

***ROS Receptor Tyrosine Kinase: A New Potential Target for
Anticancer Drugs***

Ibrahim Mustafa El-Deeb,^{1,2} Kyung Ho Yoo,¹ So Ha Lee,¹

¹Life / Health Division, Korea Institute of Science and Technology, P.O. Box 131,
Cheongryang, Seoul 130-650, Republic of Korea

²Department of Biomolecular Science, University of Science and Technology, 113 Gwahangno,
Yuseong-gu, Daejeon 305-333, Korea

Correspondence to: So Ha Lee, Life Sciences Research Division, Korea Institute of Science and Technology, P.O. Box 131, Cheongryang, Seoul 130-650, Republic of Korea, Tel.: +82 2 958 6834; fax: +82 2 958 5189; e-mail address: LSH6211@kist.re.kr

Abstract: ROS kinase is one of the last two remaining orphan RTKs with a yet unidentified ligand. The normal functions of human ROS kinase in different body tissues have not been fully identified so far. However, the ectopic expression, as well as the production of variable mutant forms of ROS kinase has been reported in a number of cancers, such as glioblastoma multiforme, and non-small cell lung cancer, suggesting a role for ROS kinase in deriving such tumors. It is thought also that c-ROS gene may have a role in some cardiovascular diseases, and the fact that homozygous male mice targeted against c-ROS gene are healthy but infertile, has inspired researchers to think about ROS inhibition as a method for development of new male contraceptives. The recent discovery of new selective and potent inhibitors for ROS kinase, along with the development of new specific diagnostic methods for the detection of ROS-fusion proteins, raises the importance of using these selective inhibitors for targeting ROS mutations as a new method for treatment of cancers harboring such genes. This review focuses on the ectopic expression of ROS and its fusion proteins in different cancer types, and highlights the importance of targeting these proteins for treatment of substantial cancers. It describes also the recent advances in the field of ROS kinase inhibition, and the potential clinical applications of ROS kinase inhibitors.

Key words: ROS; glioblastoma; receptor tyrosine kinase; cancer; NSCLC

1. INTRODUCTION

Receptor tyrosine kinases (RTKs) are important players in the process of signal transduction and cellular communication. They act as the cell surface receptors for a number of important growth factors and hormones.¹ In addition to the vital role of these RTKs as regulators for normal cellular processes, they also have another dark side presented by their key roles in the initiation and progression of a number of cancers. In these types of cancers, gene translocations resulting in kinase fusion proteins with constitutive and uncontrolled activity are so common.²

A famous example for kinase fusion proteins is the Bcr-Abl oncoprotein, a non-receptor tyrosine kinase, which was identified as the most common translocation event in chronic myeloid leukemia (CML) with an incidence percent of more than 90% in CML patients.³ A number of gene translocations leading to the production of fusion proteins in a variety of other cancers have also been reported.⁴ The identification of translocations and mutations in human cancers is of great importance, since it can lead to the development of new therapeutics that target such fusion or mutant proteins selectively, providing new more selective and less toxic anticancer drugs.

It's much helpful also in providing new selective and sensitive diagnostic tools for identifying patients harboring such gene mutations or translocations allowing for their early treatment before progression of the cancer into a more complicated phase. Referring again to the example of Bcr-Abl translocation in CML patients, it's noteworthy to mention that the development and approval of Gleevec (Imatinib mesylate, STI-571)⁵ as the first kinase inhibitor to be used as an anticancer drug that selectively targets Bcr-Abl fusion protein for treatment of CML, has opened a new stream in the field of development of new selective therapeutics for treatment of certain cancers. This drug is the first of a new class of anti-proliferative agents designed to interfere with the signaling pathways that derive the growth of tumor cells. The development of this drug represents a significant advance over the classical therapies for CML. The use of conventional chemotherapies and radiation which lack the selectivity of targeting cancerous cells, results in serious side effects and is often of limited usefulness, since it fails to specifically target the underlying causes of malignancies. Accordingly, there is a continuous desire to identify new gene translocations or mutations that are responsible for the production of fusion or mutant proteins implicated in the progression of human cancers.

There are 58 RTKs in humans that belong to 20 distinct families,^{1,2} among these kinases is 'ROS kinase', which is one of the last two remaining orphan RTKs, since its ligand is yet unidentified. During the last 25 years, extensive work has been made for the identification and characterization of ROS RTK and its encoding gene, however, the first and the only review available so far about ROS kinase was published recently by Acquaviva *et al.*⁶ That review has addressed the different roles of ROS kinase in normal and cancerous tissues, its distribution in different body organs and hypothesized some possible methods for the discovery of its unidentified ligand. In this review we focus mainly on the mutagenic transformations in ROS gene and the resulted fusion proteins as possible targets for new selective anticancer drugs. This review addresses also the recent discovery of selective ROS kinase inhibitors,^{7,8} and the possible and potential applications for such inhibitors.

2. *c-ROS GENE AND ROS PROTEIN*

c-ROS gene was first discovered in 1986 when a recombinant DNA clone containing cellular sequences homologous to the transforming sequence, v-ROS, of the avian sarcoma virus UR2⁹⁻¹¹ was isolated from a chicken genomic DNA library.^{12,13} UR2 sarcoma virus is a retrovirus of chicken that encodes for a fusion protein, P68^{gag-ROS}, having tyrosine specific kinase activity.¹⁴ The oncogene, v-ROS, of UR2 carries a kinase domain that is homologous to those present in the oncogenes of the src family.¹⁵ The c-ROS sequence appeared to be conserved in vertebrate

species, from fish to mammals, including humans.¹³ The comparison of the deduced amino acid sequence of c-ROS and that of v-ROS showed two differences; 1) v-ROS contains a three amino acids insertion within the hydrophobic domain (TM domain), presumed to be involved in membrane association, 2) The carboxyl 12 amino acids of v-ROS are completely different from those of the deduced c-ROS sequence.¹²

Early reports have indicated that the deduced amino acid sequence of the kinase domain of ROS is highly homologous to that of the kinase domain of the human insulin receptor (HIR).^{12,16} However, it was proved later on, that the amino acid sequences in the kinase domains of these two RTKs are highly different. The homology level in the amino acid sequence in the kinase domains of ROS and HIR was found to be only 48.5%.¹³ On the other hand, the overall structure of c-ROS gene showed that the encoded protein carries an extracellular domain with a potential site of *N*-linked glycosidation, a hydrophobic 24-amino acids stretch, and a tyrosine kinase domain.¹³ These structural organizations are similar to those of the c-ErbB (the gene of the epidermal growth factor receptor), the c-Fms (the gene of macrophage colony-stimulating factor receptor) and the HIR gene.^{14, 17-19} These results strongly suggested that the human ROS gene encodes for a trans-membrane molecule which may function as a receptor for cell growth or differentiation factors. The analysis of c-ROS gene sequence applied to a transcript separated from rat lung,²⁰ and later on, for a cDNA from the human glioblastoma cell line, AW-1088,²¹ indicated a homology between the putative extracellular domain of ROS and the extracellular domain of the sevenless gene product of *Drosophila melanogaster*. Sevenless is a gene required for normal eye development in the fruit fly *Drosophila melanogaster* and it also encodes a transmembrane tyrosine specific protein kinase.^{22,23} The c-ROS oncogene was proved to be a member of the src gene family,²⁴ the proteins encoded by these genes have a high degree of amino acid sequence homology and are all associated with tyrosine specific kinase activities.²⁵

3. c-ROS GENE DISTRIBUTION AND FUNCTION

The trans-membrane RTK ROS shows a specific profile of expression restricted primarily to distinct epithelial cells during embryonic development.²⁶⁻²⁹ When c-ROS was first isolated from the chicken genome, tissues at various stages of chicken development were analyzed, but only kidneys were found to contain a significant level of c-ROS DNA.¹² Shortly after the first isolation of c-ROS from chicken, the expression of c-ROS gene in rats was examined and cDNA fragments containing the whole coding sequence of the gene were molecularly cloned.²⁰ The c-ROS gene

was found to be expressed in a tissue specific manner with c-ROS transcripts of varying sizes in different tissues. The obtained transcripts were isolated from lungs, kidneys, heart and testis.²⁰

The *in vivo* expression pattern of ROS in mice was also determined, where transient ROS expression was found during development, in kidneys, lungs and intestine.²⁶ It was found also that ROS mRNA is present in the caput segment of the epididymis of adult mice.²⁷ The expression was found to be restricted to the epithelial cells of the epididymis and is initially detected at the onset of regionalization of the caput epididymis.²⁷ In humans, ROS was found to be expressed throughout the human epididymis at varying levels, while absent from the proximal caput.³⁰ A recent northern blot analysis of RNA isolated from various adult human organs has determined that the highest ROS expression was detected in the lungs. Size variants were also detected in RNA isolated from placenta and skeletal muscle tissues.⁶

The expression pattern of ROS in different organs suggests that it may play a role in the mature functions of these organs beyond the developmental role. It's important also to notice that usually cellular homologues to retroviral transforming genes play an important role in cellular growth and/or differentiation, and appear to have oncogenic potential that can be manifested after transduction by a retrovirus. The process of conversion from a normal proto-oncogene to a transforming oncogene involves either mutation and/or degradation.^{17,25,31,32}

4. ONCOGENIC EXPRESSION OF ROS

The human c-ROS gene was mapped to the human chromosome 6 region 6q16q22.³³ This region of chromosome 6 is involved in non-random chromosomal rearrangement in specific neoplasias, including acute lymphoblastic leukemia,³⁴ malignant melanoma,³⁵ and ovarian carcinomas.³⁶ c-ROS gene up-regulation and/or mutation were found mainly in brain and lung cancers, in addition to chemical-induced stomach cancer, breast fibroadenomas, liver, colon and kidney cancers.

4.1. ROS in brain tumors

A number of RTKs are characteristic as markers for nervous system tumors. In particular, the epidermal growth factor receptor (EGFR) and its associated oncogene Erb-B are noteworthy, since 45-50% of malignant gliomas show evidence for EGFR amplification.³⁷⁻⁴⁰ Other RTKs include Neu,⁴¹⁻⁴⁴ platelet derived growth factor (PDGF) receptor⁴⁵⁻⁴⁸ and ROS⁴⁹⁻⁵². In a survey of 45 different human cell lines, ROS was found to be expressed in 56% of

glioblastoma derived cell lines at high levels (ranging from 10 to 60 transcripts per cell), while not expressed at all or expressed minimally in the remaining cell lines.⁴⁹ Moreover, no expression of ROS gene was observed in normal brain tissues, thus, the high level of ROS expression in glioblastoma seems specific.

In all of the tested glioblastoma cell lines, the c-ROS encoded transcript was found to be 8.3 Kb in size, except for the cell line U-118MG, where its size was found to be only 4 Kb, which suggests that the glioblastoma cell line U-118MG produces a high level of an altered (mutated) ROS-encoded protein. These results were contradicted by the findings of Wu and Chikaraishi⁵³ where they failed to detect elevated ROS expression in a survey of 25 specimens taken from patients with astrocytomas and glioblastoma multiformes (GBM) of all histological grades. However, they did not rule out the possibility of presence of small clones in the tumor, in which ROS is expressed in significant levels, but diluted with the larger population of non-ROS or low-ROS expressing cells. They expected also that ROS expression could be induced when the astrocytomas or glioblastomas were adapted in tissue culture.

However, the over-expression of ROS in surgical specimens was proved later by two independent analyses using RNase protection and cDNA hybridization techniques, where high levels of ROS expression in 33% and 40% of glioblastoma surgical tumors has been reported.^{50,51} The failure of ROS detection in lower grade astrocytomas suggests however that ROS may play a role in tumor progression rather than initiation.⁵⁰ The expression of ROS in meningiomas was also evaluated by complete reverse transcription and polymerase chain reaction (RT-PCR) assays.⁵⁴ ROS was found to be expressed at high levels in meningiomas (55%, 17 of 33) while not expressed in non-neoplastic brain samples. Recently, the expression of ROS in malignant glioma tumors was also evaluated.⁵² ROS was found to be over-expressed in 25% of low grade and 30% of malignant gliomas. From a total of 231 astrocytic glioma samples, it was found that 23% (11 of 47) of grade II, 17% (13 of 78) of grade III and 29% (31 of 106) of glioblastoma multiformes expressed ROS.

4.2. ROS in non-small cell lung cancer (NSCLC)

In a large scale survey of tyrosine kinase activity in lung cancer, tyrosine kinase signaling was characterized across 41 NSCLC cell lines and over 150 NSCLC tumors.⁵⁵ Profiles of phosphotyrosine signaling were generated and analyzed to identify known oncogenic kinases. Surprisingly, ROS kinase was of the top ten RTKs found in both cell lines and tumors. RTKs in this survey were ranked according to phosphorylation rank (phosphorylation level/sample). The results revealed that ROS kinase was highly expressed in one tumor sample and in the NSCLC

cell line (HCC78).⁵⁵ In addition to ROS over-expression in these samples, PTPN11 (protein tyrosine phosphatase, non-receptor type 11) and IRS-2 (Insulin receptor substrate-2) previously reported to be important downstream effectors of ROS in glioblastoma⁵⁶ were found to be highly phosphorylated in ROS expressing samples.⁵⁵

Furthermore, several microarray analyses of tumor specimens revealed significantly elevated ROS expression levels in 20 – 30% of patients with NSCLC.⁵⁷⁻⁵⁹ Contrasting for brain tumors, elevated ROS expression in lung tumors was observed in both early and late stage tumors,⁶⁰ suggesting a key role for ROS in the initiation or development rather than progression of lung tumors.

4.3. ROS in stomach, breast, liver, colon and kidney cancers

c-ROS gene was found to be up-regulated in gastric cancer induced by oral administration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in rat.⁶¹ ROS gene was one of six genes found to be persistently up-regulated after 4 weeks from MNNG treatment. ROS gene was found also to be over-expressed (in a number of other genes) in fibroadenoma samples taken from breast tumors of 5 different patients. It was found to be expressed at levels more than 2 fold higher than those in normal tissues.⁶²

In liver, the induction of hepatic progenitor cells activation in a rat model of liver injury was found to be associated with over-expression of ROS. In addition, over-expression of ROS was also observed in a rat hepatoma cell line.^{63,64} Recently, a global sequencing survey of all tyrosine kinases in 254 cell lines revealed three new ROS mutations in two colon adenocarcinoma and one kidney carcinoma cell lines.⁶⁵

5. MUTANT FORMS OF ROS

The most common methods for RTKs activation in cancer cells are genomic arrangements, exemplified by gene amplifications, chromosomal translocations, inversions and deletions.² Many of these genomic aberrations have been shown to result in the formation of onco-fusion proteins. Similarly, five different onco-fusion ROS proteins have been identified and characterized so far, these fusions are v-ROS, Mcf3, FIG-ROS, SLC34A2-ROS and CD74-ROS.

5.1. v-ROS and Mcf3

The transforming gene product, P68^{gag-ROS}, encoded by UR2 sarcoma virus genome is a rearranged product of the chicken c-ROS gene.¹² Studies made on v-ROS have revealed that the 3'-change of v-ROS has little effect on activation of the transforming potential of c-ROS, while 5'-truncation (deleting all but 6 amino acids of the extracellular domain) and fusion to gag are required for activation of c-ROS transforming activity, although this activity is not as potent as that of v-ROS.⁶⁶ Combination of 5'-truncation and the 3 amino acids insertion in the TM domain is sufficient to convert c-ROS into an oncogene as potent as v-ROS.⁶⁶

To further explore the role of this 3 amino acids insertion in v-ROS, this sequence was deleted and the transforming function and signal transduction of the mutant protein were investigated.⁶⁷ The obtained results revealed that the three amino acids deletion has no effect on the protein tyrosine activity of v-ROS, but this deletion results greatly in reduced transforming and mitogenic potency, as well as an altered protein modification and substrate interaction, despite the fact that the cytoplasmic domain of the mutant protein is unaltered.⁶⁷

In mammalian species, an activated form of human c-ROS gene, *Mcf3*, has been isolated from the DNA of the human carcinoma cell line MCF-7, using a tumorigenicity assay.^{68,69} *Mcf3* probably arose during gene transfer from a normal human c-ROS gene, by the loss of a putative extracellular domain. In this transformation, all of the amino acids (except only 8) of the putative extracellular domain of c-ROS are thought to be replaced by another unknown piece of DNA.⁶⁹ The point of fusion between cellular and viral sequences in v-ROS gene was found to be located in an analogous position to that in *Mcf3* fusion, leading to the loss of most of the putative extracellular domain.¹² Accordingly, the deletion of the extracellular domain appears to be an important event in the activation of the oncogenic potential of the c-ROS gene.

5.2. FIG-ROS

Elevated expression of growth factors and/or their related tyrosine kinase receptors has been found in all grades of astrocytomas.^{39,70-73} In most of the cases analyzed, this increased expression is the result of a gene amplification. The first genomic rearrangement reported in astrocytomas that results in the creation of a fusion RTK protein is 'FIG-ROS' fusion. This genomic fusion was noticed for the first time in the survey carried out by Birchmeier *et al.*,⁴⁹ on glioblastoma-derived cell lines, where a common transcript of 8.3 Kb was separated from all over-expressing glioblastoma cell lines except U-118MG, where a shorter transcript of only 4 Kb was observed.

The point of divergence between the normal and U-118MG c-ROS genes was located to that portion of c-ROS that encodes the junction between the putative extracellular and trans-membrane domains (TM domain).⁴⁹ Interestingly, this point of rearrangement in U-118MG DNA was found to be very close to the point of rearrangement that created v-ROS and *Mcf3* genes.¹⁵ After about 15 years, the molecular details for this genetic transformation have been explored by Charest *et al.*^{74,75} They demonstrated that an intra-chromosomal homozygous deletion of 240 kDa on 6q21 is responsible for the formation of FIG-ROS transcript. FIG (Fused in Glioblastoma) is a gene that encodes a protein that peripherally associates with Golgi apparatus, and likely plays a role in Golgi function.⁷⁶ This FIG-ROS transcript is encoded by 7 FIG exons, and 9 ROS derived-exons, and is a constitutively active fusion protein (Fig. 1).

Figure 1

This was the first example for a fusion RTK protein that resulted from an intra-chromosomal deletion, and it represents the first fusion RTK protein isolated from a human astrocytoma. In order to ascertain the frequency of FIG-ROS fusion event in glioblastoma, 10 different cell lines derived from all grades of astrocytomas were screened for the presence of FIG-ROS. Two GBM cell lines (U-118MG and U-138MG) have showed expression of FIG-ROS locus,⁷⁵ and an identical breakpoint was found in the FIG-ROS transcripts taken from both of these two cell lines. These two cell lines were established concurrently from the same patient,⁷⁷ strongly suggesting that the deletion was present in the primary tumor before the establishment of these two cell lines in culture, and is less likely to represent a culture artifact.

It has been reported that FIG peripherally associates with Golgi apparatus through its second coiled coil.⁷⁶ This portion is retained within FIG-ROS fusion, suggesting that it may also target the Golgi apparatus, and this targeting would be sufficient to incur an activation of its tyrosine kinase domain.⁷⁵ These results have demonstrated that the FIG-ROS locus encodes for an in-frame fusion protein with a constitutive kinase activity, suggesting that FIG-ROS may act as an oncogene. A recent study has further proved the oncogenic potential of FIG-ROS through the *in vivo* induction of glioblastoma formation in CNS, in a genetically engineered mouse model of brain cancer.⁵⁶

5.3. *SLC34A2-ROS and CD74-ROS*

Relative to other cancer types, only a limited number of gene translocations and mutant proteins have been reported in lung cancer, such as the t(15;19) translocation involving notch3.⁷⁸ A transforming fusion gene "EML4-ALK" has been recently identified by Soda *et al.*⁷⁹ in 6.7% of smoker Japanese patients with non-small cell lung cancer. This chimeric gene which was generated by a small inversion within the chromosome 2 short arm encodes a 1059 amino acids fusion protein.⁸⁰ However, the first reported translocations in human NSCLC involving protein kinases were those of ROS.^{55,81,82} The first translocation, SLC34A2-ROS, resulted from the fusion of c-ROS to the transmembrane solute carrier protein (SLC34A2) was discovered in the NSCLC cell line HCC78.^{55,81} This translocation between chromosomes 4q15 and 6q22 produced two fusion protein variants, that combine the *N*-terminus of sodium-dependent phosphate transporter protein, with the transmembrane and kinase domains of the proto-oncogene RTK ROS (Fig. 2A). The resulting SLC34A2-ROS fusion proteins, which are 724 amino acids (long variant) and 621 amino acids (short variant) are expected to retain kinase activity, and to derive the proliferation and survival of a subset of human NSCLC tumors, in which the fusion protein is expressed.

SLC34A2 is a phosphate transporter protein that is expressed in human lung and small intestine, and which has Na-dependent activity.⁸³ Defects in SLC34A2 expression and/or activity have been previously reported in ovarian cancer.⁸⁴ In order to verify the role of this genetic fusion in the initiation and development of the cancerous cells harboring it, the ability of two different siRNAs, targeted against c-ROS, to inhibit HCC78 cellular growth was evaluated.⁵⁵ Both of the two tried siRNAs were found to be effective in reducing ROS protein expression, and inducing cell death in HCC78 cells, demonstrating a strict dependence on ROS signaling for HCC78 cells survival.

Figure 2

The second ROS fusion (CD74-ROS) was identified in a c-ROS positive NSCLC tumor, where a translocation between chromosomes 5q32 and 6q22 resulting in the fusion of the *N*-terminus of CD74 to ROS at the typical site of the short transcript of SLC34A2-ROS fusion created a fusion protein (703 amino acids length) with two transmembrane domains, as in SLC34A2-ROS fusion, (Fig. 2B).^{55,82} CD74 is a type II trans-membrane protein that functions as the receptor for the macrophage migration inhibitory factor (MIF) immune cytokine.⁸⁵ CD74 functions also as an MHC (major histo-compatibility complex) class II chaperone protein which plays a critical role during peptide presentation to CD4-positive lymphocytes.⁸⁶

6. ROS SIGNALING

It has been demonstrated by the limited number of studies made on ROS kinase, that ROS activates several signaling pathways important for cell growth and survival. However, this kind of studies about ROS signaling is so limited, because of lack of knowledge about ROS ligand. To date, it was found that ROS activation involves these downstream activators and effectors:

6.1. STAT3

Signal transducers and activators of transcription (STATs) are transcription factors that regulate many aspects of cell growth, survival and differentiation. They are involved in cytokine and growth factor receptor induced gene expression. These transcription factors are activated by tyrosine phosphorylation, and they were found to be activated in a number of primary tumors, leading to increased angiogenesis, enhanced survival of tumors and immuno-suppression.^{88,89} The seven mammalian STAT family members identified so far are STAT1, STAT2, STAT3, STAT4, STAT5 (STAT5A and STAT5B), and STAT6. It's noteworthy to mention that the constitutive activation of STAT3 has been reported in malignant brain tumors,⁹⁰ and that STAT3 activation has been associated with resistance to chemo- and radiotherapy, which is a characteristic feature in CNS cancers. It has been also reported that ROS activates predominantly STAT3 in NIH-3T3 cells, and that STAT3 is required for ROS-anchorage-independent growth.⁹¹ Accordingly, the activated function of STAT3 seems to be important for the establishment and maintenance of ROS-induced cell transformation.

6.2. VAV3

VAV proteins are guanine-nucleotide exchange factors for Rho GTPases that regulate actin dynamics and gene expression. Three VAV members have been identified, *Cel-VAV*⁹² and two mammalian VAVs (VAV2 and VAV3).^{93,94} Although all VAV family proteins have similar structural features, they display different tissue expression patterns. VAV is primarily expressed in hematopoietic lineages, while VAV2 is ubiquitously expressed.^{95,96} VAV3 has a broad but different expression profile compared to that of VAV2.^{97,98} It has been reported that stimulation of several RTKs, including ROS, leads to tyrosine phosphorylation of VAV3 as well as its interaction with the receptor protein and its downstream signaling molecules.⁹⁹ ROS-VAV3 interaction was found to

be constitutive and independent of ROS activation. The inhibition of VAV/Rho GTPase signaling was found to be successful in suppressing ROS-induced colony formation in chicken embryo fibroblasts (CEFs) and anchorage-independent growth in NIH-3T3 cells.¹⁰⁰

6.3. PI3K/AKT/m-TOR signaling cascade

The Phosphoinositide-3 kinase (PI3K) signaling pathway is a key regulator in a number of cellular processes, such as cellular proliferation, differentiation and apoptosis.^{101,102} The AKT kinase, is a major downstream target of PI3K, and is an important mediator in cell growth and function.^{103,104} The activation of PI3K/AKT pathway was found to be an important event for ROS-induced transformation, and the inhibition of PI3K signaling pathway was proved to significantly diminish the ability of ROS to induce colony formation and anchorage-independent growth of fibroblasts.^{100,105} The activation of PI3K by ROS is thought to be mediated by IRS-1 scaffold protein, since the activity of PI3K was significantly decreased using SIR-1 antibodies.^{105,106}

It has been demonstrated also that FIG-ROS can activate the PI3K/AKT/m-TOR signaling pathway in FIG-ROS induced-brain tumor.⁵⁶ In addition, the m-TOR inhibitor, rapamycin,¹⁰⁷ was capable of inducing dose dependent growth inhibition in FIG-ROS brain tumor cell cultures,⁵⁶ which suggests that m-TOR inhibitors may be effective in treating ROS-activated malignancies.

6.4. SHP-1

The SH2-domain containing protein tyrosine phosphatase SHP-1,^{108,109} is expressed in hematopoietic, and at lower levels, in epithelial cells. It has been speculated that the defects in sperm maturation in ROS^{-/-} mice (with a targeted mutation of c-ROS)²⁷ and me^v mice (with 80-90% reduction of SHP-1 activity)¹¹⁰ might be related at the molecular level. This speculation was based on the hypothesis that, if ROS and SHP-1 interact in a common signal transduction pathway, impairment of the epidermal function might result from inactivation of either gene. Indeed, the molecular analysis has revealed that ROS binds and activates SHP-1 and that SHP-1 strongly binds ROS, and regulates ROS signaling in a negative manner, through a -ve feed back inhibitory mechanism.¹¹¹ ROS and SHP-1 are co-expressed in epididymal epithelium, and elevated phosphorylation of ROS in the epididymis of me^v mice suggests that ROS signaling is under control of SHP-1 *in vivo*. The interaction between SHP-1 and ROS was found to be mediated by high affinity binding of the SHP-1 N-terminal SH2 domain, and ROS phosphopeptides binding

sites pY2267 and pY2327.¹¹² These two phosphopeptides binding sites were found to be differentially employed in binding to SHP-1, where pY2267 was found to be involved in ligand-dependent stimulation of complex formation, while pY2327 mediates constitutive ligand-independent SHP-1 association.¹¹²

A number of linear and cyclic phosphopeptides related to the pY2267 binding site of ROS kinase have been synthesized and evaluated as ligands for the amino terminal SH2 domain of the protein tyrosine phosphatase SHP-1, in order to be used as activators for ROS down-regulation.^{113,114} The synthesized derivatives displayed much lower potencies for the stimulation of SHP-1 activity, but higher affinities relative to ROS pY2267, recommending their use as SHP-1 competitive inhibitors rather than agonists for phosphatase activity.¹¹⁴

6.5. SHP-2

In addition to binding to SHP-1, ROS kinase was found to bind and activate the SH2-domain containing protein tyrosine phosphatase SHP-2.⁵⁶ PTPN11, the gene encoding for SHP-2, was found to be mutated in a number of hematological malignancies,¹¹⁵⁻¹¹⁷ and solid tumors.¹¹⁸ The constitutively active SHP-2 is hence considered to be an oncogene, and the source of its oncogenicity comes from its ability to regulate the activation of a number of RTKs to the RAS/Raf/MEK/ERK signaling cascade.¹¹⁹⁻¹²¹ It was found also that the oncogenic activity of FIG-ROS fusion protein is mediated through phosphorylation of SHP-2, suggesting that SHP-2 activation is a crucial event in FIG-ROS transformation.⁵⁶ Furthermore, in SLC34A2-ROS and CD74-ROS fusion proteins harboring cells, the phosphopeptides corresponding to the tyrosyl phosphorylation sites of SHP-2 were found to be up-regulated.⁵⁵ These results strongly suggest that the activation of SHP-2 tyrosine phosphatase is an important signaling mediator for the constitutive activity of ROS kinase.

7. *c*-ROS: A GENETIC VARIANT FOR CARDIOVASCULAR (CV) DISEASES

As mentioned before, the physiological roles of *c*-ROS gene expression in different body organs have not been fully characterized. However, a number of studies have correlated between *c*-ROS and the incidence of different cardiovascular diseases. A large-scale, gene-centric association study made on 11,053 single nucleotide polymorphisms (SNPs) from 6891 genes has suggested that the risk of myocardial infarction (MI) was associated with specific genes, so far not linked to atherosclerosis.¹²² These genes encode the cytoskeletal protein paladin (PALLD), a taste receptor (TAS2R50), an olfactory receptor (OR13G1), a zinc finger protein (ZNF627) and ROS.

However, a number of subsequent studies failed to prove such association between MI and these genetic variants,¹²³⁻¹²⁵ emphasizing the high potential of false positive results obtained from the initial study, and indicating the necessity for continued refinement of cardiovascular genetic methodologies for clinical applications.

In two other studies applied to Japanese individuals, links have been proposed between the polymorphism of c-ROS gene (in a number of other genes) and restenosis¹²⁶ and hypertension.¹²⁷ In the first study, polymorphism of BCHE, GPX1 and c-ROS genes were found to be independently associated with in-stent restenosis after bare-metal stenting of coronary arteries, suggesting a genetic risk of these gene-determinants in in-stent restenosis.¹²⁶ In the second study, polymorphism in ABCA1 and c-ROS genes were reported in hypertensive Japanese patients, suggesting the possibility of using these genotypes for the prediction of the genetic risk for hypertension.¹²⁷ The genetic risk of atherothrombotic cerebral infarction was also correlated to c-ROS gene polymorphism, where in two different studies,^{128,129} c-ROS gene was among a number of other genes whose polymorphism was proposed to be informative for the prediction of the genetic risk of atherothrombotic cerebral infarction.

These frequent observations of c-ROS gene in a number of different CV diseases, and in every time among a number of varying genes, suggest a real possible role for ROS in one or more of these CV diseases. Furthermore, the reasonable expression of ROS reported in heart²⁰ and epithelial tissues²⁶⁻²⁹ provides another reason for considering a role of this gene in the CV system. However, the lack of ROS-ligand identity is a key difficulty in the full understanding of its role in different body tissues, and the identification of such ligand may provide important answers for such questions.

8. c-ROS GENE AND INFERTILITY

Transgenic mice targeted for c-ROS gene are fertile when heterozygous (HET) but infertile when homozygous (Knockout, KO).^{27,130} The infertility of male homozygous c-ROS knockout mice, inspite of normal mating contrasts with the effectiveness of cauda epididymal sperm in fertilizing ova *in vitro*,²⁷ and the most obvious explanation of these results would be that the motility of sperms from the knockout animals is impaired, so that they fail to reach the oocytes *in vivo*.¹³¹ c-ROS knockout mice lack the prepubertal differentiation of the epididymal initial segment, and a high proportion of sperms released from the cauda epididymis of these mice display sever tail angulations at the midpiece.¹³⁰ These flagellar angulations were thought to be the reason behind the reduced sperm numbers in the oviduct of mated females, and the failure to fertilize *in vivo*.¹³⁰

However, because the majority but not all the mature epididymal sperms from the knockout mice exhibit tail angulations, these findings suggest the possibility of some motile sperms with straight tails migrating into the oviduct, so the complete infertility of knockout males remain unexplained. One crucial aspect of natural fertilization is the binding of sperms to the oviductal epithelium for survival and prevention of precocious capacitation.¹³²⁻¹³⁷ Because the initial epididymal segment is active in the synthesis of specific epididymal secretions, and this segment fails to differentiate in the sterile c-ROS knockout males, it is possible that sperms from these males are not normally endowed and consequently incapable of normal interaction with the oviductal epithelium and fail to reach the oocyte in a potentially fertilizing state.¹³¹

Hence, the infertility of c-ROS knockout male mice can be explained by the inability of sperms to enter the oviduct, as a result of the bent tails of the majority of them compromised by their inability to effectively interact with the oviductal epithelium. Because flagellar angulations usually reflect a defect in volume regulation,^{130,138} and the capacity for sperm volume regulation is developed during maturation in the epididymis,¹³⁹ it was important to verify modifications in luminal fluid of c-ROS knockout male mice. A number of studies have revealed variable changes and abnormalities in the luminal fluid of these mice.¹⁴⁰⁻¹⁴² It was found that c-ROS homozygous knockout mice don't express the glutamate transporter excitatory amino acid carrier 1 (EAA1) mRNA in the caput, and since glutamate is used as an osmolyte in somatic cells, the lack of EAA1 may disrupt osmolyte balance in the proximal epididymal lumen, affecting sperm maturation and the development of sperm volume regulatory mechanisms.¹⁴⁰ A decrease in the Na-hydrogen exchanger NHE2 in the caput, and NHE3 in the cauda, associated with increase in luminal fluid pH in the c-ROS knockout male mice was also reported.¹⁴¹ In another study, an increased K⁺ ion concentration in cauda epididymal fluid was observed.¹⁴² These abnormal changes in sperm content which result in maturational abnormalities in volume regulation might have a compromising effect to sperm tail angulations, leading finally to c-ROS knockout male mice sterility.

The much concern about c-ROS gene and its role in the infertility of c-ROS knockout mice suggests the possibility of future using of ROS inhibitors as male contraceptives. However, a number of important points should be considered. First of all, the distinct differences between the human and mice epididymis, and the corresponding variations in ROS expression pattern in the epididymis of each of them may lead to a different effect of ROS inhibition in humans, which is not necessarily a complete infertility. The other important point to be considered is that the changes in the sperms of c-ROS knockout mice which made them to be infertile are thought to be caused by

maturational defects in the epididymis before puberty. Hence, the effect of ROS-inhibition on the fertility of mature wild male mice, and consequently on adult humans, would likely be significantly different.

9. ROS-INHIBITION FOR CANCER TREATMENT

It could be concluded from what is described in the previous sections that ROS kinase (with its fusion forms) is an important driver for a number of cancers, and that the targeting and inhibition of its activity could provide more efficient and selective solutions for the treatment of substantial cancers derived by it. The ability to diagnose and identify the cancer types harboring c-ROS mutations is as important as the development of selective and potent inhibitors for ROS RTK. The ability to identify, as early as possible, cancers that are derived by a mutant ROS kinase will greatly assist in determining the suitable therapeutic, or combination of therapeutics, that is most appropriate for the patient, thus helping to avoid development of inhibitors targeting other kinases that are not, in fact, the primary signaling molecule deriving the cancer.

For example; although targeted EGFR inhibitors are currently approved for the treatment of NSCLS,⁸⁷ it's anticipated that this therapy might be partially or wholly ineffective against those patients having tumors in which, mutant ROS kinase (rather than or in addition to EGFR) is expressed and deriving the disease in whole or in part. Instead, these tumors are most likely to respond to inhibitors for the mutated ROS kinase.

Moreover; a recent discovery has proved a high potential risk if the demethylating agent, 5-aza-2'-deoxycytidine (5-aza-dc) used for the treatment of ROS-derived tumors, since the ectopic expression of c-ROS in tumors was found to be tied to hypomethylation of a CpG island in the c-ROS promoter region, which means that ROS expression could be further activated by treatment of c-ROS negative cells with the demethylating agent (5-aza-dc).⁵²

In this stream, new sensitive and selective probes for the detection of SLC34A2-ROS and CD74-ROS fusions have been patented recently.^{81,82} Furthermore, a reporter construct that recognizes the phosphorylated state of c-ROS has been also recently engineered.¹⁴³ This new construct recognizes c-ROS activity and tests the ability of any potential inhibitor to turn-off ROS kinase activity. The construct is made of six parts, cyan fluorescent protein (CFP), a ROS substrate domain, a flexible linker, a recognition domain for tyrosine phosphorylation, a yellow fluorescent protein (YFP) and a nuclear export sequence. The construct changes conformation when ROS

phosphorylates the substrate domain, and this alteration can be recognized by measuring the fluorescence resonance energy transfer (FRET) between the CFP and YFP fluorophores.

10. ROS INHIBITORS AND POTENTIAL USES

The discovery of selective ROS kinase inhibitors was an important step to verify a number of theoretical speculations about ROS, and also to test for the applicability of these inhibitors in the clinical uses presumed for ROS inhibitors. Since the approval of the first kinase inhibitor, Gleevec,⁵ for treatment of chronic myeloid leukemia, a vast growth has been noticed in the field of drug discovery for the development of new selective kinase inhibitors targeted against mutant kinases. A number of inhibitors such as Staurosporine, AST-487 and PP 2 have been reported to inhibit ROS kinase, and even with high potency in some of them (as in case of Staurosporine, IC₅₀= 0.9 nM), but all these inhibitors were unfortunately highly unselective (Fig. 3).¹⁴⁴ The lack of selectivity in these inhibitors prohibited their clinical uses against any of the kinases inhibited by them in most of the cases (Table I).¹⁴⁵

Figure 3

Table I

10.1. Recent discovery of selective and potent ROS kinase inhibitors

The first selective and potent ROS kinase inhibitors have been recently developed by our group.^{7,8} A new hit pyrazole derivative (KIST301072) was discovered during screening of new potential kinase inhibitors prepared in our laboratory over a large panel of kinases (Fig. 3). KIST301072 was initially screened over a panel of 45 different kinases at a single dose concentration of 10 μ M. At this concentration, a 94% inhibition of the enzymatic activity of ROS kinase was observed, while the inhibition in activity was below 30% in all of the other kinases.⁷ The compound was then further tested in a 10-dose IC₅₀ mode and showed an IC₅₀ value of 199 nM for ROS kinase (Table II).

Table II

Following the discovery of this compound, we have synthesized a series of structurally related compounds and evaluated their capacity to inhibit ROS kinase.⁸ A new equipotent structurally simplified hit was evolved in this study, where KIST301080 showed in Fig. (3) was found to inhibit ROS kinase activity with an IC₅₀ value of 209 nM.

10.2. Potential clinical applications of ROS kinase inhibitors

As showed in table III; a number of potential clinical applications and uses are speculated for ROS kinase inhibitors. At the top of these applications is the ability of these inhibitors to treat and inhibit tumors derived by ROS kinase. Accordingly; it is very important now to evaluate the ability of these inhibitors to inhibit the growth of cancer cell-lines that are over-expressing c-ROS or harboring ROS mutations, such as GBM U-118MG and NSCLC HCC78 cell lines. Generally, it is anticipated that compounds which can inhibit the activity of wild ROS would be also capable of inhibiting mutant forms of ROS, since the kinase domain is conserved in all ROS-fusion proteins, and the transformations in such mutants involve only the extracellular domain. The inhibitors could be also tested *in vivo* by evaluating their ability to inhibit the growth of engineered tumors derived by ROS mutations, like the previously reported FIG-ROS induced brain cancer.⁵⁶ The selective responsiveness of cells bearing ROS mutations to these inhibitors would provide a new and feasible tool for the identification of cancers that are derived by ROS, by simply evaluating the inhibitory effect of these inhibitors over a large panel of suspected cell lines.

It would be also interesting to use these inhibitors to test for the sensitivity and applicability of the newly engineered reporter construct designed to test for the ability of potential inhibitors to turn-off ROS kinase activity.¹⁴³ The emergence of these new potent and selective inhibitors could also open the way for the verification of the ability of ROS inhibitors to be used as male contraceptives. The treatment of wild adult mice with ROS inhibitors and evaluating the ability of the inhibitors to produce infertility temporarily would be of great impact on the field of development of male contraceptives. Similarly, the doubt about a genetic role for ROS in a number of cardiovascular diseases could be also verified by testing the capability of ROS inhibitors to reverse and cure such diseases.

Table III

11. OTHER METHODS FOR ROS INHIBITION

In addition to small molecular kinase inhibitors, the inhibition of ROS kinase could be achieved by some other alternative ways. These alternatives include the use of ROS-kinase targeted antibodies that specifically bind to critically catalytic or binding domains required for ROS activity, and inhibit the kinase by blocking access of ligands, substrates or secondary molecules. It's important however to note that the use of antibodies that target the extracellular domain of ROS would be useless in inhibiting mutant ROS-fusion proteins, since in these fusion proteins the extracellular domain is totally or partly lost. Instead, the antibodies targeting the newly fused part (from FIG, SLC34A2 or CD74 for example) could be helpful in inhibiting the activity of these fusion proteins.

Another possible alternative is the use of small interfering RNA (siRNA), which inhibits gene translation and hence activity of ROS kinase, through the process of RNA interference. RNA interference and selective silencing of target protein expression by introduction of exogenous small double strand RNA molecules comprising sequence complementary to mRNA encoding the target protein has been well described.¹⁴⁶⁻¹⁵⁰ The use of siRNA against ROS has been already proved to be effective in ceasing ROS expression and inducing cell death in HCC78 NSCLC cells,⁵⁵ as previously described in this review. The inhibition of ROS signaling could be also achieved by indirect ways, through the interference with its downstream effectors, such as STAT3, VAV3, SHP-1, SHP-2, and PI3K/AKT/mTOR signaling mediators. As mentioned previously, the inhibitions of m-TOR⁵⁶ and VAV/Rho GTPase signaling¹⁰⁰ were successful in suppressing ROS signals and subsequently inhibiting ROS-induced cell growth. Accordingly, the inhibitors for such signaling downstream effectors could be useful, alone or in combination with specific ROS inhibitors, for the silencing of amplified c-ROS signaling in different diseases.

12. CONCLUSION

The discovery of c-ROS gene along with the subsequent discovery of its ectopic expression in different types of cancer, and the recent discovery of its mutant forms have shed light on its importance as a new potential target for treatment of cancer. The possible relation between c-ROS genetic alleles and the incidence of different cardiovascular diseases raises a question about possible roles for this gene in diseases other than cancer. The full distribution and expression profiles for ROS in different body organs and its physiological roles in normal body functions as well as in diseases needs to be further explored. The absence of any useful information about the activating ligand of ROS is a big mystery that awaits to be solved. The possibility of presence of more than one

ligand that is involved in different functions cannot be omitted. The recent discovery of ROS selective inhibitors should however help in providing useful answers for a number of questions; the majority of which are about the applicability of using ROS inhibitors in treatment of ROS derived cancers, as well as in other speculated applications, such as male contraception. During the next few years, the success in optimizing a more potent and selective ROS kinase inhibitor with good pharmacokinetic profile, can raise the interest about ROS kinase and attract the attention of researchers from different disciplines to focus research on ROS, which may lead finally to useful answers for the yet unanswered questions about this interesting orphan RTK.

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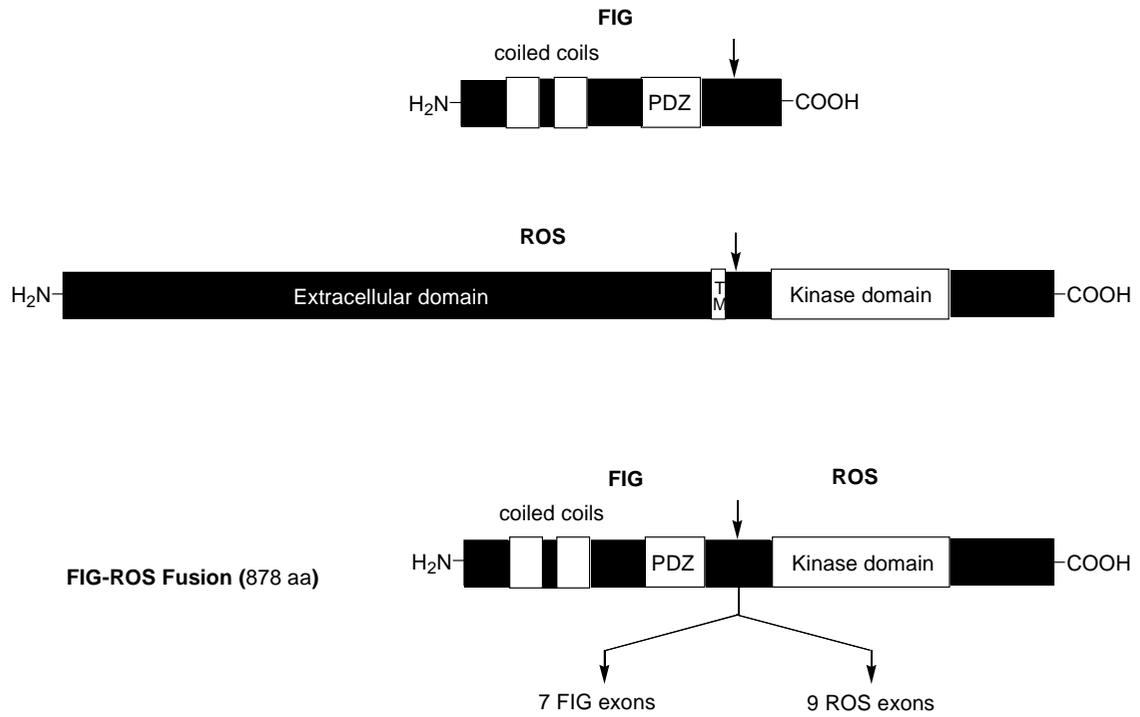


Figure 1. FIG-ROS gene translocation

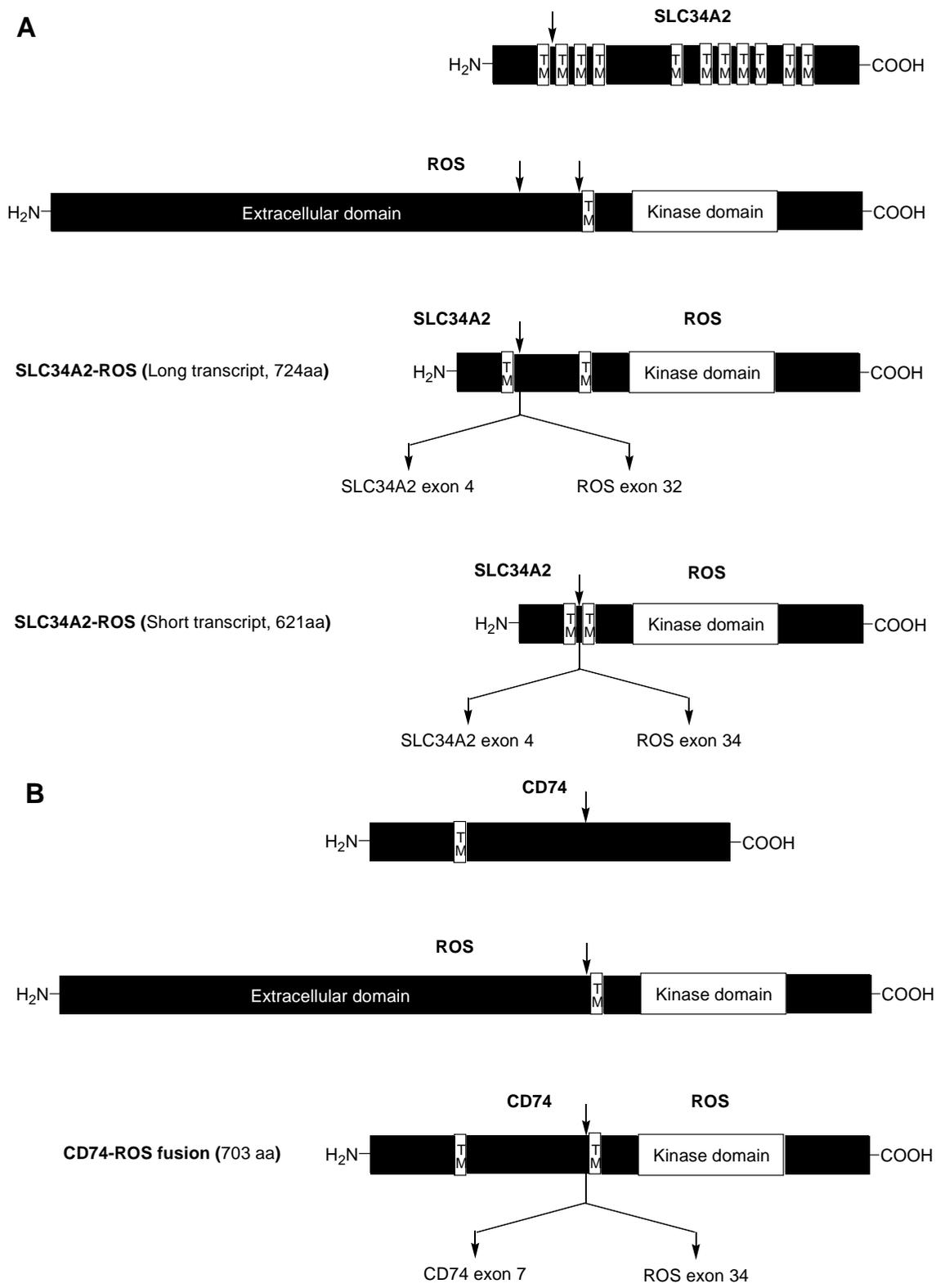
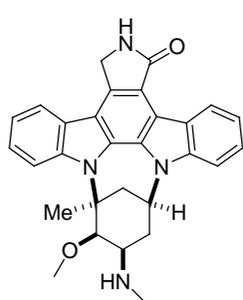
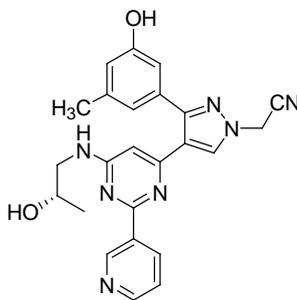


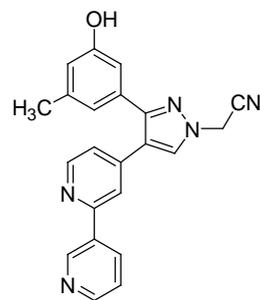
Figure 2. SLC34A2-ROS (A) and CD74-ROS (B) gene translocations



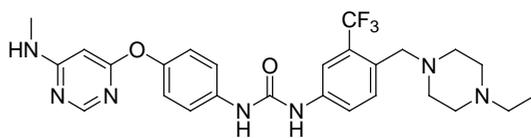
staurosporine



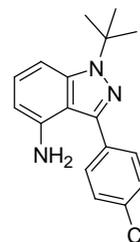
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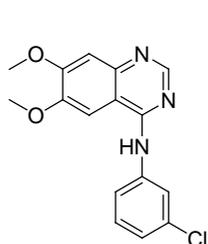
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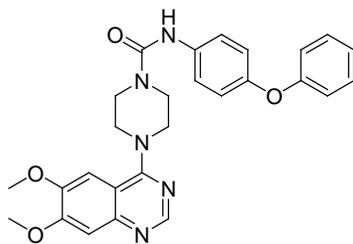
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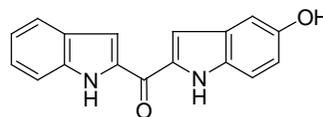
PP 2



AG 1478



PDGFR I-III



D-64406

Figure 3. The structures of compounds screened against ROS kinase.

Table I. IC₅₀ values of ROS kinase inhibitors by enzyme-based screening

Inhibitors	Selectivity	IC ₅₀ (nM)	Reference
Staurosporine	non-selective	0.9	144
KIST301072	selective	199	7
KIST301080	selective	209	8
AST-487	non-selective	1,700	143
PP 2	non-selective	5,200	144
AG 1478	non-selective	13,600	144
PDGFR I-III	non-selective	48,000	144
D-64406	non-selective	365,000	144

Table II. % Enzymatic inhibitions exerted by KIST301072

Kinase Enzyme	% Inhibition ^a	Kinase Enzyme	% Inhibition ^a
ABL1	20.44	JNK1a1	3.83
AKT1 (dPH, S473D)	-2.97	KDR/VEGFR2	-5.84
Aurora A	21.75	LCK	10.28
BRAF	8.97	LYN	-1.45
CDK1/cyclinB	3.64	MEK1	-6.97
CHK1	2.36	MST4	-1.54
CK1epsilon	9.32	MUSK	-0.42
c-Kit	2.85	P38a/MAPK14	5.27
c-MET	5.82	p70S6K	5.79
c-Src	16.18	PAK4	1.37
DAPK1	-1.17	PIM1	5.91
DNA-PK	6.56	PKCa	9.23
EGFR	-2.23	PLK1	5.85
EPHA1	23.27	RAF1	20.39
FAK/PTK2	3.43	RET	6.65
FGFR1	-8.05	ROCK1	11.72
FGR	3.34	RON/MST1R	28.82
FLT1	-1.67	ROS	93.92
FYN	-7.86	SYK	8.23
HIPK1	-3.43	TIE2/TEK	8.17
IKKa/CHUK	1.37	TRKA/NTRK1	-0.14
IR	7.01	YES	-2.59
JAK1	9.15		

^a Test compound was used in a single dose concentration of 10 μ M.

Application	Verification Methods
Anticancer drugs	Evaluate the ability of the inhibitors to inhibit the growth of cancer cell lines and in vivo engineered tumor models that are harboring ROS mutations
Discovery of cell-lines harboring ROS mutations	Evaluate the inhibitory effect of the inhibitors over a large panel of suspected cell lines utilizing the selective responsiveness of cells harboring ROS mutations
Male Contraceptives	Evaluate the ability of inhibitors to temporarily induce infertility in wild male mice similar to that produced in c-ROS knockout mice
Treatment of ROS-induced CV diseases	By testing the capability of ROS inhibitors to reverse and cure such diseases.

Table III. Potential clinical applications of ROS kinase inhibitors

Biosketch

Ibrahim Mustafa El-Deeb obtained his Bachelor of Pharmaceutical Sciences from Mansoura University, Egypt in 2001. In 2006 he earned his MS degree in Medicinal Chemistry from the same University. Now, he is a 3rd year Ph.D. student in the University of Science and Technology, South Korea, under the direction of Dr. So Ha Lee. He has been actively involved in the design and synthesis of kinase inhibitors as anticancer agents.

Kyung Ho Yoo received his Ph.D. degree in organic chemistry from Korea Advanced Institute of Science and Technology, Korea, in 1992. Subsequently, he did post-doctoral work at The Johns Hopkins University, USA. Currently he is working as a senior researcher at Korea Institute of Science and Technology, Korea. His research interests focused on the development of novel therapeutic agents for the treatment of cancer and neurodegenerative diseases.

So Ha Lee was born in 1962 in Kongju, Korea. He received his PhD from Korea University in 1999 for work on the synthesis of bisoxazine ligands and 3-substituted piperidine ligands, and their enantioselective reactions, supervised by Professor Bong Young Chung. From 1987-1989, he worked at the Korea Institute of Energy and Resources; from 1991 to the present time, he has been based at the Korea Institute of Science and Technology as a senior researcher. His research interests include the synthesis of biologically active the novel drugs utilizing chemical synthesis and natural product.