Identification of *h*Pin1 inhibitors that induce apoptosis in a mammalian Ras transformed cell line†

Elena Bayer,[a] Michael Thutewohl,[b] Claudia Christner,[c] Thomas Tradler,[c] Frank Osterkamp,[c] Herbert Waldmann[b] and Peter Bayer*[a]

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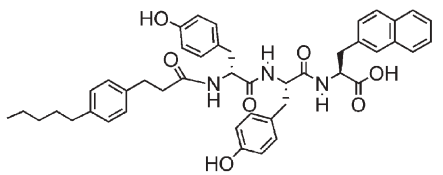
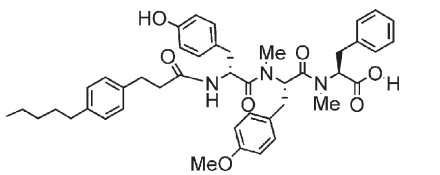
The authors have developed a class of potent inhibitors against the phosphate specific prolyl isomerase *h*Pin1, which induced apoptosis in transformed cell lines.

The development of new anti-tumor drugs is one of the great challenges of modern medicinal chemistry. Highly specific anti-cancer compounds of the second and third generation are currently developed to address various key proteins triggering cell signaling and cell division. One recently discovered major player in the regulation of mitosis, the peptidyl-prolyl *cis*/*trans* isomerase (PPIase) *h*Pin1, has become a promising new anti-cancer target. Overexpression of *h*Pin1 occurs in a number of human malignancies, *e.g.* oral squamous cell carcinoma and human breast cancer.[1] Deletion or depletion of the protein in human cancer cell lines causes mitotic arrest followed by apoptosis.[2] Mice and *Drosophila* knockouts of the Pin1 gene are viable,[3] a fact that emphasizes the advantages of *h*Pin1 as an anti-cancer target. On the molecular level *h*Pin1 is involved in several oncogenic pathways.[4] Due to its PPIase function, the protein is able to regulate the conformation of pSer/pThr–Pro moieties of a subset of mitotic proteins after they have been targeted by Ser/Thr–Pro specific kinases.[5a,b] This in turn controls the activation state of interaction partners downstream of the signaling cascade. To date only a few inhibitors of the Pin PPIase have been reported, *i.e.* juglone,[6] prolylphosphopeptides,[7a] a series of polycylic aromatic compounds[7b] and Ser–Pro mimics[7c].

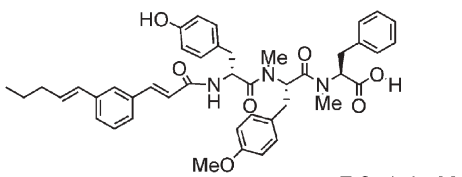
Given the biological importance of Pin1 and this lack of efficient inhibitors the identification and development of a compound class that might give rise to potent Pin inhibitors as well as insights into the mechanism that underlies the inhibition are of major importance to current medicinal chemistry and chemical biology research. In particular, it would be desirable to identify an inhibitor class with already proven biological relevance[8] that is readily amenable to combinatorial variation. In this paper we report on the identification of a new class of Pin1 inhibitors that fulfils these criteria. In the context of a program aimed at the combinatorial synthesis of peptide analogs derived from the biologically active natural product Pepticinnamin E, we synthesized a library that contained several compounds capable of inducing apoptosis of transformed cells.[9] Whereas in one case inhibition of farnesyltransferase might be the reason for this activity, for five other compounds a correlation with this inhibitory activity was not evident. In an attempt to identify the possible cellular targets of these inducers of apoptosis, the involvement of Pin1 in this process and the established link between Pin1 and the Ras signaling pathway downstream of Ras[5b] suggested to investigate the compound library for possible Pin1 inhibition. Thus, 34 Pepticinnamin analogs—among them the five apoptosis inducing compounds—were subjected to a PPIase assay.[9] Surprisingly and gratifyingly three of the apoptosis-inducing compounds turned out to be potent Pin1 inhibitors displaying IC_{50}‡ values in the nanomolar to single-digit micromolar range (Fig. 1).



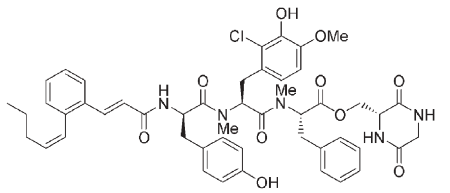
1 0.6±0.1 μM



2 1.9±0.1 μM



3 7.2±1.4 μM



Pepticinnamin E

Fig. 1 Pepticinnamin E, inhibitors and IC_{50} values of PPIase inactivation.

† Electronic supplementary information (ESI) available: Precipitation data, TOF-MS-ES$^+$ spectra, methods and peptidic library tested for *h*Pin1 inactivation. See http://www.rsc.org/suppdata/cc/b4/b414037k/

*peter.bayer@mpi-dortmund.mpg.de

In addition to the experiments on *h*Pin1 we proved exemplarily compound **1** for its selectivity against other PPIases. Inhibition constants for the human PPIases *h*Cyp18 (4.8 ± 0.1 μM) and *h*FKBP12 (4.6 ± 0.2 μM), were found to be one order of magnitude lower than that measured for inhibitor **1** of *h*Pin1. When compared with the other members of the library notably all three compounds contain a D-Tyr-L-Tyr dipeptide linked to naphthyl- or *N*-methylphenylalanine, indicating that this particular tripeptide unit might adopt a conformation that favours interaction with the peptide conformation-sensing *cis*/*trans* isomerase Pin1. The other two apoptosis-inducing compounds were one order of magnitude less active than compounds **2** and **3**, which might result from structural differences in the N-terminal elongation of the peptide chain (see ESI†). In order to explore this possibility in detail we attempted to determine the binding mode of this new class of inhibitors to the Pin1 protein by means of NMR spectroscopy techniques. To this end, increasing amounts of ligand **1** were added to a ^{15}N-labeled *h*Pin1 solution to reach a final molar ratio of 2 : 1 (ligand/protein). However, no shift differences were measured in the presence and absence of the inhibitor. Ligand binding could not be monitored, but severe precipitation of *h*Pin1 occurred in the sample (see ESI†). Only the protein but not the ligand is found in the precipitate. To test our assay we reverted to the well investigated Pin1 inhibitor, 5-hydroxy-1,4-naphthoquinone (juglone, K_i = 0.2 ± 0.1 μM),[6] as a positive control and repeated the HSQC experiments with this compound using the aforementioned experimental conditions. Juglone is known to be selectively attacked by the free thiol groups in parvulins followed by a subsequent 5-fold slower protein inactivation process, which is thought to be a partial unfolding of the active site.[6] Surprisingly, no chemical shift perturbation of amide resonances was observed in the NMR study. However, the protein precipitated in the NMR tube within one hour of spectra acquisition similar to the case of compound **1**. Dynamic light scattering experiments of Juglone–protein mixtures (data not shown) showed a rapid increase of high molecular weight particles a few seconds after the reaction was induced. Juglone and inhibitor **1** had probably caused *h*Pin1 to form high molecular aggregates and were able to lower its solubility. Inspired by this idea we asked, if induction of structural disintegration accounts for the inhibitory effect of our peptidomimetic compounds as was shown for juglone. Therefore, the potency to precipitate or accelerate precipitation of *h*Pin1 in solution was tested for the three inhibitors and juglone in a quantitative manner. A six molar excess of ligands (100 mM DMSO stock solution) was added to a 100 μM solution of *h*Pin1 and a series of 1D NMR spectra was recorded over a time period of about 45 min. Precipitation occurred in all cases, but not in the control sample. Juglone modifies the thiol groups of the two cysteines[6] by Michael addition or radical substitution and probably attacks some lysine side chains of the protein as shown by nanoESI MS. Pin1 contains two cysteine and seven lysine residues in its catalytic domain and additionally three lysine residues in its linker region and WW-domain. To prove, if induction of precipitation is a direct measure of Pin1 inhibition, its kinetics were compared to that of Pin1 inactivation.

Extensive kinetic studies of *h*Pin1 inhibition have already been performed with juglone.[6] A pseudo-first order rate for *h*Pin1 PPIase inactivation of 1.5 × 10^{-3} was found at a 10-fold excess of inhibitor. To measure induction of precipitation we followed the time course of the intensity of a methyl resonance in the 1D-NMR spectrum (Fig. 2). When neglecting the contribution of oligomers to a first approximation, a plot of resonance intensity *versus* time can be fitted to pseudo-first order reaction kinetics. The rate for juglone induced precipitation is 1.9 × 10^{-3} s^{-1} and is in excellent agreement with the rate of PPIase inactivation.[6] Although, the action of our three inhibitors on the protein could not be expressed by a simple pseudo-first order reaction rate, they seem to precipitate and destabilize *h*Pin1 in solution in a juglone-like manner, but without covalently modifying the protein.

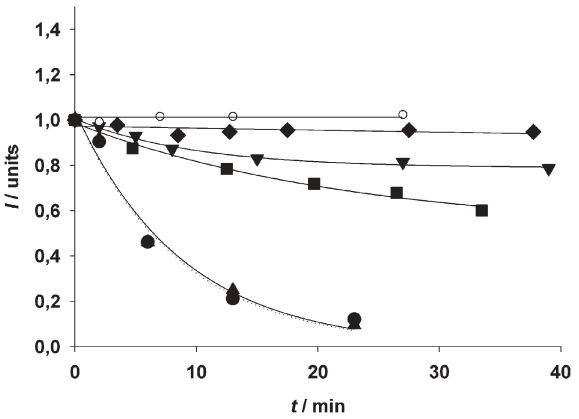


Fig. 2 Time dependency of integral of methyl resonance on inhibitor excess. ○, DMSO; ▼, inhibitor **1**; ◆, inhibitor **2**; ■ **3**, ● and ▲, juglone (measured on two different methyl resonances).

These results demonstrate that our synthesized non-phosphorylated peptidomimetica, which are biologically active and induce apoptosis in a mammalian transformed cell line, represent a new class of potent *h*Pin1 inhibitors. The inhibitors act by decreasing the thermodynamic stability of the protein and/or by lowering its solubility in the aqueous environment. The non-phosphorylated compounds exhibit exceptional advantages to highly charged peptidic *h*Pin1 inhibitors[7] as their uptake into cells is enhanced. Solid-phase synthesis of the identified compound class are established. Due to this peptidic nature the structure of this inhibitor class is readily amenable to both combinatorial variation as well as molecular-modelling-aided structure optimization and translation into non-peptidic compounds. Such compounds might be useful tools to study the influence of *h*Pin1 on the downstream signaling cascade *in vivo* and have the potential to serve as guiding structures in the development of new anticancer drugs.

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Elena Bayer,[a] Michael Thutewohl,[b] Claudia Christner,[c] Thomas Tradler,[c] Frank Osterkamp,[c] Herbert Waldmann[b] and Peter Bayer*[a]

[a]Molecular and Structural Biophysics, Max-Planck-Institut für Molekulare Physiologie, Otto-Hahn-Str. 11, D-44227 Dortmund, Germany & Interdisziplinäres Zentrum für Magnetische Resonanz, Dortmund, Germany. E-mail: peter.bayer@mpi-dortmund.mpg.de

[b]Department of Chemical Biology, Max-Planck-Institut für molekulare Physiologie, Otto-Hahn-Str.11, D-44227 Dortmund, Germany & Universität Dortmund, Fachbereich 3, Organische Chemie, D-44221 Dortmund, Germany

[c]Jerini AG, Invalidenstraße, 10115 Berlin, Germany

Notes and references

‡ IC_{50} values described in this context are a measure for the inhibitory potential of the compounds and are only valid under the sample conditions defined in the Methods section (see ESI†).

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