Entire human tyrosine kinase profiling panel will help to develop the specific kinase inhibitor.

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Protein kinases play critical roles in cell signaling pathways, related to cell proliferation and differentiation. Among more than 500 protein kinases from human genome, 90 tyrosine kinases are predicted based on their sequence analysis. These tyrosine kinases are classified into two types, transmembrane and cytoplasmic type. Former is known as receptor tyrosine kinase (RTK), which functions as mediator of environmental variations to intracellular signals. They are recognized as outstanding drug targets for aberrant cell proliferative diseases and clinically useful kinase inhibitors have been successfully developed. Since these inhibitors are known to have specific target among kinases, it is extremely important to clarify the inhibitory profile of drug candidates. This presentation is to discuss the profiling result of clinically useful tyrosine kinase inhibitors against human tyrosine kinase profiling panel. All human tyrosine kinase genes were cloned and expressed by using insect cell expression system. Most cytoplasmic tyrosine kinases have been obtained as full-length protein and cytoplasmic regions are expressed for most RTKs. For 77 out of 90 tyrosine kinases, *in vitro* phosphotransferase activities have successfully been confirmed and their assays have been developed for inhibitor profiling. Most of others such as EphB6 and CCK4, are regarded as inactive. Our entire human tyrosine kinase profiling panel (QuickScout™ TK Comprehensive Panel) is a powerful tool for developing kinase inhibitors with preferable specificity/selectivity.

Standard procedure of ELISA.

1) Compound solution, substrate solution containing Mg^{2+}/Mn^{2+} with or without ATP (w/o ATP for control), and enzyme solution were mixed and incubated in Streptavidin-coated 96-well plate.
2) After the incubation, the reaction was terminated by washing, and before the plate was blocked. 3) Incubation with HRP-conjugated antibody was followed by developing with TMB solution, and then read the absorbance at 450 nm.

Compound
↓
Substrate, ATP (+/-), Mg^{2+}/Mn^{2+}
↓
Enzyme
↓
Wash, blocking
↓
Anti phospho-Y
↓
Wash
↓
Colorimetric development for detection
↓
Stop
↓
Measure

Table 1. Seventy seven tyrosine kinases for the panel.

Name	Structure	Name	Structure	Name	Structure
ABL	Full Length	FER	Full Length	LTK	Kinase Domain
ACK	Kinase Domain	FES	Full Length	LYN(a/b)	Full Length
ALK	Cytoplasmic Domain	FGFR1	Cytoplasmic Domain	MER	Cytoplasmic Domain
ARG	Full length	FGFR2	Cytoplasmic Domain	MET	Cytoplasmic Domain
AXL	Full Length	FGFR3	Cytoplasmic Domain	MUSK	Cytoplasmic Domain
BLK	Full Length	FGFR4	Cytoplasmic Domain	PDGFRα	Cytoplasmic Domain
BMX	Full Length	FGR	Full Length	PDGFRß	Cytoplasmic Domain
BRK	Full Length	FLT1	Cytoplasmic Domain	PYK2	Full Length
BTK	Full Length	FLT3	Cytoplasmic Domain	RET	Cytoplasmic Domain
CSK	Full Length	FLT4	Cytoplasmic Domain	RON	Cytoplasmic Domain
CTK	Full Length	FMS(CSFR)	Cytoplasmic Domain	ROS	Cytoplasmic Domain
DDR2	Cytoplasmic Domain	FRK	Kinase Domain	SRC	Full Length
EGFR	Cytoplasmic Domain	FYN	Full Length	SRM	Kinase Domain
EphA1	Cytoplasmic Domain	HCK	SH3-SH2-Protein Kinase	SYK	Full Length
EphA2	Cytoplasmic Domain	HER2	Cytoplasmic Domain	TEC	Kinase Domain
EphA3	Cytoplasmic Domain	HER4	Cytoplasmic Domain	TIE2	Cytoplasmic Domain
EphA4	Cytoplasmic Domain	IGF1R	Cytoplasmic Domain	TNK1	Kinase Domain
EphA5	Kinase Domain	INSR	Kinase Domain	TRKA	Cytoplasmic Domain
EphA6	Cytoplasmic Domain	IRR	Cytoplasmic Domain	TRKB	Cytoplasmic Domain
EphA7	Cytoplasmic Domain	ITK	Full Length	TRKC	Cytoplasmic Domain
EphA8	Kinase Domain	JAK1	Kinase Domain	TXK	Kinase Domain
EphB1	Cytoplasmic Domain	JAK2	Kinase Domain	TYK2	Kinase Domain
EphB2	Cytoplasmic Domain	JAK3	Kinase Domain	TYRO3	Cytoplasmic Domain
EphB3	Cytoplasmic Domain	KDR	Cytoplasmic Domain	YES	Full Length
EphB4	Cytoplasmic Domain	KIT	Cytoplasmic Domain	ZAP70	Full Length
FAK	Full Length	LCK	Full Length		

Table 2. Inhibition Patterns of Gleevec and Iressa.

Although as it is reported that Gleevec inhibits PDGFRα, PDGFRβ, KIT and ABL, Iressa inhibits EGFR, and several other less recognized targets are inhibited by these kinase inhibitors.
ARG, DDR2, FMS, BLK, LCK, LYN(a/b) as Gleevec targets.
EphA4, EphA5, EphB4, PDGFRs, LYNa as Iressa targets.

These results are important not only this inhibitions against novel targets may help to predict/explain side-effects of the inhibitors, but also they may indicate novel therapeutic target as in the example of Gleevec, which has been developed and launched by Novartis AG for the treatment of Philadelphia chromosome positive chronic myeloid leukemia (CML). It's inhibition against Kit revealed the treatment of patients with Kit positive unresectable and/or metastatic malignant gastrointestinal stromal tumors (GIST).

pIC_{50}	Gleevec	Iressa
More than 8	PDGFRα	EGFR
Between 8 to 7	KIT PDGFRβ	EphA6
Between 7 to 6	ABL ARG DDR2 FMS BLK LCK LYNa LYNb	HER2 HER4 EphA4 EphA5 EphB4 PDGFRα PDGFRβ LYNa

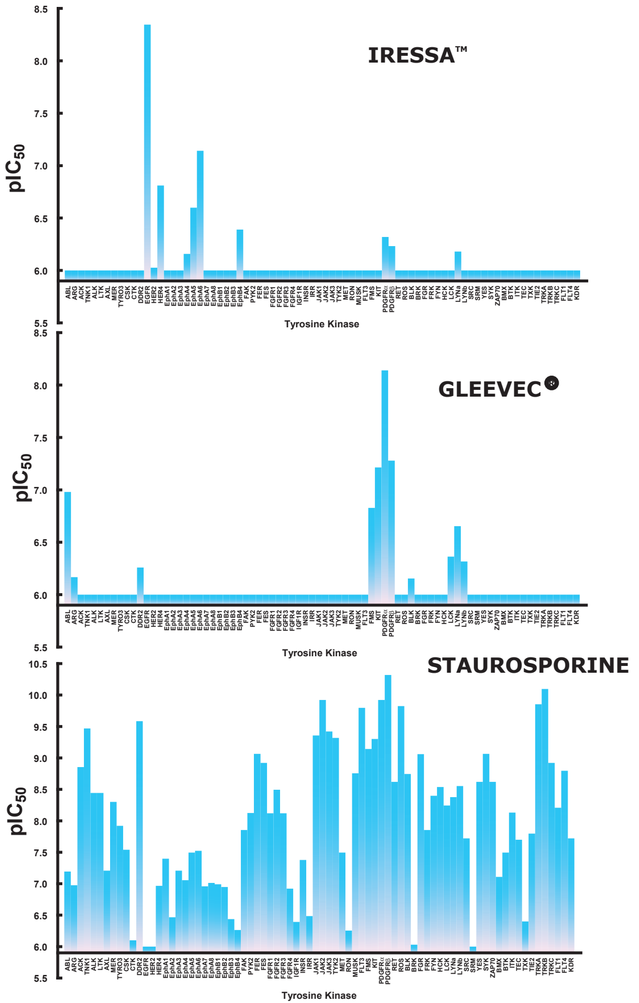
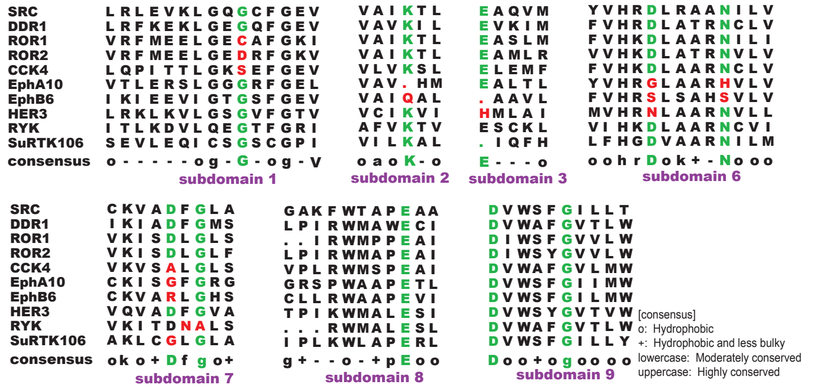


Figure 1. Profiling results for well known TK inhibitors

Three well known protein (tyrosine) kinase inhibitors were examined against 77 active tyrosine kinases (horizontal axis). Vertical axis indicates pIC_{50} ($-\log(IC_{50})$).

Figure 2. Alignment of phosphotransfer inactive tyrosine kinases.



Potential inactive kinases are aligned with Src, a typical active tyrosine kinase, showed in the top of the alignment (Fig. 2). Only important motifs for phosphotransfer reaction are shown in this figure and residues with important functions are colored green[1]. Residues supposed to be responsible to lose/weaken their activities are colored red.

In case of DDR1, although no *in vitro* phosphotransfer reaction has been observed, autophosphorylation of the kinase has been suggested from overexpression experiments in 293 cells after the stimulating with collagen[2]. This implies the unique mechanism to activate the protein. On the other hand, recent report of ROR2 is clearly demonstrating tyrosine kinase activity, *in vitro*[3] with unique activation mechanism. Learning unique properties of these two kinases, investigations are now on going at Carna Biosciences.

Others in the alignment are considered inactive. For ROR1, ROR2 and CCK4, conserved Gly in subdomain 1 is replaced by Cys, Asp and Ser respectively. This mutation might weaken affinity of kinases for ATP, depending on the type of replaced residue. For EphA10 and EphB6, highly conserved Lys at subdomain 2 is missed or replaced to Gln. The Lys is recognized as essential for maximal enzyme activity by anchoring and orienting ATP with interacting its α– and β– phosphates. EphB6 and SuRTK106 are missing Glu at subdomain 3. The Glu appears to help stabilizing the interaction between Lys (subdomain 2) and α– and β–phosphate of ATP. EphA10, EphB6 and HER3 have mutation on invariant Asp in subdomain 6. The Asp is believed to have a role as proton acceptor within phosphotransfer reaction. EphA10 and EphB6 also have mutation at the invariant Asn in subdomain 6 which is known to chelate secondary Mg^{2+} ion. CCK4, EphA10, EphB6 and SuRTK106 replace Asp in subdomain 7 to Ala, Gly, Arg and Gly respectively. The Asp chelates the primary activating Mg^{2+} ion to help γ-phosphate transfer. For RYK, a replacement of Phe-Gly in subdomain 7 to Asn-Ala shall weaken phosphotransfer activity by losing hydrogen bonding between Asp and Ala.

References
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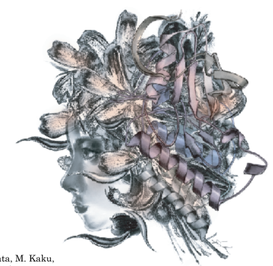
2) Vogel W, Gish GD, et al., Mol Cell. 1997 Dec;1(1):13-23. "The discoidin domain receptor tyrosine kinases are activated by collagen."
3) Kani S, Oishi I, Yamamoto H, et al., J Biol Chem. 2004 Nov 26;279(48):50102-9. Epub 2004 Sep 15. "The receptor tyrosine kinase Ror2 associates with and is activated by casein kinase Ic."

Conclusion

(1) Carna Biosciences has successfully collected 77 active human tyrosine kinases and developed their inhibitor screening systems (QuickScout™ TK Comprehensive Panel).
(2) Although Gleevec and Iressa strongly inhibit their well known targets, their novel targets ("off-targets") found by QuickScout™ TK Comprehensive Panel.
(3) For the rest of our under developed tyrosine kinases, 9 out of 13 has no activities in *in vitro* experiment (Three of LMR family members are found as serine/threonine kinase (data not shown)). Investigation of *in vitro* activity for DDR1 and ROR2 is now on going. Weak phosphotransfer activity has been observed for TIE1 and suitable conditions for inhibitor screening are under study. (Fig. 2)
(4) Our QuickScout™ TK Comprehensive Panel is useful to develop more advanced medicines.



The presenters wish to acknowledge K. Akita, T. Asami, H. Fukada, M. Gouda, N. Harada, M. Hatakeyama, E. Ishibushi, N. Iwata, M. Kaku, Y. Kawase, T. Myojin, R. Nakai, M. Nakamura, Y. Narumi, Y. Nishioka, C. Nukuzuma, K. Okita, and T. Tateishi for their contributions.