancer. Two direct targets of narciclasine have been discovered so far: ribosomes and eEF1A. Recent findings suggest that the actin cytoskeleton and thus processes like cytokinesis and cell migration are disturbed by narciclasine. This new knowledge has challenged the long standing concept of narciclasine primarily acting as an inhibitor of protein biosynthesis. Beyond cancer, a second promising field are inflammatory diseases, since the alkaloid has emerged to exhibit profound antiphlogistic properties *in vitro* and *in vivo*. Unfortunately, in particular in the area of inflammation, but also in the much better investigated field of cancer, only few studies provided in-depth insights into altered cellular functions and the underlying molecular mechanism. Moreover, the development of narciclasine is hampered by a lack of efficient supply, since total syntheses are complex and biotechnological approaches are completely missing. Thus, despite all the intriguing

findings about narciclasine, it still remains inconclusive whether narciclasine could be advanced to clinical trials.

Conflict of Interest

▼
The author declares no conflict of interest.

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