

# Chemical Genetic Blockade of Transformation Reveals Dependence on Aberrant Oncogenic Signaling

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## Summary

**Background:** Our understanding of protein kinase inhibition in the treatment of cancer is clearly limited by the lack of inhibitors that selectively block a single kinase implicated in neoplastic transformation. One approach to developing specific inhibitors is to engineer in protein kinases silent mutations that allow selective inhibition while retaining kinase activity. Because it is implicated in a large number of malignancies, EGFR provides an attractive target for such selective kinase inhibition.

**Results:** We generated an inhibitor-sensitized allele of the transforming receptor tyrosine kinase *v-erbB*. Transformation of immortalized rodent fibroblasts by sensitized versions of *v-erbB* (*v-erbB-as1*) was blocked by 1-naphthyl PP1 (NaPP1), a cell-permeable ATP-competitive inhibitor. NaPP1 also reversed morphological transformation by *v-erbB-as1*. Signaling through MAP kinase and PI(3) kinase was initially blocked by inhibitor treatment and then recovered to levels comparable to those in nontransformed cells. Surprisingly, NaPP1-treated *v-erbB-as1* cells failed to re-enter the cell cycle, showed decreased levels of D- and A-type cyclins, and showed increased levels of p27. To extend this result, we showed that NaPP1 treatment of *v-Src-as1* cells also led to cell cycle arrest. Arrested cells could be rescued with a conditional allele of *Raf* or by transduction of a constitutive allele of cyclin D1.

**Conclusions:** These data suggest that mammalian cells can become dependent on aberrant oncogenic signaling; this dependency renders them incapable of returning to a normal, proliferative phenotype.

## Introduction

The epidermal growth factor receptor (EGFR) is a prototype receptor tyrosine kinase implicated in tumors of the brain, lung, breast, head and neck, bladder, skin, and ovary [1]. Three quinazoline inhibitors of EGFR have been tested in phase 1 and 2 clinical trials. ZD1839 Iressa has a dose-limiting acne-like rash as an associated toxicity [2]. OSI-774 had dose-limiting toxicities including folliculitis, diarrhea, and mucositis [3]. These dose-limiting toxicities are presumably due to a lack of

specificity or to the unexpected role of EGFR in these processes.

EGFR is a 170 kDa protein defining the *erbB* family of transmembrane receptor tyrosine kinases, and it plays a central role in proliferation, differentiation, migration, and oncogenesis (reviewed in [4]). The identification of EGFR as the cellular homolog of the transforming oncogene *v-erbB* was one of the first links between an activated oncogene and the development of cancer [5]. *V-erbB* differs from EGFR principally through extensive deletion of the extracellular domain [6]. This mutation confers constitutive kinase activity of the receptor [7–9] and is the only mutation in *v-erbB* that is required for transformation of mammalian cells [10]. In humans, extensive deletions of the extracellular domain of EGFR have been identified in astrocytic neoplasms [11–14]. The most common of these alterations, EGFRvIII, is similar to *v-erbB* in that it shows constitutive kinase activity independent of ligand binding [13, 15, 16].

We have developed a model system for studying the cellular response to inhibition of *v-erbB* in a situation in which the kinase inhibitor is specific for the oncogene. We show that specific inhibition of *v-erbB* leads to normalization of cellular morphology and of basal cellular signaling through MAP kinase and PI(3) kinase pathways. Despite apparently reversing many features of transformation, treated cells showed sustained G<sub>0</sub> arrest and failed to re-enter the cell cycle. These data suggest that transformed cells become dependent on oncogenic signaling and are subsequently incapable of recovering normal proliferative functions.

## Results

### An Inhibitor Analog-Sensitive Allele of *v-erbB* Is Highly Transforming

Tyrosine kinases share a consensus ATP binding site that can be specifically mutated while kinase activity is retained. This creates a unique pocket that allows competitive inhibition of ATP binding with compounds such as NaPP1 [17]. To engineer *v-erbB-as1*, we mutagenized T210 to alanine. We subsequently transformed NIH3T3 cells with *v-erbB* or *v-erbB-as1* and analyzed the level and pattern of tyrosine-phosphorylated proteins in comparison to the levels of *v-erbB*. Figure 1A shows that the level and profile of tyrosine-phosphorylated proteins was identical in cells transduced with *v-erbB* and *v-erbB-as1*, suggesting that these alleles show similar kinase activities as well as similar substrate specificities. The cloning efficiency in soft agar was indistinguishable when either *v-erbB* or *v-erbB-as1* was used to transform immortalized rodent fibroblasts (Figure 1B). These observations argue that transformation by *v-erbB-as1* is comparable to that of the unmanipulated *v-erbB* allele.

### NaPP1-Treatment Can Block and Reverse Transformation by *v-erbB-as1*

Figure 1B shows that treatment of cells with 2  $\mu$ M NaPP1 completely blocked the proliferation of *v-erbB-as1*-

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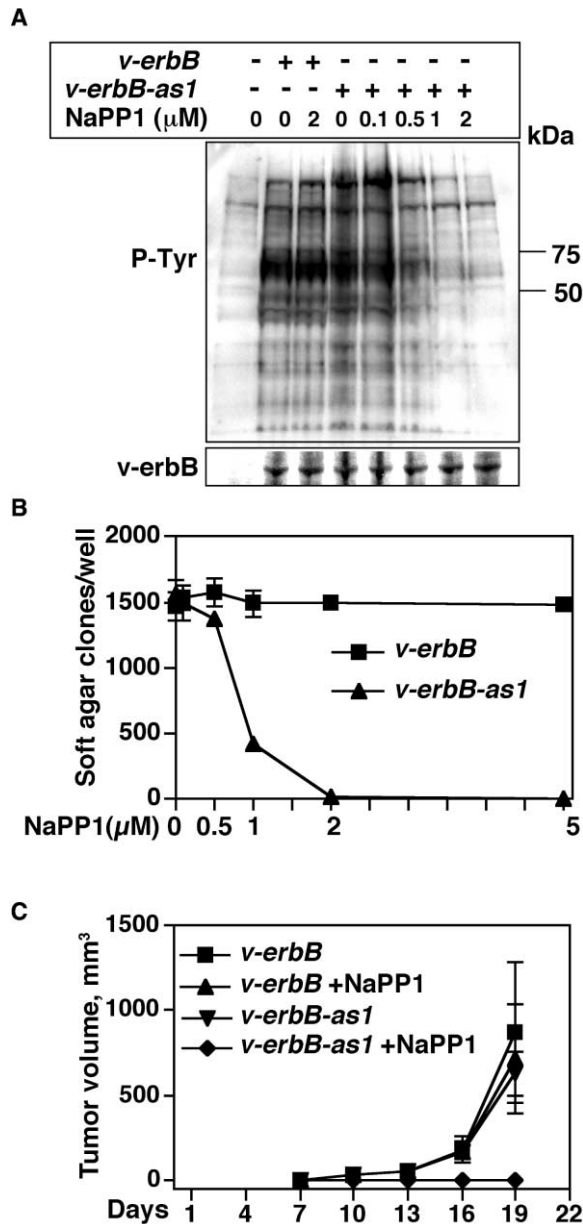


Figure 1. Characterization of *v-erbB-as1*

(A) Reversal of tyrosine phosphorylation by treatment of NIH3T3 cells with NaPP1. Vector transduced NIH3T3 cells (lane 1), *v-erbB*-transformed cells (lanes 2 and 3), and *v-erbB-as1*-transformed cells (lanes 4–8) were incubated with 1.2% DMSO (lanes 1, 2, and 4) or with NaPP1 in 1.2% DMSO (lanes 3 and 5–8) at the indicated concentrations. Cells were lysed after 24 hr, and proteins were immunoblotted with anti-phosphotyrosine antibody 4G10.

(B and C) NaPP1 treatment blocks proliferation and transformation by *v-erbB-as1* and has no effect on *v-erbB*. (B) Soft-agar colonies grown for 2 weeks in varying concentrations of NaPP1 (0–5  $\mu$ M) were stained and counted. Results represent three independent plates per experimental point. (C) Cells transduced with *v-erbB* or *v-erbB-as1* ( $1 \times 10^6$ ) were injected subcutaneously in BALB/c nu/nu mice on day 0. Animals were treated with a daily intraperitoneal injection of either 20 mg/kg NaPP1 or vehicle starting on day 1 post-implant. Each data point represents mean tumor volume  $\pm$  S.E. obtained from six mice.

transduced cells in soft agar. The  $IC_{50}$  for NaPP1-mediated growth inhibition in this assay was between 0.5 and 1  $\mu$ M and was consistent with the range of concentration required to inhibit basal *v-erbB-as1* phosphorylation (Figure 1A).

Mice injected subcutaneously with *v-erbB* or *v-erbB-as1* cells formed tumors of similar size at similar latencies (Figure 1C). NaPP1 completely blocked tumor formation in 5 of 6 mice that were injected subcutaneously with *v-erbB-as1* cells and subjected to daily intraperitoneal injections of NaPP1 for 19 days. One of 6 mice in this cohort showed only partial blockage of tumor growth and developed a tumor of approximately 20% the size of untreated controls. Tumors from mice injected with *v-erbB* cells were unaffected by treatment with NaPP1 (Figure 1C). These experiments demonstrated that *v-erbB-as1* kinase activity induced tumor formation and that tumor growth could be blocked by the selective inhibitor NaPP1.

We next asked whether NaPP1 could reverse pre-established transformation mediated by *v-erbB-as1*. Figure 1A shows that NaPP1 reversed tyrosine phosphorylation of *v-erbB-as1*-transformed cells in a dose-dependent manner. At 2  $\mu$ M NaPP1, cells transduced with *v-erbB-as1* showed full reduction of overall tyrosine phosphorylation to the level seen in cells transduced with empty vector (Figure 1A). There was no detectable decrease in the phosphorylation of cells transduced with *v-erbB* in response to treatment with 2  $\mu$ M NaPP1.

#### NaPP1 Transiently Blocks Signaling Downstream from *v-erbB* and Reverses Morphologic and Cytoskeletal Abnormalities

Signaling by *v-erbB* is mediated at least in part through the activation of MAP kinase/extracellular signal-regulated kinase 1,2 (ERK 1,2) and PI3/Akt kinase pathways (reviewed in [18]). We therefore examined the status of these pathways in cells transduced with *v-erbB* and *v-erbB-as1*. Figure 2 demonstrates that phospho-ERK was undetectable after 3 hr of treatment with NaPP1 and that the levels and cytosolic localization of phospho-ERK return by 24 hr in parallel with normalization of actin stress fibers. Western blotting with antibodies against active (phosphorylated) and total ERK and Akt (Figure 2E) demonstrated that NaPP1 treatment of *v-erbB-as1* cells led to transient decreases in the levels of phospho-ERK and phospho-Akt, which recovered by 24 hr to levels comparable to those in nontransformed cells (Figure 2J). Levels of total ERK and total Akt were unchanged.

#### Inhibition of MAP Kinase but Not Akt Leads to Stress Fiber Loss in NIH3T3 Fibroblasts Expressing *v-erbB-as1*

To further characterize the morphological changes induced by transformation with *v-erbB* or *v-erbB-as1*, we analyzed the actin cytoskeleton by immunofluorescence microscopy. Actin stress fibers were clearly reduced in *v-erbB-as1*-transformed NIH3T3 as compared with nontransformed controls (Figures 2A and 2D). In response to 48 hr NaPP1 treatment, cells transformed with *v-erbB-as1* reverted to a pattern comparable to that of

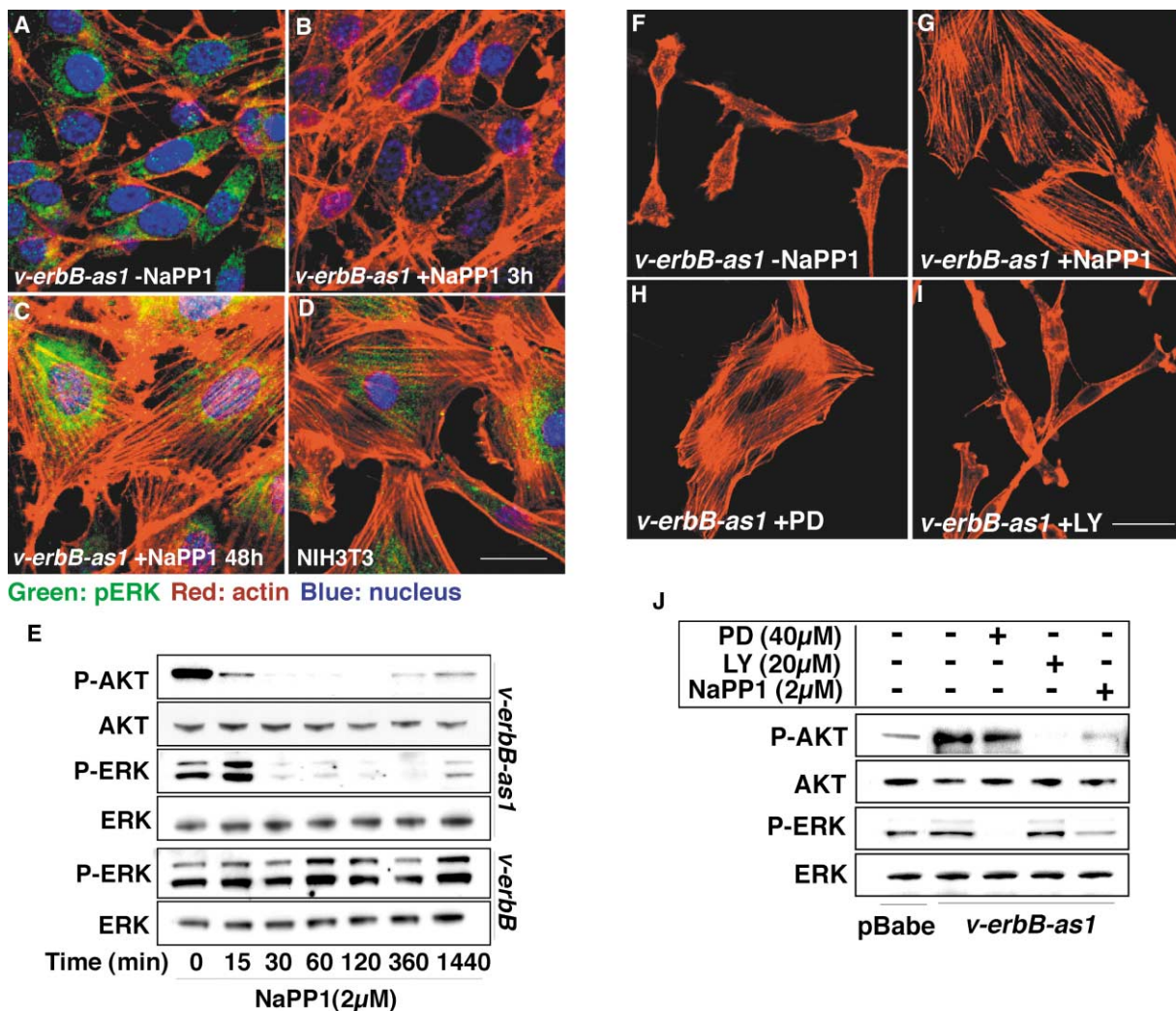


Figure 2. Selective Inhibition of *v-erbB-as1* Correlates with Morphologic Reversal of Transformation

(A–D) NIH3T3 cells and *v-erbB-as1* cells were treated with 2 μM NaPP1 for the indicated time periods and incubated with anti-pERK antibody (green). F-actin was visualized with rhodamine-phalloidin (red), and nuclei were labeled with DAPI (blue). Phospho-ERK was undetectable after 3 hr of treatment with NaPP1 and returned by 48 hr in parallel with normalization of actin stress fibers.

(E) Cells transduced with *v-erbB* or *v-erbB-as1* were treated with NaPP1 (2 μM), harvested at the indicated times, and blotted for total and phosphorylated ERK and Akt.

(F–I) *v-erbB-as1* cells were treated with 1.2% DMSO (F), 2 μM NaPP1 (G), 40 μM PD 098059 (H), or 20 μM LY 294002 (I). Cells were stained with rhodamine-phalloidin at 48 hr and visualized by confocal laser scanning microscopy.

(J) Vector and *v-erbB-as1*-transduced cells were incubated with vehicle, 40 μM PD098059, 2 μM NaPP1, or 20 μM LY294002. Cells were harvested at 6 hr post-treatment, lysed, and immunoblotted for total ERK (tERK), phosphorylated ERK (pERK), and phosphorylated Akt (pAKT).

nontransformed NIH3T3 cells (Figures 2C and 2D). In contrast, cells transformed with *v-erbB* retained a transformed morphology after treatment with NaPP1 (data not shown).

To explore the role of these signaling pathways in morphological transformation, *v-erbB-as1* cells were treated with the monospecific *v-erbB-as1* inhibitor NaPP1, with LY294002 (a relatively selective inhibitor of PI(3) kinase [19, 20]), or with PD098509, a relatively selective inhibitor of MEK1 [21]. PD098509 restored levels of actin stress fibers in a manner comparable to NaPP1 (Figures 2G and 2H). In contrast, LY294002, at a dose sufficient for inhibiting Akt activity (Figure 2J), showed no effect on actin stress fibers (Figure 2I). These

observations suggest that the rounding up of cells during transformation by *v-erbB-as1* is more strongly influenced by MAP kinase than by PI(3) kinase.

#### NaPP1 Treatment of *v-erbB-as1* and *v-Src-as1* Cells Leads to G<sub>0</sub>-G<sub>1</sub> Arrest

In response to NaPP1, fibroblasts transformed by *v-erbB-as1* showed morphological reversal of transformation and recovery of basal signaling through MAP kinase and PI(3) kinase. Surprisingly however, these cells did not proliferate, even after 48 hr when the activities of mitogenic kinases were restored (Figure 2). Cells transformed with *v-erbB-as1* accumulated in G<sub>0</sub>-G<sub>1</sub> phase of the cell cycle after 24 hr of exposure to NaPP1,

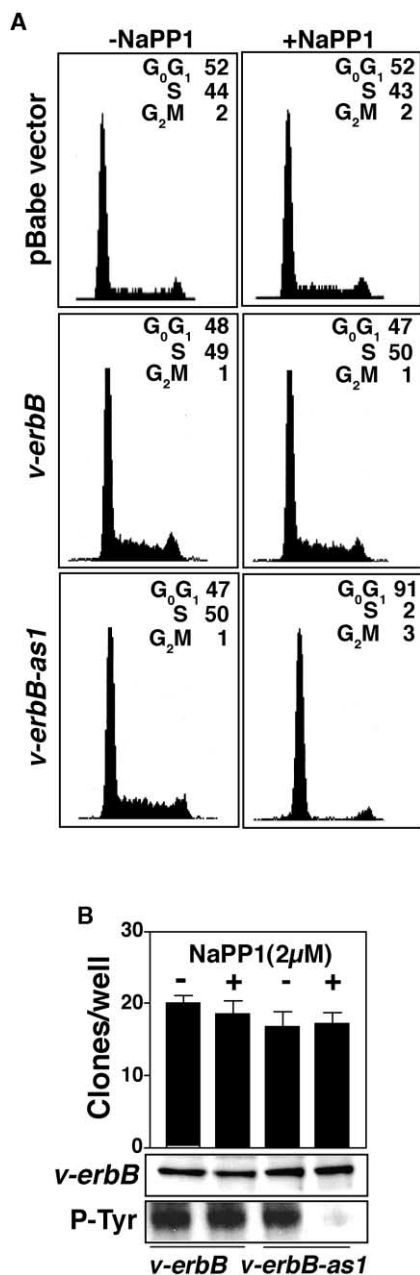


Figure 3. Reversibly Transformed *v-erbB-as1* Cells Recover Normal Signaling but Remain Arrested in G<sub>0</sub>/G<sub>1</sub>

(A) Equal numbers of cells were transduced as indicated and grown at subconfluence in 2 μM NaPP1 or in 1.2% DMSO. Cells were harvested at 48 hr and analyzed by flow cytometry.

(B) To rule out a dominant-negative cause of growth arrest, cells were grown in the absence or presence of NaPP1 (2 μM) and subsequently transduced with retroviral vectors carrying *v-erbB* or *v-erbB-as1*. Results represent the mean of three 6-well plates per experimental point.

and did not re-enter S phase during a 48 hr observation period (Figure 3). In contrast, G<sub>0</sub>-G<sub>1</sub> arrest was not observed in NaPP1-treated cells transduced with empty vector or with *v-erbB* cells (Figure 3).

To distinguish the requirement for *v-erbB* in the initiation and maintenance of transformation, we transduced

cells with *v-erbB-as1* and added NaPP1 at 0, 3, 6, 12, 24, and 48 hr after transduction. Puromycin was added at 48 hr, and puromycin-resistant cells were counted at day 10. Addition of NaPP1 up to 12 hr after transduction of *v-erbB-as1* had no effect on proliferation, whereas addition of NaPP1 at 24 hr and at later points resulted in proliferation arrest (our unpublished data).

This proliferation arrest was surprising because normal 3T3 cells proliferate in the absence of *v-erbB-as1*. One would therefore predict that NaPP1-treated *v-erbB-as1* cells should behave like normal 3T3 cells and continue to proliferate. If *v-erbB-as1* heterodimerized with a growth factor receptor required for basal growth, then NaPP1 treatment of this heterodimer could lead to growth arrest. This scenario is unlikely because signaling through MAP kinase and PI(3) kinase pathways recovers after NaPP1 treatment, and such recovery should not occur if a growth factor receptor required for basal growth were blocked. Nevertheless, to address this concern further, we transduced retroviruses containing *v-erbB* or *v-erbB-as1* into 3T3 cells grown continually in the presence of 2 μM NaPP1 such that the *v-erbB-as1*-transforming kinase was continuously inhibited. Puromycin-resistant *v-erbB-as1* clones were readily selected from cells grown and transduced in the presence of NaPP1 (Figure 3B), suggesting that growth arrest was not secondary to a dominant-negative effect of NaPP1 in a *v-erbB-as1* background.

To determine whether cell cycle arrest could be a general response to kinase inhibitor therapy, we performed flow-cytometric analysis of NIH3T3 cells transformed with *v-Src-as1* [22]. NaPP1 treatment of *v-Src-as1* cells again resulted in arrest at the G<sub>0</sub>-G<sub>1</sub> check point, with blockade of *v-Src-as1* kinase activity similar to that seen with *v-erbB-as1* (Figure 4). Unlike *v-erbB*, *v-Src* is thought to signal as a monomer (reviewed in [23]). Therefore, heterodimerization with other Src family members is not a likely mechanism by which NaPP1 treatment of *v-Src* cells could block progression through the cell cycle. In additional experiments (see Supplementary Material available with this article online), we also demonstrated that PDGF was sufficient to support growth of *v-erbB-as1* cells in low serum and that cell cycle arrest mediated by NaPP1 treatment of these cells occurred through a PDGF-independent pathway.

#### Decreased Levels of D and A Type Cyclins and Increased Levels of p27 in NaPP1-Treated *v-erbB-as1* Cells

Cyclins are required for progression through the cell cycle (reviewed in [24]). If levels of cyclins were low or if levels of cyclin-dependent kinase inhibitors were high in NaPP1-treated *v-erbB-as1* cells, then these cells would be unable to progress through the cell cycle. To address the state of cyclins and the cyclin-dependent kinase inhibitor p27 in NaPP1-treated *v-erbB-as1* cells, we performed Western blotting against cyclins A, D1, and D2 and against p27 (Figure 5). These experiments demonstrated that NaPP1-treated *v-erbB-as1* cells were quiescent at least in part because all three cyclins were reduced and because levels of p27 in the same cells were high. The low level of D type cyclins (and in

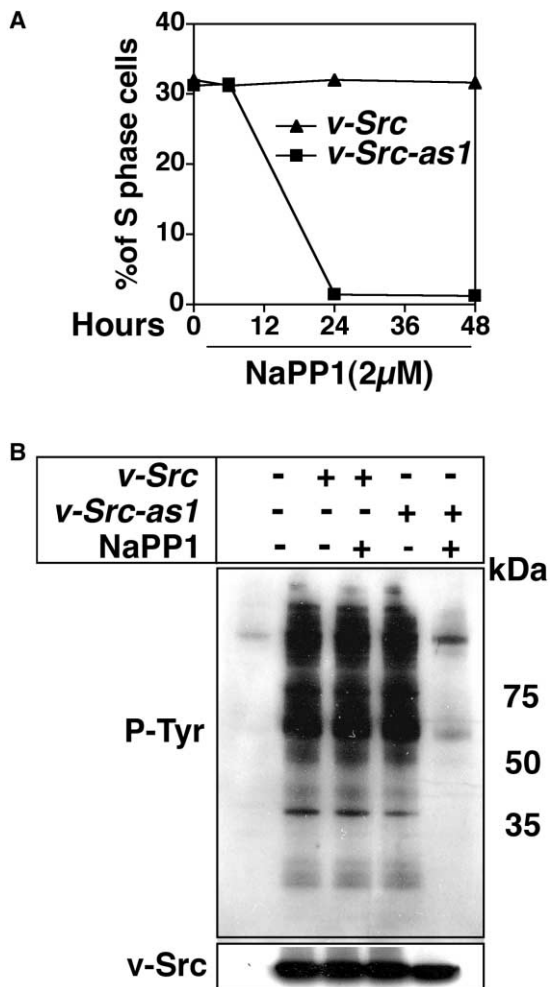


Figure 4. NaPP1 Treatment of Cells Transformed by *v-Src-as1* Also Results in Cell Cycle Arrest

(A) NIH3T3 cells transduced with *v-Src* or *v-Src-as1* were incubated with 1  $\mu$ M NaPP1 for 0, 6, 24, and 48 hr, labeled with BrDU, and analyzed by flow cytometry.

(B) A portion of cells from (A) were harvested and immunoblotted with 4G10.

particular the absence of cyclin D1) in cells transduced with *v-erbB-as1* and treated with NaPP1 argues that these cells are arrested in a  $G_0$  rather than a  $G_1$  state. Others have observed high levels of p27 in response to treating cells with inhibitors of EGFR [25, 26]. The reduction in cyclin D1/Cdk4 complexes presumably leads to increased free p27, which binds and inactivates Cdk2 and thereby blocks its ability to phosphorylate and degrade p27 [27].

To rule out the possibility that low levels of cyclin D1 were the result of cell cycle phase differences, we incubated *v-erbB-as1* cells with antisera to cyclin D1 and visualized these by confocal microscopy. Figures 6A and 6B show that nuclear cyclin D1 levels were reduced dramatically by NaPP1 treatment. We next transduced cyclin D1 into *v-erbB-as1* cells. NaPP1 treatment of these cells did not arrest proliferation, nor did it impact cyclin D1 levels. These experiments argue that cell cycle

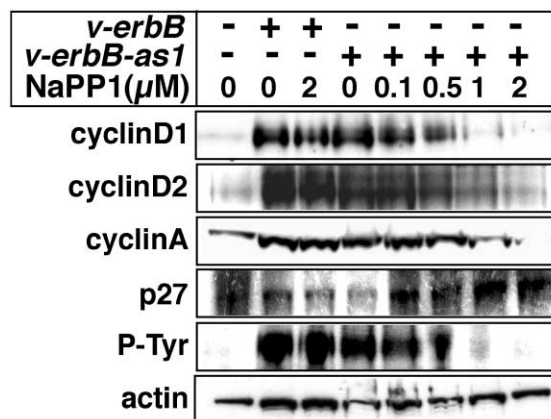


Figure 5.  $G_0$  Arrest in *v-erbB-as1*-Transduced Cells after Treatment with NaPP1

Cells were transduced as indicated, grown to subconfluence, treated with NaPP1 for 48 hr, and immunoblotted.

arrest in NaPP1-treated *v-erbB-as1* cells results from low levels of cyclin D1.

The  $G_0$ - $G_1$  arrest observed after NaPP1 treatment of *v-erbB-as1* or *v-Src-as1* cells suggests that reversal of an initiating oncogenic event by drug treatment was insufficient to allow reentry into the cell cycle. To better characterize the mechanism of cell cycle arrest, we asked whether proliferation could be restored with a conditional allele of *Raf*. We transduced cells with *v-erbB-as1* or *v-Src-as1* and with  $\Delta$ Raf1-ER, a fusion protein of the kinase domain of Raf-1 with the hormone binding domain of the human estrogen receptor, and EGFP [28]. NaPP1-mediated blockade of *v-erbB-as1* resulted in growth arrest that could be rescued by subsequent hydroxytamoxifen-mediated activation of  $\Delta$ Raf1-ER (Figure 7). Identical results were observed in NaPP1-arrested *v-Src-as1* cells (our unpublished data).

## Discussion

### An Allele of *v-erbB* Shows Specific Inhibition by a Selective Inhibitor

We used a chemical genetic strategy to generate alleles of *v-erbB* that could be selectively inhibited by cell-permeable ATP-competitive inhibitors. The *v-erbB-as1* (T210A) mutant showed transformation efficiencies and patterns and levels of cellular phosphotyrosine that were indistinguishable from those of the parental *v-erbB* allele (Figure 1). Injection of *v-erbB*- or *v-erbB-as1*-transduced NIH3T3 cells into immunocompromised mice led to tumor formation with comparable latencies. These three experiments argue that the transforming potentials of *v-erbB* and *v-erbB-as1* are functionally equivalent.

Treatment of transduced 3T3 or Rat1a cells with NaPP1 blocked transformation by *v-erbB-as1* and had no demonstrable effect on *v-erbB*. Treatment of mice injected with these cells similarly impacted *v-erbB-as1* with no effect on *v-erbB*. These data demonstrate that NaPP1 selectively inhibited *v-erbB-as1* but had no discernible effect on signaling mediated by *v-erbB*. Importantly, NaPP1 could also reverse both tyrosine

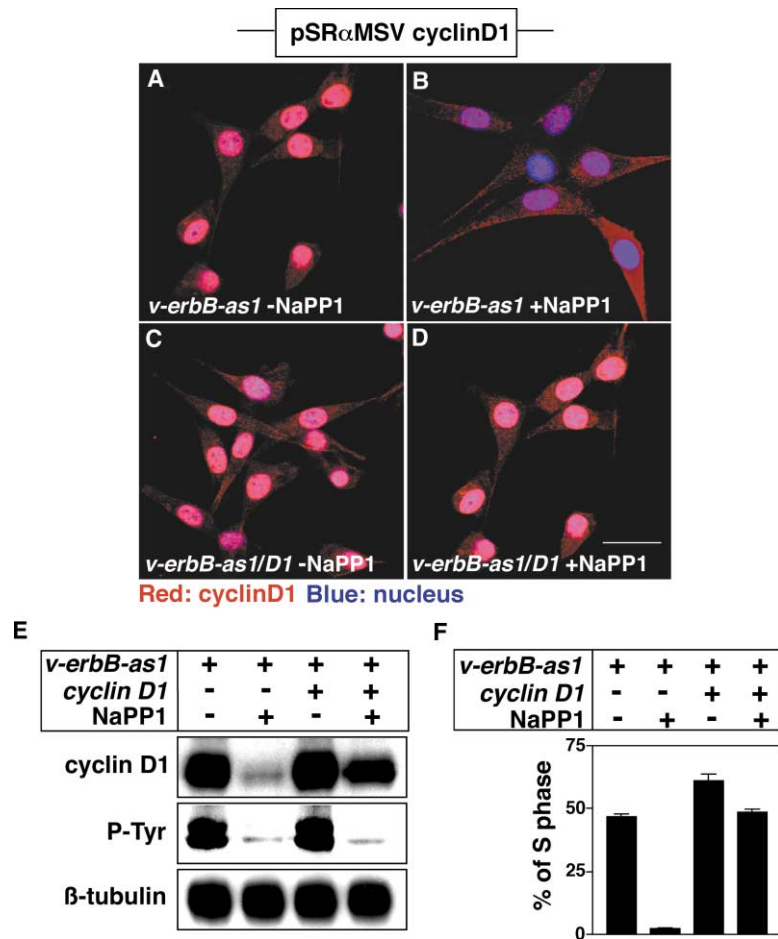


Figure 6. Rescue of Proliferation Arrest by Cyclin D1

*v-erbB-as1* cells were transduced with cyclin D1 and incubated with 2  $\mu$ M NaPP1.

(A–D) Cells were incubated with antisera to cyclin D1 (red), counterstained with To-Pro-3 iodide, and visualized by confocal microscopy. Pink color in overlay indicates nuclear localization of cyclin D1. NaPP1 treatment of *v-erbB-as1* cells leads to reduction of D1 (Figure 6B), which was not observed in *v-erbB-as1* cells transduced with cyclin D1.

(E and F) BrDU-labeled *v-erbB-as1* cells or *v-erbB-as1/D1* cells were either untreated or treated with NaPP1 (2  $\mu$ M). Aliquots of cells were analyzed for cyclin D1 or 4G10 (E), and three replicates were analyzed by flow cytometry (F).

phosphorylation and the transformed morphology associated with *v-erbB-as1* transduction of 3T3 cells (Figure 2).

#### G<sub>0</sub> Arrest in Reversibly Transformed Cells Suggests that Cells Become Dependent on Aberrant Oncogenic Signaling

After treatment with NaPP1, transformed *v-erbB-as1* cells showed reduced signaling through MAP kinase and PI(3) kinase, with subsequent recovery of basal mitogenic signaling through both pathways. Despite the apparent normalization of morphology and signaling in these cells, NaPP1 treatment of cells transduced with *v-erbB-as1* led to G<sub>0</sub> arrest, with reduced levels of D and A type cyclins and high levels of p27. Similar blockade and subsequent recovery of MAP kinase signaling has been described in cells made quiescent (in G<sub>0</sub>) by growth in low serum [29]. Quiescence in serum-starved cells was associated with cytoplasmic sequestration of phosphorylated ERK, as well as with recovery of proliferation upon addition of serum to the media [29]. Our results with NaPP1-treated *v-erbB-as1* cells demonstrate that phospho-ERK was distributed in the cytoplasm and nucleus (Figure 3), suggesting that cytoplasmic sequestration did not play a major role in the G<sub>0</sub> arrest observed in these cells.

To exclude a dominant-negative mechanism of cell

cycle arrest in NaPP1-treated *v-erbB-as1* cells, we demonstrated that NaPP1 had no impact on vector-transformed cells, that NaPP1 treatment did not affect the transduction of *v-erbB-as1*, and that NaPP1 treatment of *v-Src-as1* also caused proliferation arrest. Additionally, PDGF efficiently supported growth of serum-starved *v-erbB-as1* cells, and signaling through the PDGF receptor was intact in these cells even after NaPP1 treatment arrested their growth. These data collectively argue that G<sub>0</sub> arrest observed after NaPP1 treatment of cells transformed with either *v-erbB-as1* or *v-Src-as1* reflects the cells' dependence on aberrant signaling by the transforming oncogene and that reversal of the initiating oncogenic event by drug treatment was insufficient to allow re-entry into the cell cycle.

We hypothesize that NaPP1-treated *v-erbB-as1* and *v-Src-as1* cells failed to proliferate because some aspect of signaling has not been restored in these cells. Further examination of reversible transformation in this simple *v-erbB-as1*-driven system should allow us to identify the critical determinants that underlie cell cycle arrest in response to NaPP1 treatment. In two experiments aimed at understanding the mechanism of cell cycle arrest in this system, we have shown that both *cyclin D1* and *Raf* restored proliferation in NaPP1-treated *v-erbB-as1* cells (Figures 6 and 7). These observations suggest the block in cell cycle signaling lies



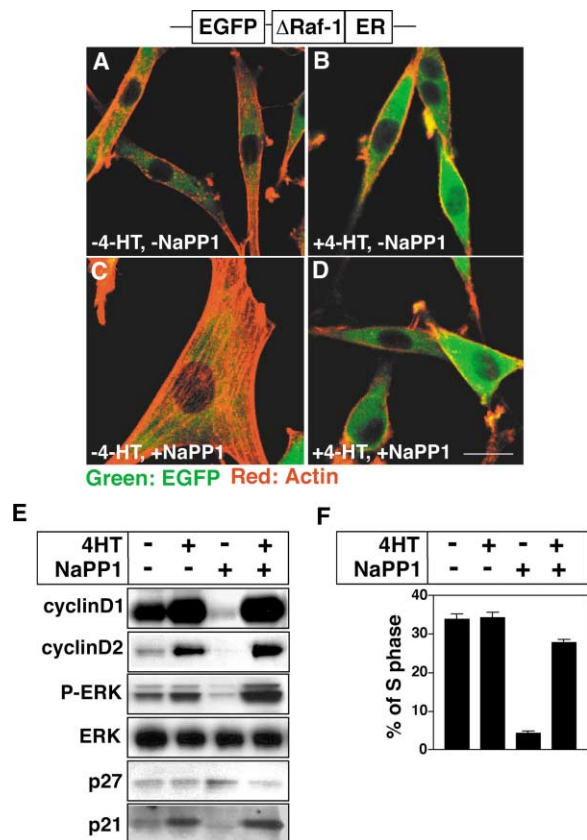


Figure 7. Activation of GFP $\Delta$ Raf1-ER Rescues NaPP1-Mediated G<sub>0</sub> arrest

NIH3T3 cells were transduced with *v-erbB-as1* and GFP $\Delta$ Raf1-ER. Cells were untreated (A), treated with 15 nM 4-hydroxytamoxifen (4-HT) for 48 hr (B), or treated with 2  $\mu$ M NaPP1 in the absence (C) or presence (D) of 4-HT. Rhodamine-phalloidin-stained cells were visualized by confocal laser scanning microscopy. Green signal is from the EGFP epitope on the  $\Delta$ Raf1-ER chimera. (E and F) BrDU-labeled cells were either untreated or treated with NaPP1 (2  $\mu$ M) plus 25 nM 4-HT for 48 hr as indicated. Aliquots of cells were analyzed for indicated cell cycle proteins (E), and three replicates were analyzed by flow cytometry (F).

between the growth factor receptor and Raf. Of course, it is possible that supraphysiological activation of the conditional *Raf* allele could overwhelm the cell cycle signaling machinery and override a block in cell signaling downstream of Raf. Experiments are currently in progress to distinguish between these possibilities.

Interestingly, others have used a modestly selective EGFR inhibitor (AG-1478) to treat breast and embryonal cancer cell lines and have also observed growth arrest [27, 30]. The full extent of molecular lesions contributing to transformation in these human cell lines remains unknown. In addition, the spectrum of intracellular kinases impacted by AG-1478 could be broad. Nevertheless, the observation that inhibition of EGFR in human cancer cell lines also led to growth arrest argues that this may be a general phenomenon.

## Conclusions

The remarkable efficacy of Gleevec in the treatment of chronic myelogenous leukemia has raised expectations

that other molecularly targeted agents may have similar efficacy. EGFR is an obvious candidate for kinase inhibitor therapy because it is implicated in a broad range of malignancies. Because most commonly used kinase inhibitors (including the new EGFR inhibitors now in clinical trials) also inhibit endogenous signaling pathways, one cannot differentiate between true oncogene-dependent events (such as those mediated by *v-erbB*) and the similar normal pathways (mediated through EGFR and other cellular kinases). By using analog-selective alleles of *v-erbB* and *v-Src*, as well as monoselective inhibitors of these alleles, we selectively blocked oncogenic signaling and could accurately distinguish normal from aberrant signaling.

Using this approach, we showed that cells adapted rapidly to oncogenic signaling prior to the acquisition of secondary mutations in additional oncogenes or tumor suppressor genes. This adaptation shifted cells from normal growth factor control to oncogene-dependent growth. Others have shown that tumor growth in transgenic mice required continued signaling by the initiating oncogene [31–34]. We here extend and in part explain these observations by showing that the original oncogene has really co-opted the cell signaling machinery. This adaptation happened on an extremely fast time scale and explains the continued dependence on signaling by the initiated oncogene (i.e., the cell has become addicted to the aberrant oncogenic signaling [35]).

Our observations in rodent fibroblasts may help to explain the remarkable therapeutic index associated with Gleevec, a drug for which the maximum tolerated dose in human patients has still not been reached [36]. Gleevec's lack of overt toxicity is somewhat surprising because it is not selective for the BCR-ABL gene product specific to chronic myelogenous leukemia (CML) but also blocks c-Abl, c-Kit (stem cell factor), and PDGFR [37]. If BCR-Abl has co-opted the signaling machinery of CML cells, then one could achieve cell cycle arrest even through partial inhibition of BCR-Abl by using a dose of Gleevec that does not impact basal signaling in normal cells. By analogy, our data suggest that selective inhibition of EGFR may effectively block proliferation of cancer cells in vitro. It is likely that normal cells will require higher levels of EGFR inhibition to block basal signaling and that the therapeutic index for EGFR inhibitors may be favorable.

As new kinase inhibitors become available in the clinical treatment of patients with cancer, it becomes increasingly important to understand the effect of selectively inhibiting specific kinases. This chemical genetic strategy can help to achieve an improved understanding of selective inhibition and can provide information that will help distinguish specific from nonspecific effects associated with nearly all kinase inhibitors now being used in or being developed for clinical applications.

## Experimental Procedures

### Construction of Analog-Sensitive Alleles of *v-erbB*

Overlap extension PCR [38] with Expand high-fidelity polymerase (Roche, Indianapolis, IN) was used for making analog-sensitive alleles of the ES-4 allele of *v-erbB*. Four synthetic oligonucleotides were used for generation of *v-erbB-as1*.

Primers were as follows: primer 1, 5'-CTGCAGAACCAATGCATTGGCTGGCTCGAGGGTGGATCA-3'; primer 2, 5'-GCATAAGCTGGG

CGATGAGCTGCAC-3'; primer 3, 5'-GTGCAGCTCATCGCCAGCT TATGC-3'; and primer 4, 5'-GAGCGGGTTCGACGATCACTCAAGA TACTCAGGGTT-3'. Primers 1 and 4 encompass *v-erbB* and introduce BstXI and Sall sites. Primers 2 and 3 contain the T210A mutation. PCR products were digested with BstXI and Sall and ligated into pBabe puro vector [39].

#### Cell Culture, Retrovirus Production, Infection, and Kinase Inhibitors

NIH3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum. *v-erbB*, *v-erbB-as-1*, *v-Src*, and *v-Src-as1* [22] were cloned into pBabe puro [39] and transfected into Bosc23 cells [40] to produce ecotropic virus. GFP $\Delta$ Raf1-ER [28] and pSR $\alpha$ MSVtkneocyclin D1 (from Charles Sherr) were transfected similarly. Pools of transduced rodent fibroblasts were selected by addition of puromycin or G418 at 48 hr. Cells were selected for 2 weeks. 1-Naphthyl PP1 was synthesized as described [41]. LY294002 and PD098509 were from Sigma.

#### In Vitro and In Vivo Growth Assays

For soft agar experiments, cells were plated in 0.35% Sea Plaque agarose (FMC Bioproducts, Rockland, ME) in triplicate. Colonies were stained with MTT (Sigma Chemical Company, St. Louis, MO) and counted with a digital camera image analyzer. For nude-mice allografts, cells were injected subcutaneously in BALB/c nu/nu mice (Harlan Sprague Dawley, Madison, WI). Beginning 1 day postimplant, six mice per group were treated by daily IP injection with 20 mg/kg NaPP1 in 50% DMSO (Sigma Chemical Company, St. Louis, MO) or 50% DMSO. Tumor diameters were measured at 3 day intervals, and volumes were calculated [30].

#### Immunofluorescence Microscopy

Cells were fixed, permeabilized, and incubated with anti-pERK or cyclin D1 (Santa Cruz, Biotechnology, Santa Cruz, CA), washed, and incubated for 1 hr in Alexa Four 488/568 secondary antibodies (Molecular Probes, Eugene, OR). F-actin was visualized by incubating cells with phalloidin Alexa 594 (Molecular Probes). Nuclei were labeled with To-Pro-3 iodide (Molecular Probes). Cells were observed with a fluorescent microscope (TET300; Nikon) equipped with a confocal laser scanning system (MR-1024; Bio-Rad).

#### Labeling Cells with Anti-BrdU and Propidium Iodide for Flow-Cytometric Analysis of Cell Cycle Distribution

Cells were plated at  $5 \times 10^5$  cells/cm<sup>2</sup>. BrdU (Sigma) was added to 10  $\mu$ M. Cells were then fixed in 70% ethanol, and DNA was denatured with 2 N HCl in 0.5% Triton X-100 v/v, then neutralized in 0.1 M Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> and 10 H<sub>2</sub>O (Sigma) at pH 8.5. Cover slips were centrifuged and resuspended in 0.5% Tween 20/1% BSA containing anti-BrdU FITC (Becton Dickinson, San Jose, CA). Nuclei were stained for with 5  $\mu$ g/ml propidium iodide (Sigma) containing 125 units/ml protease-free RNase (Calbiochem, San Diego, CA) and analyzed in a FACS Calibur flow cytometer (Becton Dickinson).

#### Immunoblot Analyses

Equal amounts of total protein were loaded for 4%–12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, membranes were blotted with 4G10 (Upstate Biotechnology, Waltham, MA), PY20, p27, phosphorylated ERK, phosphorylated MAPK, cyclin D1, cyclin D2, cyclin A, and actin (Santa Cruz Biotechnology, v-erbB [42], Ser473-phosphorylated Akt, phosphorylation state-independent Akt, phosphorylation state-independent ERK1 (New England Biolabs, Beverly, MA), or v-Src antibody GD11 (from Sally Parsons). Antibodies were detected with HRP-linked anti-mouse or anti-rabbit Ig (Amersham Pharmacia Biotech, Arlington Heights, IL), followed by enhanced chemiluminescence (Amersham).

#### Supplementary Material

Three additional figures are available with the online version of this article at <http://images.cellpress.com/supmat/supmatin.htm>.

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