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K252a inhibits the oncogenic properties of Met, the HGF receptor

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The ATP analog K252a is a potent inhibitor for receptor tyrosine kinases of the Trk family. Here we show that nanomolar concentrations of K252a prevent HGF-mediated scattering in MLP-29 cells (30 nM), reduce Met-driven proliferation in GTL-16 gastric carcinoma cells (100 nM), and cause reversion in NIH3T3 fibroblasts transformed by the oncogenic form of the receptor, Tpr-Met (75 nM). K252a inhibits Met autophosphorylation in cultured cells and in immunoprecipitates and prevents activation of its downstream effectors MAPKinase and Akt. Interestingly, K252a seems to be more effective at inhibiting the mutated form of Met (M1268T) found in papillary carcinoma of the kidney than the wild type receptor. Pretreatment of both Tpr-Met-transformed NIH3T3 fibroblasts and of GTL-16 gastric carcinoma cells with K252a results in loss of their ability to form lung metastases in nude mice upon injection into the caudal vein. These observations suggest that K252a derivatives, which are active *in vivo* as anti-cancer drugs in models of Trk-driven malignancies, should also be effective for treatment of Met-mediated tumors.
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Introduction

Cancer arises from an alteration in the rate of cell proliferation versus cell death. Growth factors and their receptors are largely responsible for keeping this balance in check. Constitutive activation of receptor tyrosine kinases (RTKs), due to ectopic autocrine/paracrine loops, overexpression, or point mutations often occurs in human cancers (Blume-Jensen and Hunter, 2001). This can be one of the events leading to tumor development. RTKs thus represent potential targets for anti-cancer drugs. Different approaches have been described to interfere with the RTK signal, such as receptor-directed antisense oligonucleotides or monoclonal antibodies, and specific RTK inhibitors (Gibbs, 2000a,b).

RTK inhibitors are small molecules that compete for the substrate or the ATP binding pocket (Obeidi and Lam, 2000). They suppress the tyrosine kinase activity of the receptor, thereby preventing the propagation of the signal to downstream effectors. One of the problems in the development of inhibitors is that of specificity. So far, most of the compounds show a broad range of activity. Specificity may ultimately be achieved by chemical modifications based on the high resolution structural data that will become available for each receptor.

Last year, the tyrosine kinase (TK) inhibitor STI-571 (Druker and Lydon, 2000) has gained the attention of the media after the FDA approved it for treatment of Chronic Myeloid Leukemia (CML). The original compound was selected by random screening for inhibitors of protein kinases. Subsequently, it was chemically modified into a series of related molecules. One of these was found to be active at submicromolar concentrations on v-Abl, and on the c-Kit and PDGF receptors. The molecule suppressed proliferation of Bcr-Abl expressing cells *in vitro* and *in vivo* (Druker *et al*., 1996). A formulation of the same which can be given orally was shown to possess significant anti-leukemic activity in patients with CML (Druker *et al*., 2001a,b; Goldman and Melo, 2001). In the case of STI-571 the relatively broad specificity probably contributes to the efficacy of the drug. In fact the c-Kit and PDGF receptors are both expressed by myeloid precursors (Druker and Lydon, 2000). Thus, high selectivity is not always a requirement for therapeutic efficacy.

K252a is a member of a group of natural alkaloids (including Staurosporin), which act as kinase inhibitors by competing with the binding of ATP to the catalytic domain (Ruggeri *et al*., 1999). It was originally described as a serine/threonine kinase inhibitor. Subsequently it was found to act as a potent and selective inhibitor of Trk family members (Tapley *et al*., 1992) and as a partial inhibitor of the PDGF receptor (IC_{50} in the low versus high nanomolar range, respectively) (Nye *et al*., 1992). In this range of concentrations the compound was inactive on the EGF, FGF and IGF-1 receptors (Nye *et al*., 1992; Chin *et al*., 1997; Ruggeri *et al*., 1999).

Trk receptors become oncogenic when activated by rearrangement, or by autocrine and paracrine mechanisms. Constitutive activation of Trk receptors has been implicated in a number of human cancers of neuronal and non-neuronal origin, such as neuroblastomas, medulloblastomas or glioblastomas, as well as prostatic adenocarcinomas, colon cancers and papillary thyroid carcinomas (Ruggeri *et al*., 1999).

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K252a has been reported to inhibit many biological actions of NGF and other neurotrophins. For example it reversibly and selectively inhibited the NGF-induced morphological transformations of proliferating PC12 cells, whereas similar effects of EGF and bFGF were not blocked (Matsuda and Fukuda, 1988; Koizumi *et al*., 1988). Furthermore, treatment with 100 nM K252a resulted in reversion of the phenotype of Trk-transformed rodent fibroblasts (Tapley *et al*., 1992). These results suggested that K252a could be of potential therapeutic value as an anti-cancer drug. The compound itself, however, did not show any anti-tumor activity *in vivo* (Akinaga *et al*., 1992). This prompted an effort to develop synthetic analogs which could still be potent TrkA inhibitors and also show *in vivo* activity (Camoratto *et al*., 1997; Ruggeri *et al*., 1999). One of these, CEP-751 developed by Cephalon, Inc., was shown to significantly inhibit the growth of xenografts of NIH3T3-TrkA transfectants implanted s.c. in athymic mice, while it had no effect on tumors deriving from the SK-OV-3 ovarian carcinoma over-expressing the erb2-HER receptor (Camoratto *et al*., 1997).

In some human prostatic carcinomas, TrkA-positive prostate epithelial cells respond with proliferation and motility to a NGF-like factor produced by the tumor's stromal cells. A similar paracrine loop was also found in several human pancreatic ductal carcinomas (PDAC). CEP-751 and CEP-701 (another K252a derivative which can be given orally) were shown to exhibit pronounced anti-tumor efficacy on xenografts of cells derived from these cancers (Ruggeri *et al*., 1999).

The tyrosine kinase Met is the receptor for hepatocyte growth factor (HGF). HGF induces proliferation, motility and protection from apoptosis in epithelial and non-epithelial cells (Boros and Miller, 1995; Zarnegar and Michalopoulos, 1995). HGF and Met are involved in mediating epithelial-mesenchymal transitions during embryonic development (Rosen *et al*., 1994; Birchmeier and Gherardi, 1998). In cultured epithelial cells Met activation confers the ability to invade extracellular matrices (Jeffers *et al*., 1996). In tumorigenesis deregulated Met activity may contribute to the acquisition of the metastatic phenotype (Jiang *et al*., 1999). c-Met was first cloned as the normal counterpart of the transforming gene Tpr-Met, which arose by rearrangement in a human osteosarcoma cell line treated with a chemical carcinogen (Park *et al*., 1986). In the hybrid Tpr-Met protein, Tpr sequences replace the extracellular domain of the receptor, causing constitutive dimerization of the kinase domain (Rodrigues and Park 1993). This form of activation is rarely found in human cancers (Soman *et al*., 1991; Yu *et al*., 2000). The Met receptor is more frequently activated in carcinomas and sarcomas by overexpression, or by the presence of an ectopic HGF loop (Rong *et al*., 1993; Cortner *et al*., 1995). Met can also be activated by distinct point mutations in hereditary and sporadic papillary carcinoma of the kidney (Schmidt *et al*., 1999) and in childhood hepatocellular carcinoma (Park *et al*., 1999). All mutations are located in the kinase domain of the receptor. Two of them hit highly conserved residues previously found mutated in oncogenic forms of the Ret and Kit receptors, suggesting a common mechanism of activation.

A small molecule active as a Met inhibitor has not yet been described. In a 1997 study K252a was shown to inhibit proliferation of U87 glioma cells (Chin *et al*., 1997). These cells express TrkC, but they show no evidence for a neurotrophin-mediated autocrine loop. It was hypothesized that in this case K252a could act by blocking PDGF receptor-mediated signaling (Chin *et al*., 1997). However, it was later shown that the tumorigenic properties of U87 cells could be abrogated by a ribozyme targeting c-Met expression (Abounader *et al*., 1999). This suggested to us that the effect of K252a on U87 cells could be due to inhibition of the Met receptor.

Here we show that nanomolar concentrations of K252a inhibit the oncogenic properties of Met, including its ability to induce lung metastases in nude mice. K252a inhibits the Met kinase and prevents activation of downstream signaling pathways. Importantly, it seems more effective on the mutated than on the wild type form of the receptor. These results suggest that K252a derivatives may be active as anti-cancer drugs on Met-mediated tumors.

Results

K252a inhibits HGF-induced scatter and interferes with Met signaling in MLP-29 cells

One of the biological effects of HGF/SF is the induction of cell motility. Epithelial cells exposed to HGF dissociate from each other and disperse throughout the culture dish (scatter). This response plays important roles both in physiological and pathological situations. For example, it mediates cell movement during embryogenesis (Birchmeier and Gherardi, 1998) and it contributes to the invasiveness of neoplastic cells (Jiang *et al*., 1999). To assess if a tyrosine kinase inhibitor is active on Met the simplest approach is to test its effect in a HGF-induced scatter assay.

The mouse liver cell line MLP-29, which expresses physiological amounts of Met receptor and grows on plastic forming tight islands of cells, has been previously used to evaluate HGF-mediated scatter (Medico *et al*., 1996). Figure 1a shows the effect of increasing concentrations of K252a on cell scattering. MLP-29 cells were treated overnight with 50 U/ml of HGF, alone or with the indicated concentrations of K252a. A partial block of the scatter response was already apparent at 11 nM. Thirty-three nM K252a completely prevented cell dissociation. There were no signs of drug-induced toxicity up to 100 nM K252a.

To evaluate biochemically the effect of K252a on Met auto-phosphorylation and signaling, MLP-29 cells were kept at low serum for 24 h, pre-treated with increasing concentrations of K252a for 25 min and

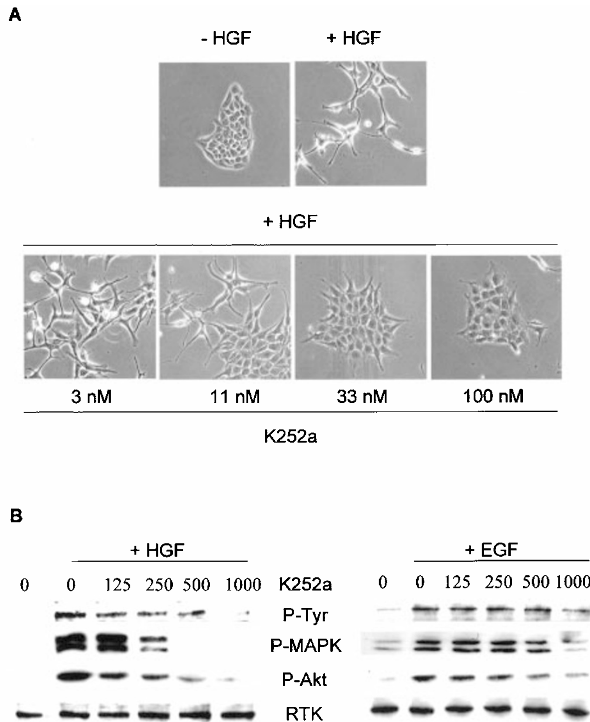

Figure 1 K252a inhibits HGF-induced scatter in MLP-29 cells and interferes with Met signaling. (**a**) Upper panel: MLP-29 cells starved in 0.1% FBS for 24 h were treated with 50 U/ml of HGF for 12 h. Lower panel: the indicated concentrations of K252a were added together with HGF. (**b**) MLP-29 cells were starved in 0.1% FBS for 24 h. Cells were pre-treated with K252a at the indicated nM concentrations for 25 min, and were then stimulated with 200 U/ml of HGF or with 200 ng/ml of EGF for 20 min. Cells were lysed and 20 μg of total protein were loaded on a 8% SDS–PAGE for Western blot analysis. Membranes were incubated with the indicated primary antibodies. RTK: left, anti-Met; right, anti-EGF receptor

finally stimulated with 200 U/ml of HGF for 20 min. As a control for the specificity of the inhibitor we used EGF, a growth factor whose receptor was previously described as insensitive to K252a (Nye *et al*., 1992; Chin *et al*., 1997; Ruggeri *et al*., 1999). After stimulation, cells were lysed and analysed for phosphorylation of the RTKs and of downstream effectors. Figure 1b shows that after such a short pre-treatment, Met phosphorylation is only partially inhibited by K252a lower than 1 μM. However, phosphorylation of downstream effectors, such as MAPKinase and Akt, appears to be more sensitive to the inhibitory effect of K252a. At 250 nM inhibitor MAPKinase phosphorylation is already drastically reduced. In the case of stimulation with EGF, submicromolar concentrations of the inhibitor are ineffective in reducing phosphorylation of both receptor and downstream effectors.

K252a induces morphological changes, reduces BrdU incorporation and inhibits constitutive Met phosphorylation in GTL-16 cells

GTL-16 cells are a highly tumorigenic cell line clonally derived from MKN-45 cells (Giordano *et al*., 1989). The latter were originally established from a poorly differentiated gastric adenocarcinoma (Motoyama *et al*., 1986). In both cell lines c-*MET* is amplified and the receptor is overexpressed (Rege-Cambrin *et al*., 1992). Met overexpression causes its constitutive activation (Ponzetto *et al*., 1991). It has been shown that proliferation of MKN-45 cells can be inhibited by an antisense against *MET*, suggesting that its deregulated activity sustains the transformed phenotype (Kaji *et al*., 1996).

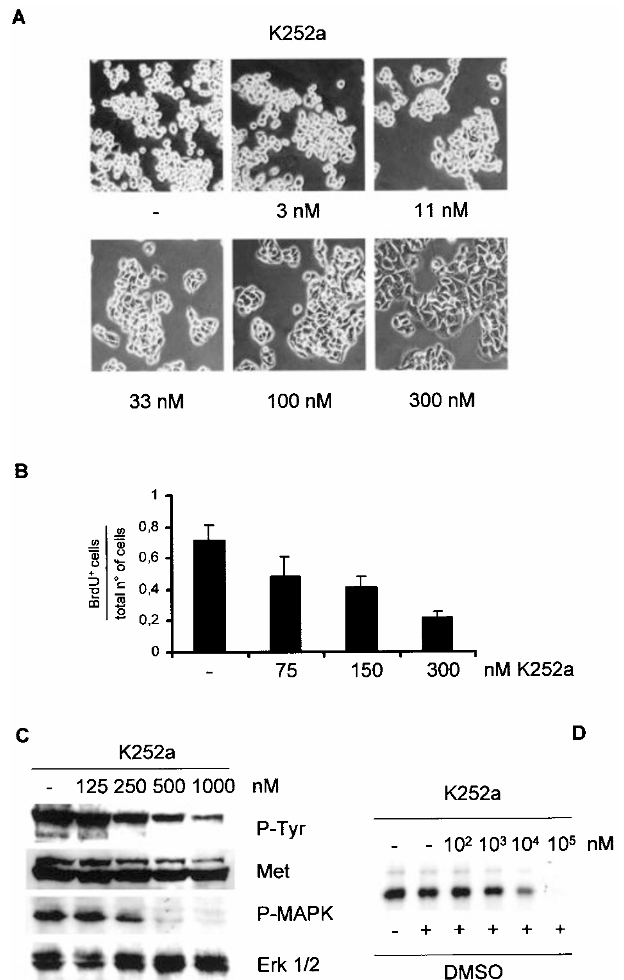

Figure 2 K252a induces morphological changes, reduces BrdU incorporation and inhibits constitutive Met phosphorylation in GTL-16 cells. (**a**) GTL-16 cells were treated for 20 h with the indicated concentrations of K252a in 5% FBS. (**b**) GTL-16 cells were kept for 5 h in 0.1% FBS. Cells were then incubated for 17 h with BrdU and the indicated concentrations of K252a and were finally fixed and stained with anti BrdU antibody. Bars represent the mean ($\pm$s.d.) of the ratios between the number of BrdU-positive cells and the total number of cells. Proliferation was evaluated in three separate experiments. (**c**) GTL-16 cells, treated as described in **a**, were lysed and 20 μg of total protein were loaded on 8% SDS–PAGE for Western blot analysis. Membranes were incubated with the indicated primary antibodies. (**d**) Lysates of GTL-16 cells (a semiconfluent 10 cm dish/lane) were immunoprecipitated with anti-Met antibodies for 3 h. Immunoprecipitates were pre-incubated with the indicated amounts of the drug (in a final concentration of 1% DMSO) for 10 min at 37°C in kinase buffer before starting the kinase reaction by addition of [γ^{32}P]ATP

Figure 2 shows the effects of K252a treatment on GTL-16 cells at the biological and biochemical level. GTL-16 cells, which in culture exhibit a highly birifrangent transformed morphology, were treated for 20 h with the indicated concentrations of inhibitor. At 100 nM K252a the cells began spreading on the dish and resumed cell-to-cell contacts. At 300 nM K252a, cells reverted to a normal epithelial phenotype without showing any sign of increased cell death. The effects of K252a on GTL-16 proliferation was evaluated by measuring BrdU incorporation after a 16 h treatment. Figure 2b shows that K252a in the same range of concentrations as in Figure 1a reduced the proliferative index of GTL-16 cells in a dose-dependent manner.

Figure 2c shows the effect of K252a on constitutive phosphorylation of the Met receptor. Protein extracts of the cells shown in Figure 2a were separated on an SDS gel and blotted with antiphosphotyrosine antibodies. Met phosphorylation was reduced by the drug in a dose-dependent manner. Again, as seen in Figure 1a and b, the effects of K252a on cell phenotype, proliferation and on downstream effectors were detectable at concentrations lower than those necessary to completely abrogate Met phosphorylation.

To verify whether K252a indeed directly inhibited Met activity, the effect of the drug was examined on anti-Met immunoprecipitates in an *in vitro* kinase assay. Under the non-physiological conditions of the assay, 1 μM K252a caused an 18% inhibition of the auto-kinase activity of Met. Fifty-five per cent inhibition was obtained at 10 μM and complete inhibition at 100 μM (Figure 2d). The lower inhibitory effect shown by K252a *in vitro versus in vivo*, is probably due to different receptor/drug ratios in the two experimental conditions.

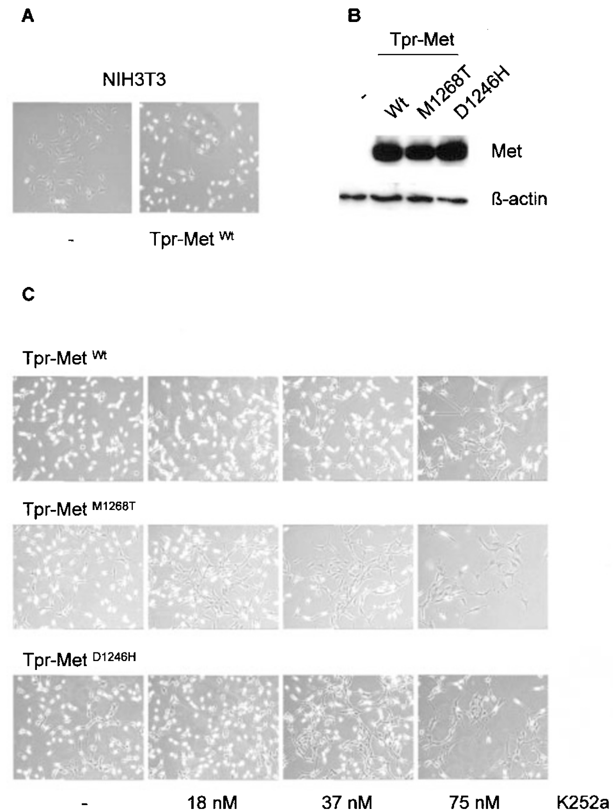

Figure 3 K252a reverts the phenotype of NIH3T3 cells transformed by Tpr-Met. (**a**) Tpr-Met-mediated transformation causes a change of cell shape from flat-polygonal to birifrangent, spindle-like. (**b**) Stable clones of transformed NIH3T3 cells were lysed and analysed by Western blotting (20 μg of total protein/lane) to compare the level of expression of wild type and mutated Tpr-Met protein. (**c**) The clones shown in **b** were seeded at a density of 1.5×10^5 cells/plate (on a six-well plate). After 5 h, the medium was supplemented with the indicated concentrations of K252a and changed at 24 h. Pictures were taken after 48 h of incubation with the drug

K252a reverts the phenotype of NIH3T3 cells transformed by Tpr-Met

Tpr-Met is an oncogenic form of Met in which the kinase domain is constitutively dimerized because of two leucine zippers present in the Tpr moiety (Rodrigues and Park, 1993). This rearrangement has recently been found in some patients with gastric cancer (Soman *et al.*, 1991; Yu *et al.*, 2000). Tpr-Met is highly transforming in NIH3T3 fibroblasts. Upon Tpr-Met-mediated transformation, the cell phenotype changes from polygonal and flat to spindle-shaped and birifrangent (Figure 3a). NIH3T3 fibroblasts transformed by Tpr-Met form tumors in nude mice when injected subcutaneously and lung metastases when injected into the caudal vein (Giordano *et al.*, 1997).

In previous work we studied the mechanism of activation of two mutations (M1268T and D1246H) found in Met in papillary carcinoma of the kidney (Maritano *et al.*, 2000). M1268 and D1246 are highly conserved residues, which were originally found mutated in oncogenic forms of the Ret and Kit receptors (van Heyningen, 1994; Furitsu *et al.*, 1993; Tsujimura *et al.*, 1994; Kitayama *et al.*, 1995). These mutations when inserted in the context of the full size Met receptor increase the activity of the Met kinase, which however is still dependent on ligand stimulation. We previously showed that their activating effect can also be demonstrated within Tpr-Met, the constitutively active form of the Met kinase. Tpr-Met^{M1268T} and Tpr-Met^{D1246H} transform fibroblasts at higher efficiency than Tpr-Met^{Wt} (Maritano *et al.*, 2000).

We used NIH3T3 cells transformed by Tpr-Met^{Wt} and by the Tpr-Met^{M1268T} and Tpr-Met^{D1246H} mutants to test whether K252a had a differential effect on the wild type *versus* the mutated form of the Met kinase (Figure 3b). Transformed NIH3T3 cells were treated for 48 h with the indicated concentrations of K252a (Figure 3c). The growth of all Tpr-Met transformed fibroblasts was clearly inhibited by treatment with 75 nM K252a, as shown by the lower number of cells in the fourth panel of each series. Cells stopped dividing, but did not die. No signs of toxicity were detectable using up to 300 nM K252a (not shown). Figure 3c shows that 75 nM K252a induced reversion of most Tpr-Met^{Wt} transformed cells to the 'untransformed' flat phenotype. Interestingly, cells transformed

by the Tpr-Met^{M1268T} began to revert at 18 nM K252a, showing higher sensitivity to the drug. Cells transformed by Tpr-Met^{D1246H} showed intermediate sensitivity. As a control for these experiments we used v-Src-transformed NIH3T3 fibroblasts. The phenotype of these cells did not revert even at 300 nM K252a (not shown).

K252a is a stronger inhibitor for mutated than wild type Met signaling

The experiments described in Figure 3c suggested that K252a may be a more potent inhibitor for the mutated M1268T receptor than for wild type Met. On the other hand, the biochemical experiments shown in Figures 1 and 2 indicated that the reduction in phosphorylation of downstream effectors is a more sensitive measure of the inhibitory activity of K252a than phosphorylation of the receptor itself. We thus verified in a stimulatable system the effect of the K252a inhibitor on ligand-induced MAPKinase and Akt activation downstream of wild type and mutated Met. To this end we generated MLP-29 cells expressing Trk-Met chimeras, with the extracellular domain of Trk and the intracellular domain of Met bearing the oncogenic point mutations. We selected MLP-29 clones expressing the same amount of chimeric receptors (Figure 4a). Wild type MLP-29 cells do not express endogenous Trk and are insensitive to NGF (not shown). The clones shown in Figure 4a were treated as those in Figure 1b, except for the fact that NGF was used for stimulation rather than HGF. Figure 4b shows that in cells expressing the Trk-Met chimera with the M1268T mutation, K252a inhibits the MAPKinase response at concentrations lower than those required for cells expressing the wild type form of Met (500 *versus* 1000 nM). The sensitivity of the D1246H Met mutant to the drug appears to be similar to that of wild type Met. Inhibition of Akt phosphorylation downstream of the wild type and mutant receptors showed a similar pattern, although the difference between wild type Met and the M1268T mutant was less pronounced.

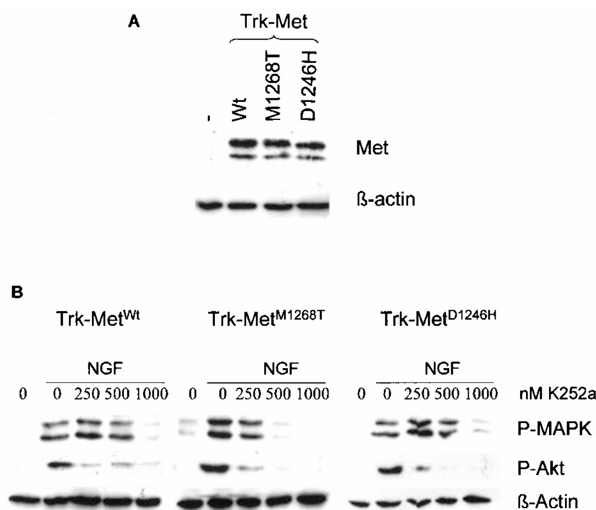

Figure 4 K252a is a stronger inhibitor of signaling triggered by mutated than by wild type Met. (**a**) Stable MLP-29 clones expressing wild type and mutated Trk-Met chimeras were lysed and analysed by Western blotting to compare the level of expression of wild type and mutated Trk-Met proteins. (**b**) The MLP-29 clones shown in **a** were starved for 24 h in 0.1% FBS, pre-treated for 25 min with the indicated concentrations of K252a, and finally stimulated with 150 ng/ml of NGF for additional 20 min. Twenty μg of total protein were loaded on 8% SDS–PAGE for Western blot analysis with the indicated antibodies

Pre-treatment with K252a interferes with the ability of Tpr-Met transformed fibroblasts and of GTL-16 cells to form lung metastases in nude mice

Having shown that K252a reverts the phenotype of both GTL-16 and Tpr-Met transformed fibroblasts, we wanted to test whether these morphological changes were associated to loss of invasiveness. To this end we performed an experimental metastasis assay, using GTL-16 cells and Tpr-Met or v-Src-transformed NIH3T3 fibroblasts. In this assay, following injection into the caudal vein of nude mice, cells are trapped in the lungs. Here, according to their invasive ability, they either cross the capillary wall and home into the lung parenchyma where they form metastases that ultimately kill the animal, or fail to cross the capillaries and are rapidly cleared away by the macrophages.

Injections were performed with an equal number of cells (1×10^6), either untreated or pretreated with 200 nM K252a for 24 h. Animals showing obvious signs of dispnea were all sacrificed by day 40. Animals with no signs of dispnea were sacrificed at day 60. Table 1 shows that all the mice injected with untreated Tpr-Met and v-Src-transformed cells developed lung metastases. Five out of six of those injected with untreated GTL-16 cells developed lung metastases as well. Mice injected with K252a-treated v-Src-transformed cells did develop metastases. All other animals injected with treated cells did not show any sign of distress, were sacrificed at the end of the experiment and were metastasis-free at autopsy. These results indicate that, after K252a pre-treatment, Tpr-Met or Met-transformed cells failed to invade the lungs. Importantly the effect of K252a was specific for Met-transformed cells, since v-Src-transformed fibroblasts caused lung metastases whether the cells were pre-treated or not (see Table 1).

Table 1 Inhibition of Met-mediated metastasis by K252a pre-treatment

Cell type	*K252a*	*Metastasis*[a]
NIH3T3 Tpr-Met	−	6/6
	+	0/6
NIH3T3 v-Src	−	6/6
	+	6/6
GTL-16	−	5/6
	+	0/6

[a]Nude mice were injected into the tail vein with 10^6 either untreated (−) or pre-treated with 200 nM for 24 h (+)

Discussion

In this work we showed that K252a, a potent Trk inhibitor whose derivatives are active as anti-tumor drugs in pre-clinical models of human prostatic and pancreatic ductal carcinomas (Ruggeri *et al*., 1999), is also a strong inhibitor of the oncogenic properties of Met.

We evaluated the effects of K252a on HGF/Met signaling in physiological and pathological models. As an example of the former we used MLP-29 cells, a murine liver cell line with normal levels of Met receptor, which responds to HGF with motility (scatter) (Medico *et al*., 1996). As a model for the oncogenic properties of Met, we used GTL-16 cells, a cell line derived from a gastric carcinoma, in which Met is amplified, over-expressed, and constitutively activated (Giordano *et al*., 1989; Ponzetto *et al*., 1991). We also used NIH3T3 fibroblasts transformed by the oncogenic form of the receptor, Tpr-Met, and by two Tpr-Met variants carrying in the Met kinase point mutations found in papillary carcinoma of the kidney (Maritano *et al*., 2000). As a stimulatable system to evaluate the effect of the drug on effectors downstream of Met and Met point-mutants, we used MLP-29 cells stably expressing wild type and mutated Trk-Met chimeras.

In general, Met-mediated biological effects were inhibited by K252a concentrations lower than 100 nM. K252a inhibited HGF-induced scatter of MLP-29 cells at 30 nM. It reverted the phenotype of GTL-16 and Tpr-Met transformed cells (and significantly inhibited their growth) at concentrations below 100 nM. Reversion occurred also using Staurosporin at the same concentrations, but this drug was highly toxic to cells (not shown), while K252a did not compromise cell viability. In both GTL-16 and Tpr-Met-transformed cells phenotypic reversion was associated with loss of invasiveness in an experimental metastasis assay. In fact K252a-treated cells failed to colonize the lungs of nude mice following injection in the tail vein. Presumably the effect of K252a pre-treatment on malignant cells (preventing them from crossing the capillary wall) lasted for a time sufficient for the macrophages to clear them away. Unfortunately, given the inactivity of K252a *in vivo* (Akinaga *et al*., 1992) this was the only type of experiment which could be done with this reagent. If K252a analogs such as CEP-751 and/or CEP-701 (which are capable of inhibiting growth of Trk-positive tumors) retain the ability to inhibit Met, these compounds would be the most appropriate to be tested *in vivo* as drugs against Met-driven tumors.

To evaluate the effects of K252a on phosphorylation of the receptor and of downstream effectors, which occur within minutes from stimulation, we used shorter K252a pre-treatments and higher concentrations of the drug. In these conditions, the reduction in phosphorylation tended to be more drastic for the downstream effectors than for the receptor itself. This suggests that the drug could also directly inhibit molecules downstream of the receptor. However in MLP-29 cells the same concentration of K252a did not affect phosphorylation of the signaling molecules following an EGF stimulus. Results similar to ours were obtained by others using K252a to inhibit PDGF receptor signaling (Nye *et al*., 1992). Also in this case receptor phosphorylation was only partially blocked in conditions in which PDGF-dependent ERK2 phosphorylation was completely inhibited. The number of receptors remaining active in the presence of the inhibitor may be below the threshold necessary to elicit the downstream response. Alternatively, residual phosphorylation may be confined to tyrosine residues (such as those in the activation loop) which are major phosphorylation sites (Ferracini *et al*., 1991), but are not directly involved in signaling. Phosphorylation of these tyrosines might turn over at a slower pace compared to those which are responsible for effector binding (Ponzetto *et al*., 1994).

Interestingly, K252a seems to be a more potent inhibitor for Met with the M1268T mutation than for wild type Met. K252a reverted the phenotype of NIH3T3 cells expressing Tpr-Met^{M1268T} at a concentration fivefold lower than that necessary for reversion of NIH3T3 cells transformed by Tpr-Met^{Wt}. Similarly, in cells expressing the Trk-Met^{M1268T} chimera, K252a inhibited HGF-induced MAPKinase activation at a concentration lower than that required in cells expressing Trk-Met^{Wt}.

The M1268T substitution is considered the 'strongest' in terms of its activating potential among the numerous tumorigenic mutations identified in the Met kinase domain (Jeffers *et al*., 1998). In a previous study we hypothesized that this mutation may result in stabilization of the catalytic domain in the 'open' configuration. The fact that K252a inhibits signaling of this mutant more efficiently than wild type Met strengthens the idea that the catalytic pocket of the mutated enzyme may be more accessible to ATP and its analogs. Thus, in the case of tumors with this genetic lesion, it may be possible to find a dose of a K252a derivative sufficient for anti-tumor activity but devoid of side effects potentially arising from inhibition of the wild type receptor.

K252a, an inhibitor of the Trks, the PDGF receptor, and Met, appears to be an ATP analog endowed with partial selectivity. As shown for STI-571, which inhibits both Bcr-Abl and c-Kit in myeloid precursors, partial selectivity may not be detrimental in terms of therapeutic efficacy.

In conclusion, in this work we have shown that K252a represents a good tool to inhibit the activity of the Met receptor and the biological effects of HGF on cultured cells. Importantly, in interpreting results of experiments in which K252a is given to cells to inhibit Met, the issue of partial selectivity should be kept in mind as a *caveat*. On the other hand, this may turn out to be a strong point in terms of the possible applications of K252a derivatives as anti-tumor drugs. First, like STI-571, which besides being used for CML is also used to treat patients with c-Kit-positive gastrointestinal stromal tumors (Joensuu *et al*., 2001),

such drugs may be appropriate to treat a wide spectrum of tumor types. Furthermore, since both Trk and Met are expressed in epithelial cells it is not unconceivable that the two receptors might be co-expressed in the same carcinoma. Likewise, given that Met can be ectopically expressed in sarcomas (Rong *et al*., 1993) it is not unlikely that, as in the case of the U87 glioma mentioned in the Introduction, Met could be co-expressed with PDGF receptor. In this case, hitting more than one target kinase in the same neoplastic cell may ultimately be advantageous, both in terms of eliminating possible alternative pathways which could drive malignancy, and of reducing the chances of developing resistance to the drug (Gorre *et al*., 2001).

Materials and methods

Reagents, cells and antibodies

All reagents, unless specified, were from Sigma Chemical Co. K252a was from Calbiochem-Novabiochem Intl., (CA, USA).

Cells MLP-29 (Medico *et al*., 1996), GTL-16 (Giordano *et al*., 1989), and Tpr-Met transformed NIH3T3 cells (Maritano *et al*., 2000) were cultured in DMEM medium supplemented with 5% Fetal Bovine Serum, in a 5% CO_2-water saturated atmosphere.

Polyclonal antibodies Anti mouse Met (SP260; #sc-162), anti human Met (C-12; #sc-10) anti EGF-receptor (1005; #sc-03) were purchased from Santa Cruz Biotechnology, Inc. Polyclonal anti Phospho-Akt (Ser 473) were from Cell Signaling Technology (New England Biolabs). Monoclonal anti Phospho-MAPK (M-8159) and anti β-Actin (A-5316) were from SIGMA and monoclonal anti PTyr (PY-99; #sc-7020) was from Santa Cruz Biotechnology. Anti BrdU monoclonal antibody (# 1170376) was from Boehringer Mannheim.

Cloning of cDNA constructs, transfection and establishment of MLP-29 cells expressing Trk-Met chimeras Point mutations were inserted by sub-cloning in previously described Trk-Met constructs (Ponzetto *et al*., 1996). Tpr-Met constructs: wild type and mutated Tpr-Met plasmids were previously described (Maritano *et al*., 2000).

To establish MLP-29 cells expressing chimeric Trk-Met constructs, 5×10^6 cells were electroporated at 290 V and 750 μF with 20 μg of Trk-Met plasmid and 1 μg of pSV2Neo. Two days after electroporation, MLP-29 cells were cultured in G418-containing medium till resistant clones could be picked. Clones were screened for the expressions of chimeric proteins by Western blotting with anti-Met antibodies.

Western blotting

Cells were lysed with a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM β-glycerolphosphate and 1 mM orthovanadate and Protease Inhibitor Cocktail (Sigma, # P8340). Protein concentration was determined using the Bio-Rad Dc Protein Assay. Twenty μg of total protein were loaded on 8% SDS–PAGE and transferred to Hybond-ECL membranes (Amersham). Filters were probed with the appropriate antibodies and specific binding was detected by the Enhanced Chemiluminescence System (ECL, Amersham). The experiments shown are representative of results obtained in at least three separate experiments.

Immunoprecipitation and in vitro *kinase assay*

GTL-16 cells were lysed with EB buffer (10 mM Tris pH 7.4, 150 mM EDTA, 10% glycerol, 1% Triton X-100) supplemented with 1 mM PMSF and Protease Inhibitor Cocktail (Sigma, # P8340). For immunoprecipitation, lysates normalized for protein content were incubated with 2 μg/ml of anti-human Met and 30 μg of packed ProteinA-Sepharose beads for 3 h at 4°C. Beads were collected by centrifugation, washed three times with EB buffer, once with kinase buffer (KB, 50 mM HEPES pH 7.5, 150 mM NaCl, 12.5 mM $MgCl_2$) and incubated in 50 μl of KB in the presence of 10 μCi of [γ^{32}P]ATP. The reaction was carried out at 37°C for 20 min and stopped by adding 1 ml of cold EB buffer. After two washes with EB buffer, concentrated Laemmli buffer was added to the beads and samples were heated for 10 min at 95°C. The eluted proteins were subjected to 8% SDS–PAGE. Radioactive gels were dried and the signal was detected by autoradiography. The autoradiography shown is representative of results obtained in at least three separate experiments.

Biological assays

Scatter assay MLP-29 cells were seeded at low density in a six-well plates and cultured for 1–2 days until they formed tightly packed colonies. After 24 h in medium with 0.1% FBS, cells were supplemented with the indicated concentrations of K252a and stimulated overnight with 50 U/ml HGF.

K252a-mediated reversion of the transformed phenotype GTL-16 cells or Tpr-Met-transformed NIH3T3 cells were plated at a density of 1×10^5 cells/plate in a six-well plates in 5% FBS. After 5 h, the medium was supplemented with the indicated concentrations of K252a. The medium was changed every 24 h. Pictures were taken after 20 (GTL-16 cells) or 48 h (NIH3T3 cells).

Inhibition of GTL-16 proliferation GTL-16 cells were seeded at low density in twelve-well plates in 5% FBS for 24 h. Cells were starved for 5 h in 0.1% FBS and incubated with the indicated concentrations of K252a and BrdU for 16 h. Cells were then washed in PBS, incubated with 2 M HCl for 10 min at room temperature, fixed with Met-OH at −20°C for 10 min, washed in PBS and stained with anti-BrdU. BrdU-positive cells were counted using a fluorescence microscope.

Experimental metastasis assay

Experimental metastasis assays were carried out in nude mice, using Tpr-Met or v-Src transformed NIH3T3 cells and GTL-16 gastric carcinoma cells. Cells (either untreated or pre-treated with 200 nM K252a for 24 h) were washed with PBS, harvested by trypsinization, resuspended in sterile PBS and counted. Cell viability was verified by Trypan blue exclusion. 1×10^6 cells were injected into the caudal vein of nude mice (six animals for each group). Animals with obvious signs of dispnea were sacrificed and autopsied. The experiment was terminated at day 60 by sacrificing the animals which had not developed signs of respiratory

discomfort. The presence of lung metastases was evaluated at autopsy and confirmed by histopathological examination of the lungs.

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