Structure-based drug design of small-molecule Ras inhibitors having anti-tumor activity

Small GTPases H-Ras, K-Ras and N-Ras (collectively called Ras) are the products of the *ras* proto-oncogenes and function as a molecular switch by cycling between GTP-bound active and GDP-bound inactive forms (Ras·GTP and Ras·GDP, respectively) in a variety of intracellular signaling pathways controlling cell growth, differentiation and apoptosis. Ras·GTP binds directly and activates downstream effectors such as Raf kinases, phosphoinositide 3-kinases (PI3Ks), RalGEFs and phospholipase Cε. Conversion of Ras·GDP to Ras·GTP is catalyzed by guanine nucleotide exchange factors (GEFs) such as Son-of-sevenless (Sos) and induces allosteric conformational changes in two flexible regions, termed switch I (residues 32-38) and switch II (residues 60-75), both of which constitute a principal interface for effector recognition. Ras are presumed to be one of the most promising targets for anti-cancer drug development because they are frequently activated by point mutations in human cancers. Nonetheless, there is no effective molecular targeted therapy for Ras at present now that farnesyl transferase inhibitors, which block posttranslational farnesylation of Ras necessary for membrane targeting, have been unsuccessful. Although recent success in drug discovery using structure-based drug design (SBDD) for AIDS and influenza has boosted hopes for its application to anti-cancer drug development, Ras have been presumed refractory to this approach because they lack apparently "druggable" pockets on their surface. Recently, by X-ray crystallography we solved novel crystal structures of the GTP-bound forms of M-Ras, a close homologue of Ras, and its mutant, which corresponded to a novel conformation called state 1, a GTP-bound inactive form, possessing surface pockets suitable for drug binding [1,2] (Fig. 1). This led us to apply SBDD to target Ras·GTP by utilizing the structural information on the surface pockets and obtain small-molecule Ras inhibitors [3].

The screening method used is summarized in Fig. 2. First, we applied the Molecular Mechanics Poisson-Boltzman surface area (MMPB-SA) method with an AMBER96 force field to carry out a computer docking screen of a virtual library containing 40,882 compounds based on the high-resolution (1.35 Å) crystal structure of M-Ras^{P40D}, carrying an H-Ras-type amino acid substitution at residue 40, in complex with a non-hydrolyzable GTP analogue guanosine 5'-(β,γ-imido)triphosphate (GppNHp), which had been determined using beamlines **BL38B1** and **BL41XU**

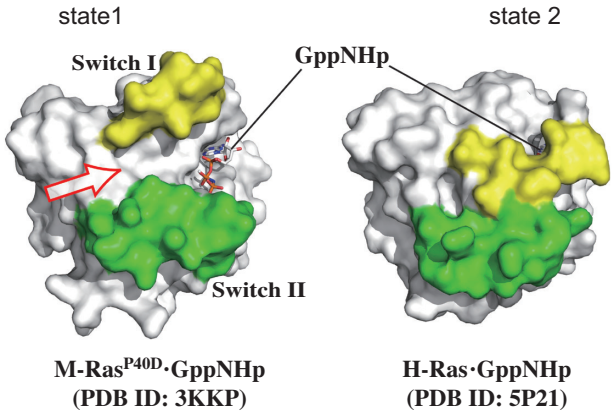

Fig. 1. Novel crystal structure solved with M-Ras^{P40D}·GppNHp in state 1 possessing a drug-accessible surface pocket (arrow) surrounded by the guanine nucleotide and the two switch regions (Switch I, yellow; Switch II, green). By contrast, the previously determined crystal structure of H-Ras·GppNHp in state 2 did not have such a pocket.

[2]. The selected compound Kobe0065, N-(3-chloro-4-methylphenyl)-2-{2,6-dinitro-4-(trifluoromethyl) phenyl}hydrazinecarbothioamide, and its analogue Kobe2602 (Fig. 2), identified by a computer-assisted similarity search, efficiently inhibited binding of H-Ras·GTP to Raf kinases both *in vitro* and at the cellular level. The compounds effectively inhibited both anchorage-dependent and -independent growth and induce apoptosis of mouse fibroblast NIH3T3 cells transformed by the mutationally activated H-*ras*^{G12V} gene, which was accompanied by downregulation of downstream molecules such as MEK/ERK downstream of Raf kinases, Akt downstream of PI3Ks and RalA downstream of RalGEFs. Moreover, they inhibited the upstream regulator Sos by interfering with Ras·GTP-binding to its allosteric regulatory site. The IC_{50} values of Kobe0065 and Kobe2602 for the inhibition of anchorage-independent growth of H-*ras*^{G12V}-transformed NIH3T3 were approximately 0.5 and 1.4 μM, respectively. The compounds were capable of inhibiting the anchorage-independent growth of human cancer cell lines carrying various activating mutations of the H-*ras*, K-*ras* and N-*ras* genes but not those carrying the mutationally activated *raf* or *PI3K* gene, indicating their specific inhibitory action on all the three Ras isoforms irrespective of the nature of the activating mutations. Furthermore, the compounds exhibited a potent anti-tumor activity, which was comparable to that of the approved anti-cancer drug sorafenib, on a xenograft of human colon

carcinoma SW480 cells carrying the mutationally activated *K-ras*^{G12V} gene by oral administration. The NMR structure of a complex of a Kobe0065-analogue Kobe2601 with H-Ras^{T35S}·GppNHp, exclusively adopting the state 1 conformation [4], confirmed its insertion into one of the surface pockets and provided a molecular basis for binding inhibition toward multiple Ras·GTP-interacting molecules [3] (Fig. 3). The fluorobenzene ring of Kobe2601 was located in close proximity to the side chains of Lys5, Leu56, Met67, Gln70 and Tyr74 of H-Ras. These six residues formed a hydrophobic surface pocket in the neighborhood of switch I, indicating that the fluorobenzene ring was inserted into the pocket through hydrophobic interaction. This study proved the effectiveness of our novel strategy for SBDD to target Ras·GTP and the resulting Kobe0065-family compounds may serve as a novel scaffold for the development of Ras inhibitors with higher potency and specificity.

Since the middle '90s, ^{31}P-NMR spectroscopic studies on Ras by Dr. Kalbitzer's group have unveiled their novel structural feature, i.e. conformational dynamics of their GTP-bound forms exhibiting equilibrium between two distinct states, state 1 and state 2, representing "inactive" and "active" conformations, respectively. Recently, we have determined a number of crystal structures of M-Ras·GppNHp, H-Ras·GppNHp and their mutants representing state 1, state 2 and their intermediates to show that hydrogen-bonding interactions of Thr35 (in switch I) and Gly60 (in switch II) with the γ-phosphate of GTP are abolished in state 1, which results in formation of a surface pocket (1, 2, 5). The pocket structure of M-Ras^{P40D}·GppNHp was successfully used for the discovery of the Kobe0065-family compounds as described above. Moreover, we clarified the molecular basis for the conformational dynamics of Ras·GTP by X-ray crystallography and NMR spectroscopy (2, 4, 5). This information could be utilized not only for the structural optimization of the Kobe0065-family compounds but also for the discovery by SBDD of a novel class of small-molecule Ras inhibitors.

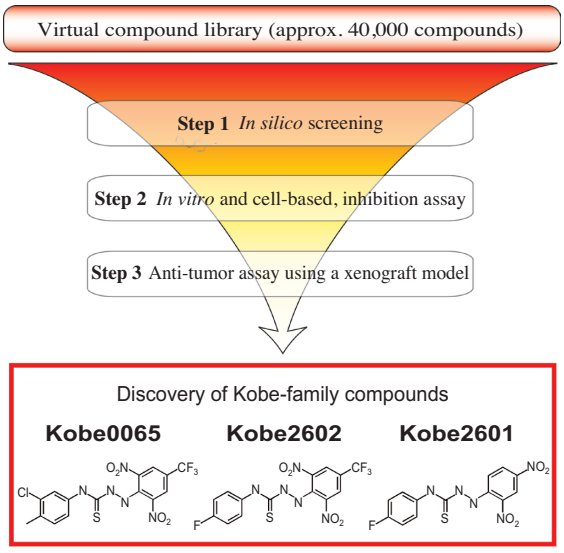

Fig. 2. Strategy for the development of Ras inhibitors used in the present study. Step1: screening for candidate compounds by computer-docking simulation targeting the surface pocket found in M-Ras^{P40D}·GppNHp. Step2: assays for inhibitory activities of the compounds on *in vitro* Ras/Raf-binding and proliferation of cancer cell lines carrying the activated *ras* oncogenes. Step3: assays for anti-tumor activity of the compounds on a xenograft of human colon cancer cells carrying the activated K-*ras* gene (K-*ras*^{G12V}).

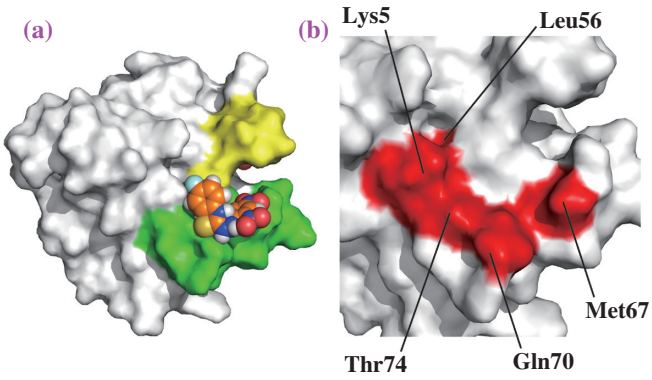

Fig. 3. Molecular basis for the interaction of the Kobe0065-family compounds with H-Ras. **(a)** The lowest energy solution structure of the complex between H-Ras^{T35S}·GppNHp and a Kobe0065-analogue Kobe2601 is shown by a surface model (Switch I, yellow; Switch II, green) while Kobe2601 is shown by a space filling model (C, orange; O, red; N, blue; H, gray; S, gold; F, light blue). **(b)** A close-up view of the compound-binding pocket in the complex (right), where the residues showing the intramolecular Nuclear Overhauser Effects are highlighted in red.

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