Medelamines, Novel Anticancer Agents which Cancel RAS2Δ19 Induced Heat Shock Sensitivity of Yeast

Sir:

Missense mutations of ras oncogenes (K-ras, H-ras and N-ras) at the amino-terminus 12th amino acid are often found in colon and pancreas cancer. Compounds which suppress function of mutated ras oncogene products have been screened for a use in treatment of these cancers1. Inhibitors of ras farnesyltransferase has also been screened since farnesylation of ras proteins is needed for normal localization and function of ras proteins2. Biochemical and molecular analysis of ras proteins have revealed structural and functional similarity between human and yeast, Saccharomyces cerevisiae3. According to amino acid sequence alignment, since yeast ras2 protein had 7 redundant amino acids at the terminal region, a corresponding mutation was introduced to yeast ras2 gene at the amino-terminus 19th amino acid of the protein4. This mutation ras2Δ19, i.e. a missense mutation (glycine→valine) at the 19th amino acid induced abnormal signaling and caused the definite phenotypic changes, such as heat shock sensitivity of strain5. We report a screening system for compounds which cancel heat shock sensitivity of the yeast mutant strain and isolation of novel compounds, medelamine A and B. We also report their cytotoxicities and enzyme inhibitory activities against ras farnesyltransferase.

For the screening system yeast strain carrying the ras2Δ19 mutation was used4. Strain was inoculated with 30ml of YPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose and 0.9% agar purified [Difco]) into a square plastic plate (10cm x 14cm). Thick paper discs with 6mm diameter were immersed with sample solutions and applied to the agar plate. For sample diffusion the plate was incubated at 4°C overnight. This plate was incubated at 30°C for 8 hours for primary cultivation. Then, heat shock was carried out by floating the plate on hot water (50°C) for 10 minutes. To detect survived strain after this treatment the plate was incubated further for 16 hours at 30°C. Sample which gave growth zone around the disc was selected as a candidate which cancelled the heat shock sensitivity of yeast. Finally, an assay without heat shock treatment was done and a sample which did not promote yeast growth was selected. An example of this assay was shown in Fig. 1.

Microbial cultured broth were applied to the assay. Among 4000 samples, one culture, Streptomyces sp. NK14819 strain, which was isolated from soil collected in Saitama Prefecture, Japan, was selected. The active compound was produced by rotary shaking culture in a medium containing 2% soluble starch, 0.1% glucose, 0.3% peptone, 0.5% yeast extract, 0.3% meat extract and 0.2% CaCO₃. The maximum productivity was attained after 4 days cultivation. The compound was extracted from mycelia with MeOH and purified by silica gel column and Sephadex LH-20 column chromatographies (Fig. 2). TLC analysis of the active fraction 3 shown in Fig. 2 gave single spot detected by phosphomolybdic acid/sulfuric acid and ninhydrin reaction. However its NMR and Mass spectrometric data showed the sample was composed of two major congeners (FAB-MS (m/z): (M+H)+ 214 and 228) and also showed the congeners had a amino group. To separate and detect congeners easily, a proton of amino group was substituted with β-methoxylbenzyl S-4,6-dimethylpyrimidin-2-ylthiocarbonate (PMZS) reagent (Fig. 2). The N-PMZ derivatives thus obtained were applied to preparative HPLC and detected with UV absorption at 254 nm of PMZ group (Fig. 3). The compound in Peak A and B were isolated and its PMZ group was removed in acidic condition (Fig. 2). The compounds were named medelamine A and B, respectively. From 10 liters of cultured broth 15mg of medelamine A and 35 mg of medelamine B were obtained as a colorless syrup. A mixture of 3 mg of medelamine A and 7 mg of medelamine B gave the same NMR and FAB-MS spectrometric data as the active fraction 3. This suggested that unexpected reactions had not occurred during the chemical conversions.

Medelamine A and B were soluble in MeOH, CHCl₃ and EtOAc. They gave positive color reaction with phosphomolybdic acid/sulfuric acid reagent and ninhydrin. On a silica gel TLC (Merck Art No. 5715) developed with BuOH-AcOH-H₂O, 10:1:2, each medelamine gave single spot at Rf 0.55. Molecular formulas and weights of medelamine A and B were es-

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Fig. 1. Positive response in the assay.

Thick paper disc with 6 mm diameter was immersed with 1 μg/ml (upper) or 10 μg/ml (lower) medelamine A solution and applied to the assay.
Fig. 2. Purification of medelamines.

Cultured broth (10 liters) 
filtration
Mycelia
MeOH extraction, Dry up
Residue
BuOH extraction (pH=8.0), Dry up
Residue (5.7 g)
	silica gel column (CHCl₃-MeOH, 4 : 1)
Active fraction 1 (450 mg)
LH-20 column (MeOH)
Active fraction 2 (122 mg)
	silica gel column (BuOH-H₂O)
Active fraction 3 (63 mg)
PMZS reagent, Et₃N/MeOH-Dioxane, 1 : 1 
25°C, 3 hours
N-PMZ derivatives
HPLC [See Fig. 3]

Peak A
N-PMZ medelamine A (29 mg)
MeOH-6N-HCl, 3 : 1 
25°C, 12 hours
Medelamine A (15 mg)

Peak B
N-PMZ medelamine B (67 mg)
MeOH-6N-HCl, 3 : 1 
25°C, 12 hours
Medelamine B (35 mg)

Active fraction 3 was treated with 7-methoxybenzyl S-4,6-dimethylpyrimidin-2-ylthiocarbonate (PMZS) reagent for conversion to N-PMZ derivatives. N-PMZ medelamine A and B were separated and prepared by HPLC as shown in Fig. 3. Finally, PMZ group was removed in acidic condition.

Established as C₁₄H₃₁N (213) and C₁₅H₃₃N (227) respectively based on HRFAB-MS and ¹H and ¹³C NMR spectra. Structures of these compounds were determined by means of chemical and spectrometric methods. Each medelamine has novel alkylamine structure (Fig. 4). The name of medelamine was derived from the structure of 12-methyltridecylamine of A and 12-methyltetradecylamine of B. Structural determination and total synthesis of the medelamine will be described in detail elsewhere.

The activity of medelamines to cancel the heat shock sensitivity of yeast was examined as demonstrated in Fig. 1 and summarized in Table 1. Both medelamines showed nearly the same activity in this assay. Growth inhibitory activities of medelamines against cultured cells were investigated with rat normal kidney cell line, NRK⁶ and its transformant cell line, K-NRK⁷. K-NRK cell had been constructed by introduction to NRK cell of v-H-ras gene which had a missense mutation at the amino-terminus 12th amino acid⁷). As shown in Table 2, both medelamines showed growth inhibitory activities against both cells. There observed a tendency that K-NRK cell was slightly more susceptible than NRK cell. However, several anticancer drugs such as 5-fluorouracil, pepleomycin, vincristine, cisplatin, adriamycin and etoposide showed diverse selectivities to K-NRK cell, and some of them showed further selectivities than medelamines. Therefore, anti-ras activity of medelamines could not be concluded by the cytotoxicities. In
Table 2. Biological activities of medelamines.

<table>
<thead>
<tr>
<th>Assay</th>
<th>IC_{50} ± SD (μg/ml)</th>
<th>A</th>
<th>B</th>
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<tbody>
<tr>
<td>Cell toxicity¹ NRK</td>
<td>2.10 ± 0.82a</td>
<td>1.96 ± 0.81c</td>
<td></td>
</tr>
<tr>
<td>K-NRK</td>
<td>1.20 ± 0.33b</td>
<td>1.13 ± 0.40d</td>
<td></td>
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<tr>
<td>p = 0.075 (av sb) p = 0.093 (cv sd)</td>
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</table>

Ras farnesylation¹¹ 4.9 4.1

¹ NRK and K-NRK cells were cultured in 5% FCS-Dulbecco-MEM medium. Cells were inoculated into 96 well microtiter plate. 24 hours later samples were added, and cells were cultivated for 3 days. Cell growth was monitored by MTT method. Each experiment was performed in triplicate and was carried out independently 3 times. Each value is the mean of IC_{50} ± SD of 3 independent experiments. P value was calculated by Student’s t-test.

¹¹ Ras farnesytransferase assay was carried out as reported. Enzyme was purified from bovine brain and human ras protein was produced in recombinant E. coli strain. Commercially available 3H-farnesylpyrophosphate (Amersherm) was used as substrate and its incorporation into trichlor-acetic acid insoluble fraction was measured.

order to investigate a possible mode of action, an inhibitory activity against ras farnesy transferase was also examined. As shown in Table 2 both medelamines inhibited ras farnesylation reaction in cell free system.

Acute toxicity of medelamine A was examined. Medelamine A hydrochloride salt was dissolved in 5% glucose solution and administered to mice for 5 days (once a day). In po administration death was not observed even at a top dose of 200 mg/kg, though body weight decreased slightly. On the other hand, in ip administration, LD_{50} value was estimated between 25 mg/kg and 50 mg/kg.

Novel microbial metabolites, medelamine A and B were isolated from the Streptomyces cultured broth using chemical conversion reactions. Medelamines cancelled heat shock sensitivity of yeast caused by ras^{va119} mutation. We showed that medelamines had the inhibitory activities against ras farnesytransferase. Therefore interruption of farnesylation of ras^{va119} protein may be speculated as a possible action of medelamines in this assay. However their precise biological properties should be demonstrated in detail since medelamines also inhibited protein kinase C and A in our preliminary experiments. Medelamine A showed cytotoxicity and moderate acute toxicity in mice. Its anticancer activity in vivo is now under examination and will be reported elsewhere.

References


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