Intracellular calcium is an important regulator of subsets of transmembrane signaling categories of ion fluxes, phosphorylation, and guanine nucleotide-binding protein-mediated second messenger production (Berridge et al., 2003; Berridge et al., 1998; Mooren and Kinne, 1998). Recently, alterations of Ca\(^{2+}\) homeostasis have been involved in bcr-abl-driven development and progression of myeloid leukemia (Soboloff et al., 2002; Vichalkovski et al., 2006). Carboxyamidotriazole (CAI), an inhibitor of calcium-mediated signal transduction, is one of the first cytostatic signal inhibitory anti-cancer drugs; it has been tested in solid tumor patients in Phase I and II clinical trials at the National Cancer Institute (Kohn et al., 2001; Hussain et al., 2007). Although imatinib mesylate (IM) has revolutionized the treatment of chronic myeloid leukemia (CML), some patients develop resistance with progression of leukemia. Alternative or additional targeting of signaling pathways deregulated in bcr-abl-driven CML cells may provide a feasible option for improving clinical response and overcoming resistance. In this study, we show that carboxyamidotriazole (CAI), an orally bioavailable calcium influx and signal transduction inhibitor, is equally effective in inhibiting the proliferation and bcr-abl dependent- and independent-signaling pathways in imatinib-resistant CML cells. CAI inhibits phosphorylation of cellular proteins including STAT5 and CrkL at concentrations that induce apoptosis in IM-resistant CML cells. The combination of imatinib and CAI also down-regulated bcr-abl protein levels. Since CAI is already available for clinical use, these results suggest that it may be an effective addition to the armamentarium of drugs for the treatment of CML. J. Cell. Physiol. 215: 111–121, 2008. © 2007 Wiley-Liss, Inc.

Chronic myeloid leukemia is a myeloproliferative disorder in which leukemic cells display a distinctive shortened chromosome, the Philadelphia (ph) chromosome generated from a reciprocal t(9:22) (q34;q11) translocation (Rowley, 1973). This translocation results in the head-to-tail fusion of the breakpoint cluster region (BCR) gene on chromosome 22 with the ABL proto-oncogene on chromosome 9 (Ben-Neriah et al., 1986). The encoded chimeric bcr-abl oncoprotein exhibits constitutively active tyrosine kinase activity that stimulates a number of downstream signaling cascades including those related to the Ras/Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, the phosphoinositide 3-kinase (PI3K) pathway, Janus kinase 2 pathway and the signal transducer and activator of transcription 5 pathway (STAT-5) (Nieborowska-Skorska et al., 2000; Sonoyama et al., 2002). Oncogenic activation of these signaling effectors in turn causes unrestrained proliferation, protection from apoptosis, and augmentation of invasion abilities (Arlinghaus and Sun, 2004). Imatinib mesylate (IM), is a selective well tolerated inhibitor of the bcr-abl tyrosine kinase that has significantly improved the prognosis of patients with chronic phase CML and, to a lesser extent, of patients with accelerated and blast phase disease (Druker et al., 1996; Kantarjian et al., 2002). Despite this remarkable progress, a major problem associated with the administration of imatinib, is acquired resistance occurring with an annual incidence of approximately 4% (Kantarjian et al., 2004). BCR/ABL gene amplification, increased expression of bcr-abl protein, point mutations in the bcr-abl tyrosine kinase domain and recently, inhibition of phosphotyrosine phosphatase 1B have been reported as mechanisms of resistance to imatinib (Shah et al., 2002; Koyama et al., 2006). There is an urgent need for new anticaner agents and combinations that could improve responses and survival rates for CML. Moreover, the use of a combination of biochemically based agents to target alternative signaling may yield a higher therapeutic success accompanied by less toxicity.
Exposure to CAI concentrations of 1–10 μM (0.4–4.0 μg/ml) has anti-tumor activity, inhibiting tumor cell calcium-mediated signal transduction and proliferation in vitro (Alessandro et al., 1996; Wu et al., 1997; Moody et al., 2003), inhibiting invasion in vitro and in vivo (Jacobs et al., 1997; Lambert et al., 1997), and reducing both the number of spontaneous metastases and solid tumor burden in mice receiving oral drug administration (Kohn et al., 1994). In addition, CAI has been shown to have anti-angiogenic capabilities both in vitro and in vivo (Kohn et al., 1995). The present study is designed to assess the potential of CAI in defeating the in vitro imatinib resistance in CML cell lines. We have demonstrated that addition of CAI to imatinib-resistant cells induces a marked decrease in cell viability and a stronger apoptosis, events associated with down-regulation of bcr-abl protein, inhibition of tyrosine phosphorylation of bcr-abl, STAT5, CrkL, as well as inhibition of ERK1/2 phosphorylation. These findings suggest the potential clinical use of CAI in the treatment of patients with IM-resistant forms of chronic myelogenous leukemia.

Materials and Methods

Cell culture and reagents

LAMAB4R, K562R, and KCL22R are imatinib resistant cells kindly provided by Dr. P. Vigneri, University of Catania. Cells were cultured in RPMI 1640 medium (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal bovine serum (BioWhittaker), 2 mM l-glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin (Euroclone, Paignton, UK). Imatinib resistance was induced in these cell lines that were initially imatinib-sensitive (LAMAB4R, K562, and KCL22) by continuous culture exposure up to 1 μM. All experiments were performed using logarithmically growing cells (4–6 x 10⁵ cells/ml). Imatinib, kindly provided by Dr. R. Bertieri (Novartis, Milan, Italy), was prepared as a 10 mM stock solution in sterile phosphate-buffered saline (PBS) and kept at -20 °C. PD 98059 was from Calbiochem (Darmstadt, Germany). CAI (40 μM) and PD98059 stocks were prepared in DMSO and kept at -80 °C. Serial dilutions of all drug stocks were performed in culture medium the same day of use. For all experiments, cells were constantly exposed to 1 μM imatinib and to the different CAI concentrations. MTT uptake was assayed daily to ensure exponential growth of the untreated cells. Means and standard deviations generated from 3 to 4 independent experiments are reported as the percentage of growth versus control. Cell proliferation curves were derived from these data by using Microsoft Excel software.

FISH analysis and sequencing of the ABL kinase domain

In order to detect the BCR/ABL rearrangements, FISH analyses were performed using the commercially available LSI BCR/ABL dual color dual fusion translocation probe (Vysis, Abbott, England) and to the different CAI concentrations. MTT (5-(3,4-dimethyldihydroimidazol-2-yl)-2,5-diphenyloxazolium bromide), trypan blue, propidium iodide, RNase A, proteinase K and acridine orange were purchased from Sigma Chemical Co. (St. Louis, MO).

Proliferation assay (MTT assay)

For the methyl-thiazol-tetrazolium (MTT) assay, cells were plated in triplicate or quadruplicate at 1.5 × 10⁵ per well and exposed to escalating doses of CAI (1–40 μM) and PD 98059 (10–50 μM) for up to 5 days. MTT uptake was assayed daily to ensure exponential growth of the untreated cells. Means and standard deviations generated from 3 to 4 independent experiments are reported as the percentage of growth versus control. Cell proliferation curves were derived from these data by using Microsoft Excel software.

Apoptosis assays

Imatinib-resistant CML cells were treated with varying concentrations of CAI and the 0.1% DMSO as control. After 96 h of culture, cells were harvested and apoptosis assays were performed as described. (A) Morphological identification of apoptotic cells: Cells were collected, washed, and stained with acridine orange, according to the method of Saydam et al. (2003). At least 200 cells/field were counted in each experiment. The number of apoptotic cells was plotted as a percentage of increase of apoptosis versus control. (B) DNA ladder agarose-gel electrophoresis assay: After treatment, CML cells were fixed in suspension in 70% ethanol and the cell pellets resuspended in 80 μl of phosphate-citrate buffer, consisting of 192 parts of 0.2 M Na₂HPO₄ and 8 parts of 0.1 M citric acid (pH 7.8), at room temperature for 30 min. A 6 μl aliquot of Nonidet NP-40 was then added, followed by 6 μl of a solution of RNase A (1 mg/ml). After 30 min incubation at 37 °C, 6 μl of a solution of proteinase K (1 mg/ml) was added and the extract was incubated for an additional 30 min at 37 °C. DNA samples were then precipitated, resuspended in water and an aliquot electrophoresed on a 1.5% agarose gel: DNA bands were visualized using a Kodak EDAS 290 device.

Reverse transcription and TaqMan PCR

BCR/ABL fusion gene transcript levels were measured by reverse transcription (RT) and TaqMan real-time quantitative polymerase chain reaction (RQ-PCR). In brief, RNA was isolated with the Rneasy Mini kit (QIAGEN) according to the manufacturer’s instructions. Five micrograms of total RNA from each cell line were used for first strand cDNA synthesis using the 1st Strand cDNA Synthesis Kit (Amersham). The PCR amplification mixture (25 μl) contained cDNA. BCR forward primer ENF501 (5’- TTCAGAAGCTTCTCCTCGCATC-3’) and BCR reverse primer 4065 (5’-CTCTCTTAGCTAGTACAGCTGG-3’). Nested reactions were performed using BCR F4 forward (ACAGCATTCCGCTGACCATAA) and reverse U396 (GCCATAGTTGACATTTTCC). PCR products containing the kinase domain were sequenced with both forward primer 3306F (TGTTTCATCATCATTCAACGG) and reverse primer 4000R (GGACATGCCATAGTAGTACA) using Big Dye Terminator chemistry and ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA). The obtained sequences were compared with the one deposited in GeneBank (accession no. M14752).

JOURNAL OF CELLULAR PHYSIOLOGY
Western blot analysis

Total protein cell lysates were obtained and analyzed by SDS-PAGE followed by Western blotting as previously described (Alessandro et al., 2005). Briefly, imatinib-resistant CML cells were treated as indicated, then washed and lysed in lysis buffer (300 mM NaCl, 50 mM Tris HCl pH 7.6, 0.1% Triton, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 4 mM EDTA, 2 mM sodium orthovanadate, 10 mM NaPPi, 100 mM NaF) on ice for 1 h. The cell lysates were clarified at high speed centrifugation for 15 min and an aliquot of the supernatant was assayed to determine protein concentration by the Bradford method. Proteins were separated by 6% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Protran Schleicher & Schuell, Dassel, Germany). The membrane was incubated in block solution (5% non-fat dry milk, 20 mM Tris, 140 mM NaCl, 0.1% Tween-20), and...
probed overnight at 4°C with specific antibodies against c-Abl, phospho-Abl, phospho-Stat-5, phosphor-CrkL, EIf4, actin, p44/42 MAP, phospho-p44/42 MAP kinase (all from Cell Signalling Technology, Beverly, MA), anti-phosphotyrosine and anti-Stat5 (Santa Cruz Biotechnology, Santa Cruz, CA). After three washes with 20 mM Tris, 140 mM NaCl, 0.1% Tween-20, (WS), the membrane was incubated with horseradish peroxidase-conjugated secondary antibody and proteins detected by the enhanced chemiluminescence detection system (Super Signal, Pierce, Rockford, IL).

Statistical analysis
Statistically significant differences between mean values (from three independent experiments at least) were determined using two tailed Student’s t-test. Differences were considered statistically significant at \( P < 0.05 \).

Results and Discussion
Characterization of CML cells
To evaluate the status of the BCR/ABL fusion gene at the genomic level, the three cell lines were subjected to FISH analysis. Using two probes representing BCR and ABL, the presence of more than 1 copy of the Philadelphia chromosome was detected in all the samples and precisely: the KCL22R cell line shows 3 copies of the fused signals (Fig. 1A, arrows) and both the K562R and LAMA84R cell lines show two extra signals compatible with amplification elements (Fig. 1B,C respectively, arrows). As concerns the presence of point mutations in the BCR/ABL fusion gene, sequencing of ABL kinase domain showed the presence of the D276G mutation in the K562R cells while the other two cell lines possess a wild type BCR/ABL allele.

Effects of CAI on growth of human CML cells
MTT assays were performed to determine the antiproliferative effects of CAI on three imatinib-resistant CML cell lines. Data presented in Figure 2 show results of 5-day treatments. CAI inhibits cell growth of LAMA84R, K562R, and KCL22R in the low micromolar range in a dose dependent fashion. Plasma concentrations of 2–10 \( \mu \)M CAI are attainable in steady state after oral administration. The results herein show a 40% growth reduction of the CML lines within this therapeutic window.

Although initial imatinib CML clinical results are encouraging (Kantarjian et al., 2002), the development of drug resistance and
the more limited responsiveness of patients with blast crisis and BCR/ABL^+ acute lymphoblastic leukemia represent persistent challenges. Such considerations have prompted attempts to circumvent resistance by developing strategies combining imatinib with other agents active against BCR/ABL^+ cells. Simultaneous inhibition of two signal transduction targets important in CML could offer significant therapeutic advantages.

Identification of promising candidates for combinatorial therapeutics requires strategic selection. Ca^{2+} ions are involved in the regulation of several cellular functions including proliferation, apoptosis and kinase-mediated signaling.

Fig. 4. CAI treatment inhibits tyrosine phosphorylation in IM-resistant CML cells. Whole cell lysates were prepared from IM-resistant CML cells treated with increasing doses of CAI for 24, 72, and 96 h and subjected to Western blot analysis with antibodies against phosphorylated tyrosine as described in Materials and Methods. Protein loading was controlled by probing the stripped membrane with a polyclonal anti-beta actin antibody (lower parts). These results were confirmed by three independent experiments for each cell line. Ctrl: cells treated with DMSO and imatinib 1 \mu M.
Furthermore, it has been shown that alterations of Ca$^{2+}$ homeostasis are involved in bcr-abl-driven development and progression of myeloid leukemia (Soboloff et al., 2002; Vichalkovski et al., 2006). CAI inhibits calcium-mediated signal transduction, is well-tolerated and orally bioavailable and has been evaluated in clinical trials of solid tumor patients in the United States (Hussain et al., 2003). This has led to our hypothesis that the combination of CAI and imatinib may be investigated for chemotherapeutic applications. We showed that within 5 days, the addition of 10 μM CAI induced a 40–50% decrease in net cell proliferation in the three imatinib-resistant CML lines with LAMA84R cells showing the higher sensitivity and KCL22R cells the minor sensitivity to CAI addition. Several studies have indicated that the mechanisms leading to STI571 resistance differ between different lines. The most commons are overexpression of the bcr-abl protein due to amplification of BCR/ABL gene, reduced intake mediated by P-glycoprotein, mutation in the bcr-abl kinase domain and acquisition of compensatory mutations in other genes (Xavier Mahon et al., 2000; Walz and Sattler, 2006). Our FISH analyses demonstrate that both LAMA84R and K562R have amplification of the BCR-ABL gene while KCL22R cells show supranumerary copies of the gene. Interestingly, the higher resistance of KCL22R to chemotherapeutic compounds have been observed by other groups and related to not still defined alternative signaling (Xavier Mahon et al., 2000). We have observed by a proteomic approach that KCL22 cells have a molecular profiling correlated with an intrinsic resistance to apoptosis in general and to imatinib treatment in particular (paper in press, Journal of Proteome Research) and further studies are ongoing to better characterize the effectors of this phenotype.

An elevation in cytosolic Ca$^{2+}$ is commonly noted in transformed cells and has been proposed to be important in cell proliferation through signaling mechanisms mediated by Ras GTase activity, transcription factor NFAT and mitochondrial activity (Berridge, 1995; Holmuhammerov et al., 2002; Kupzig et al., 2005). Our results suggest that inhibition of CAI-sensitive signals in these resistant cells may open a new avenue in the management of patients refractory to therapy with imatinib.

**CAI induces apoptosis in imatinib-resistant CML cells**

Consistent with its anti-proliferative action, the effects on apoptosis induction by CAI was observed after a 96 h exposure to CAI at different doses. As shown in Figure 3A, treatment of cells with CAI leads to increased apoptotic cell death in a dose dependent manner; CAI concentrations of 5 and 10 μM resulted in a net increase in apoptotic cells (up to 300% induction with 10 μM CAI concentration; \( P < 0.001 \)). Morphological identification of apoptosis (acridine orange/ethidium bromide staining) is shown in Figure 3B. The number of apoptotic cells in CAI-treated samples is increased with respect to control cells. DNA fragmentation is considered to be a distinctive trait of apoptosis, and produces 180- to 200-base pair internucleosomal DNA ladder gel patterns. DNA fragmentation was seen with 1 μM CAI, becoming more prominent with 5 μM (Fig. 3C). These apoptosis results mirrored the findings in the MTT assays.

One measure of efficacy of chemotherapeutic agents is their effectiveness at inducing apoptosis in malignant cells. It is widely recognized that the mechanism by which bcr-abl may promote accumulation of leukemic cells is mainly by inhibition of apoptosis, rather than by increasing the rate of cell division (Rowley et al., 1996). A number of reports suggest that agents causing a reduction in bcr-abl protein expression, determine the onset of apoptosis, as has been shown with proteasome inhibition. Dysregulation of intracellular Ca$^{2+}$ concentrations has been associated with the induction of apoptosis in several cell types (Dowd, 1995). Depending upon the cell type, both high and low intracellular Ca$^{2+}$ concentrations have been shown to lead to apoptosis. Gommerman and Berger (1998) have shown that blockade of Ca$^{2+}$ influx reverses the ability of
Steel factor to protect a myelomonocytic cell line from apoptosis, thus highlighting the role of Ca$^{2+}$ influx in Steel factor-dependent cell survival. Petranka et al. (2001) showed that lowering cytosolic Ca$^{2+}$ reduces the activation of NF-kb and confers susceptibility to apoptosis in preneoplastic cells. In the present study, we demonstrate that CAI may trigger apoptosis in all three imatinib-resistant CML lines independently by their BCR/ABL gene condition. In line with our results, Waselenko et al. (2001) provided evidence that CAI induced apoptosis in vitro in human B-cell chronic lymphocytic leukemia cells at drug concentrations attainable in vivo while Mikkelsen’s group showed that CAI determined glioma cancer cell apoptosis in micromolar concentrations achievable in brain tissue in vivo (Ge et al., 2000). Further studies will be necessary to investigate if CAI is also effective in inducing apoptosis in cells that acquire resistance to Imatinib by distinct mechanisms including BCR/ABL mutation (T315I, E255K, M351T, and others).

Effects of CAI on bcr-abl mediated tyrosine phosphorylation

To investigate the biochemical basis of the antiproliferative effects of CAI, the global phosphotyrosine signature, phosphorylation of p210 bcr-abl, and phosphorylation of selected bcr-abl downstream targets was evaluated in whole cell extracts of the LAMA84R, K562R, and KCL22R after 24, 72, and 96 h of drug treatment. Cells were incubated with increasing concentrations of CAI, harvested and subjected to immunoblotting with reagents that recognize phosphotyrosine, p210bcr/abl, STAT5, and CrkL. As shown in Figure 4, several heavily tyrosine-phosphorylated proteins were observed in the untreated imatinib-resistant cells. A dose-dependent inhibition of the overall pattern of tyrosine phosphorylated proteins were observed in all three cell lines, starting at 5 μM CAI.

All three CML-resistant cell lines displayed a reduction in levels of phosphorylated bcr-abl after 24, 72, and 96 h of CAI exposure (Fig. 5). Consistent with this conclusion, CAI

![Image of immunoblots showing effects of CAI on tyrosine phosphorylation](image-url)
inhibited the phosphorylation of selected targets of bcr-abl kinase. Tyrosine phosphorylation of STAT5 was reduced by 5 \( \mu \)M CAI treatment and ablated in all imatinib-resistant cells by the addition of 10 \( \mu \)M CAI (Fig. 6). A similar dose-response effect was observed for the tyrosine phosphorylation of CrkL, another substrate of the oncogenic bcr-abl kinase (Fig. 6).

Several reports have linked agonist-induced and oncogene-driven \( \text{Ca}^{2+} \) mobilization with activation of tyrosine kinases in normal and cancer cells (Alessandro et al., 1998; Furst et al., 2002; D’Amato et al., 2003). In line with this finding, it has been reported that removal of calcium from the extracellular medium inhibits the activation of tyrosine kinases in BCR/ABL+ leukemic cells (Vichalkovski et al., 2006).

Thus, there may be multiple kinase-inhibitory compounds with which to target bcr-abl kinase activity in different fashions. Identifying the proteins whose phosphorylation levels change after CAI treatment will be necessary to discover novel drug targets within IM-resistant CML.

### Downregulation of bcr-abl expression by CAI in imatinib-resistant CML cells

Our observation that activation of bcr-abl and its signaling pathway is downregulated led to the question as to whether the protein itself is regulated by CAI exposure. Immunoblot analysis was used to assess the expression of the p210 bcr-abl fusion protein in imatinib-resistant CML cells. As shown in Figure 7A, expression of bcr-abl was downregulated by CAI at a concentration slightly higher than the dysregulation of its phosphorylation, but commensurate with its antiproliferative and proapoptotic activity. Actin expression was stable at doses up to 10 \( \mu \)M CAI. This observation rules out the possibility that CAI-induced down-regulation reflects non-specific events. To

---

**Fig. 7.** Exposure of CML cells to CAI diminishes bcr-abl expression. A: LAMA84R, K562R, and KCL22R were treated with increasing doses of CAI for 24, 72, and 96 h after which protein lysates were prepared and subjected to Western blot analysis as described in Materials and Methods using anti-bcr-abl antibodies. Blots were subsequently reprobed with antibodies against beta actin to ensure equal loading as well as to check for CAI specificity. In (B) is showed that treatment of CML cells with CAI does not change the mRNA levels of BCR/ABL as quantitated by RealTime PCR assay. Ctrl: cells treated with DMSO and imatinib 1 \( \mu \)M.
Further elucidate the mechanism responsible for the changes in the amount of p210 bcr-abl protein, the levels of p210 bcr-abl mRNA were determined. In contrast to the protein levels, the p210 bcr-abl mRNA expression levels, as calculated by a real-time RT-PCR assay, had no significant change after treatment of all three cell lines with CAI (Fig. 7B). This result suggests that down-regulation of bcr-abl by CAI is post-translational. Whereas exposure of BCR/ABL cells to imatinib has not in general been associated with down-regulation of the bcr-abl protein, several other agents have been shown to act in this way, including arsenic trioxide (Porosnicu et al., 2001), geldanamycin (Nimmanapalli et al., 2001), proteasome inhibitors (Dou et al., 1999), and the kinase inhibitor AG957 (Mow et al., 2002). Interestingly, BAG3, a protein that was shown to be upregulated upon prolonged cell exposure to CAI, abrogates the geldanamycin-driven protein degradation in human cancer cells (Doong et al., 2003). Further experiments are necessary to elucidate the exact mechanism by which CAI down-regulates bcr-abl expression.

**Ras-MAP kinase signaling in CML is inhibited by CAI treatment**

Bcr-abl activates Ras-MAP kinase signaling, which is involved in cellular proliferation and differentiation. We examined effect of CAI treatment on ERK1/2 activation. The results showed phosphorylation of ERK1/2 was markedly inhibited by CAI treatment, with no remarkable change in the total amount of ERK1/2 in all three IM-resistant CML cells (Fig. 8). In contrast, neither phosphorylated ERK1/2 nor total ERK1/2 were changed following treatment even with high doses of imatinib (Fig. 8). In order to evaluate if MEK/MAPK pathway is involved in the proliferation and/or survival of leukemia cells, we performed cytotoxicity experiments with PD98059, a specific MEK/MAPK inhibitor. As shown in Figure 9 this compound inhibits cell growth of LAMA84R and K562R in a dose/time dependent fashion, but has weak effect on KCL22R growth. These results showed that the cytotoxic effect of CAI on LAMA84R and K562R cells is strictly related to inhibition of ERK1/2, while in KCL22R the anti-proliferative activity of CAI is mainly due to partial inhibition of bcr-abl.

The Raf/MEK1/2/ERK1/2 pathway is known to exert cytoprotective actions in diverse neoplastic cell types, particularly those of hematopoietic origin (Chang et al., 2003). In this context, it is notable that several laboratories have developed pharmacologic MEK1/2 inhibitors such as PD184352 to increase the lethality of imatinib. MEK1/2 inhibitors have been shown to induce apoptosis in BCR/ABL cells and to potentiate the activity of imatinib (Allen et al., 2003; Chunrong et al., 2002). Ohmine et al. (2003) showed that ERK1/2 mediated intracellular signaling was not disturbed by treatment with high doses of imatinib in IM-resistant KCL22 cells. Others researchers have reported activation of MAPK signaling in response to imatinib treatment, a potential pathway inducing resistance (Chu et al., 2004); inhibitors that counteract MAPK activity may be therefore of clinical value to overcome imatinib resistance. Evidence from several experimental models suggest that activation of ERK1/2 may occur through Ca$^{2+}$-dependent as well as Ca$^{2+}$-independent mechanisms. Our results suggest that in IM-resistant CML cells,
CAI, an inhibitor of calcium influx, may reduce ERK activity concomitantly to bcr-abl driven signaling and thus create a strong rationale to investigate the in vivo activity of this compound in imatinib-resistant BCR-ABL \textsuperscript{+} leukemias.

Acknowledgments
M.G. is a PhD student in Immunopharmacology at the Universita\' of Palermo, C.C. is a fellow of Italian Association for Cancer Research (AIRC), P.C. is a fellow of the Universita\' di Palermo. This work was supported by Italian Association for Cancer Research (AIRC) to G.D.L. and R.A., Universita\' di Palermo (International Cooperation) to R.A. and A.M.F; ex 60\% MURST to R.A., A.M.F. and to G.D.L.

Literature Cited
EFFECTS OF CAI ON IMATINIB-RESISTANT CML CELLS


