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Design, synthesis and evaluation of new RDP inhibitors

Hallur Gurulingappa, Phillip Buckhaults, Srinivas K. Kumar, Kenneth W. Kinzler, Bert Vogelstein and Saeed R. Khan*

The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD 21231, USA

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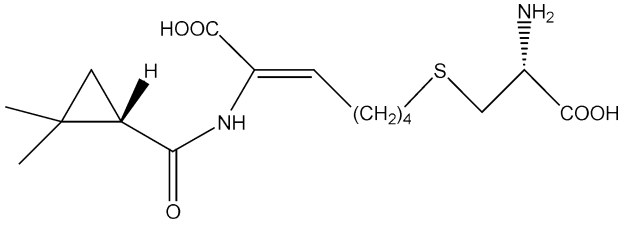
Abstract—Aminophosphinic acid derivatives were synthesized as potential inhibitors of renal dipeptidase, an enzyme overexpressed in benign and malignant colon tumors. Several compounds showed potent enzyme-inhibitory activity.

Colon cancer is the second most common cancer in the US and kills more than 50,000 people each year, but it is also one of the most preventable cancers. Screening provides the best prevention. With regular screening, precancerous polyps can be detected and removed, thus preventing the development of colon cancer. Current screening tests such as sigmoidoscopy, colonoscopy and detection of fecal occult blood have significant problems, which have stimulated the search for more specific non-invasive tests for the early detection of colorectal cancers. In recent Serial Analysis of Gene Expression (SAGE) studies performed on normal, adenomatous and cancerous colonic epithelium, the enzyme Renal Dipeptidase (RDP) was found to be overexpressed in both benign and malignant tumor compared with normal colonic epithelium.[1]

RDP has been extensively analyzed with respect to its catalytic mechanism and inhibition kinetics by a variety of synthetic inhibitors.[2–4] The crystal structure of human renal dipeptidase showed it to be a homodimer with each subunit consisting of a 369 amino acid residue peptide (42 kDa).[5] RDP is a zinc-containing hydrolytic enzyme that shows preference for dipeptide substrates with dehydro amino acids at the carboxyl position. Moreover, it can accommodate substrates with both D- or L-amino acids at that position, providing an excellent opportunity for the development of specific probes for its detection in vivo.[6] α-Aminophosphinic acids, the phosphorous analogues of natural occurring α-aminocarboxylic acids, have received increasing interest in medicine[7] and synthetic organic chemistry.[8–10] The crystal structure of RDP–cilastatin complex[5] has demonstrated that the dipeptidyl moiety of cilastatin is sandwiched between the negatively charged and positively charged sidewalls. Both ends of the moiety are clamped tightly by hydrophobic interactions. Certain aminophosphinic acid derivatives bind to the active site of RDP similar to dipeptides.[12] Based on these findings we designed and synthesized alkylaminophosphinic acid derivatives with C-terminal residue mimics incorporating cyclohexyl and *p*-substituted phenyl groups and tested their ability to act as inhibitors of RDP, in order to use them as biomarkers to detect early stage colon tumors.



Cilastatin

We report here the synthesis and biological evaluation of new RDP inhibitors. The method employed for the preparation of aminophosphinic acid derivatives is outlined in Scheme 1. Compound **1** was prepared by the condensation of cyclohexylacetaldehyde, hypophosphoric acid and aminodiphenylmethanehydrochloride,[11] followed by protection of the NH_2 and P–OH groups with Boc and CH_2N_2,[12] respectively. Reaction of **1** with trimethyl-2-phosphonoacrylate in the presence of NaOMe as base gave an intermediate, which subsequently underwent a Wittig–Horner olefination[13] with aldehydes to give mixtures of **E** and **Z** isomers, which

* Corresponding author. Tel.: +1-410-614-0200; fax: +1-410-614-8397; e-mail: khansa@jhmi.edu

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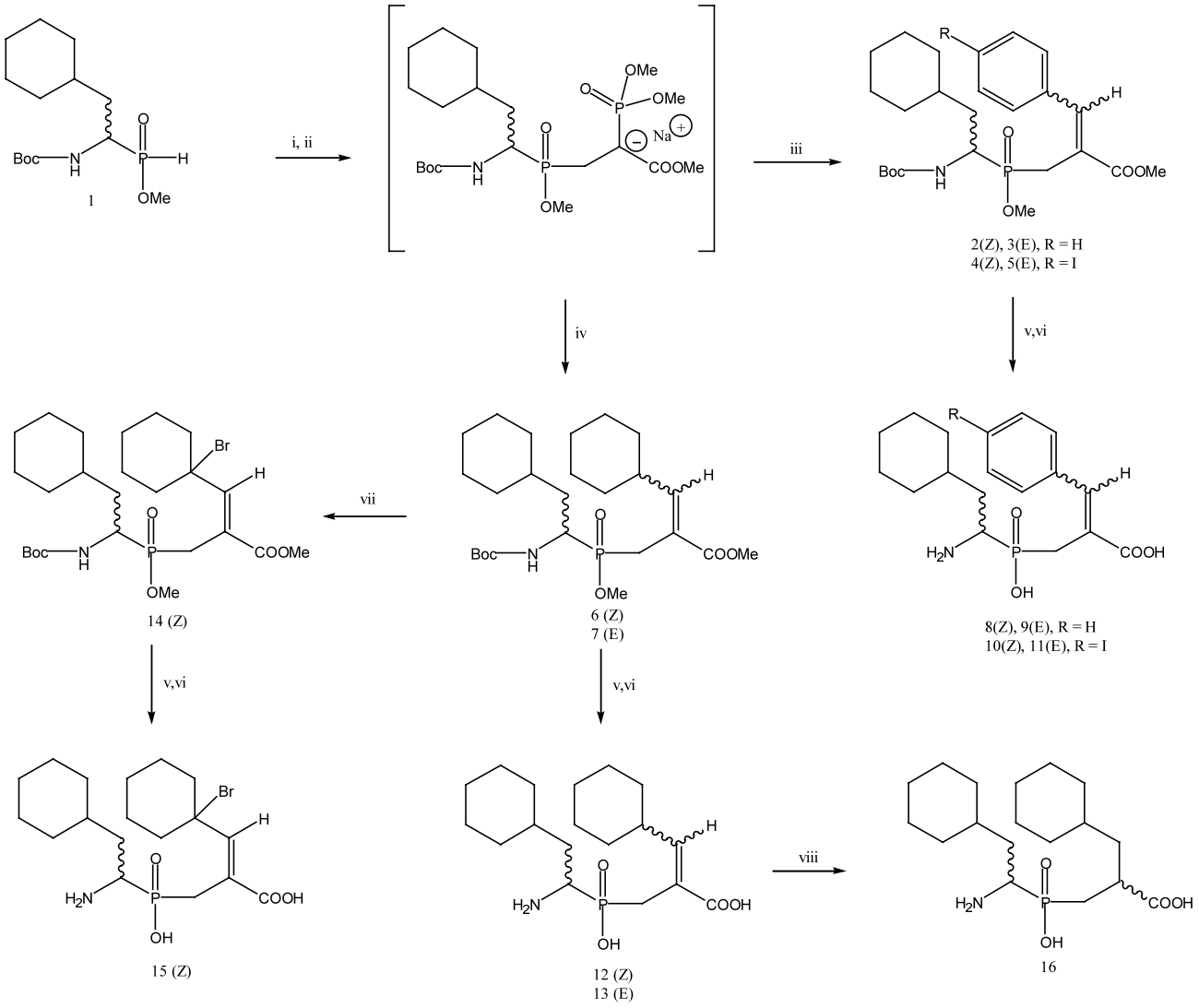

Scheme 1. *Reagents and conditions*: (i) NaoMe, MeOH, 0°C, 10 min; (ii) trimethyl-2-phosphonoacrylate, 0°C, 30 min; (iii) R-Ph-CHO, rt, 1 h; (iv) cyclo-$C_6H_{11}CHO$, rt, 1 h; (v) CF_3COOH, CH_2Cl_2, rt, 30 min; (vi) conc. HCl, 50°C, 18 h; (vii) NBS, BzOOBz, CCl_4, reflux, 2.5 h; (viii) H_2, Pd/C, MeOH, 30 min.

were separated by column chromatography over silica gel. The yields were 30–40% for the **Z** isomers and 20–30% for the **E** isomers. The protecting groups were removed by treatment with CF_3COOH followed by conc. HCl,[12] and the crude products were purified by column chromatography over silica gel to give **8**–**11**. Known compounds **6** and **7** were prepared[12] for comparison. The reaction of **6** with NBS gave a mixture of products from which **14** was isolated in 17% yield. Removal of the protective groups of **14** gave the corresponding free acid **15**.[15] The dihydro derivative **16** was prepared by catalytic hydrogenation of **12** and **13**. All compounds were purified by column chromatography over silica gel and their structures were unambiguously confirmed by spectroscopic methods.[16,17]

The RDP inhibition activity of these compounds was determined using crude lysates prepared from human colon cancers. The results are expressed as the concentration of inhibitor needed to inhibit enzyme activity by 50% (IC_{50}). The data for the synthesized compounds are shown in Table 1. Human colon cancer extracts were prepared by homogenizing 1 cm^3 of frozen colon tissue in 10 ml of 20 mM Tris, pH 8.0, 10 μM $ZnCl_2$, 0.1% Triton X 100. The extract was clarified by centrifugation at 13,000×*g* for 5 min at 4°C and, for each

Table 1. RDP inhibition activity[a] of compounds

Compound	Olefin geometry	IC_{50} (nM)
8	*Z*	5.5
9	*E*	300
10	*Z*	8
11	*E*	25
12	*Z*	3.5
13	*E*	60
15	*Z*	45
16		300

[a] IC_{50} values were determined using colon cancer lysate.

measurement, 20 μl was diluted into 158 μl of 20 mM Tris, pH 8.0, 10 μM $ZnCl_2$. 20 μl of the synthesized compounds were added to each reaction to obtain final concentrations ranging from 0 to 10 μM. The mixtures were incubated at room temperature for 30 min to allow enzyme-inhibitor complex formation, and the reactions were initiated by the addition of 2 μl of 1 mM substrate (∈DNP-L-Lys-D-Amp).[14] While incubating at 37°C, fluorescence ($\lambda_{ex}=320$ nm, $\lambda_{em}=405$ nm) measurements were determined at 30 s intervals and the relative reaction rate was taken as the rate of increase of fluorescence over time.

From the data in Table 1, it is apparent that compounds **8**, **10** and **12** are potent RDP inhibitors while **15** and **16** are far less active. In general, compounds with the **Z** configuration (**8**, **10**, and **12**) are significantly more active than their **E** counterparts (**9**, **11** and **13**).

Our rationale for the preparation of these compounds was based on the previous studies that the tetrahedral geometry of the phosphorous in phosphinic acid is important to mimic the presumed tetrahedral transition state to inhibit dipeptide hydrolysis.[9] We envisioned that the use of aromatic side chains in such compounds would substantially stabilize enzyme-inhibitor complex formation by hydrophobic interactions.

In conclusion, we have designed small molecules as inhibitors of RDP. Structure–activity relationship studies of this new class of compounds are continuing and will be reported in due course.

Acknowledgements

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15. Compound **11**: ^{1}H NMR (400 MHz, CD_3OD): δ 7.02 (m, 1H), 3.44 (m, 1H), 3.21 (m, 2H), 0.80–1.85 (m, 23H); ^{13}C NMR (125 MHz, CD_3OD): δ 168.3, 145.0, 125.6, 43.5, 39.5, 38.5, 35.3, 32.8, 31.6, 30.5, 29.2, 28.2, 26.5, 25.2, 24.5, 22.5, 21.6, 19.5; LC–MS m/z 436 $[M]^+$, 438 $[M+2]^+$.
16. Compound **4**: ^{1}H NMR (400 MHz, CD_3OD): δ 7.83 (d, 1H, $J=4$ Hz), 7.78 (d, 2H, $J=8$ Hz), 7.46 (d, 2H, $J=8$ Hz), 3.63 (m, 1H), 3.07 (m, 2H), 0.4–1.82 (m, 13H); ^{13}C NMR (125 MHz, CD_3OD): δ 168.6, 138.5, 137.4, 136.8, 134.2, 128.3, 127.5, 126.2, 95.8, 46.3, 32.4, 31.8, 30.6, 29.2, 28.4, 25.3, 24.5, 19.6; LC–MS m/z 477 $[M]^+$.
17. Compound **5**: ^{1}H NMR (400 MHz, CD_3OD): δ 7.67 (d, 2H, $J=8$ Hz), 7.14 (d, 2H, $J=8$ Hz), 6.95 (d, 1H, $J=4$ Hz), 3.62 (m, 1H), 3.10 (m, 2H), 0.88–1.81 (m, 13H); ^{13}C NMR (125 MHz, CD_3OD): δ 169.2, 137.5, 137.2, 136.6, 135.2, 127.5, 126.5, 125.8, 95.3, 46.5, 32.4, 30.8, 31.2, 29.3, 28.2, 25.4, 24.8, 23.6; LC–MS m/z 477 $[M]^+$.