PKC Potentiates Tyrosine Kinase Inhibitors STI571 and Dasatinib Cytotoxic Effect

ARACELI TOBÍO, AMPARO ALFONSO and LUIS M. BOTANA

Pharmacology Department, School of Veterinary Sciences, University of Santiago de Compostela, University Campus, Lugo, Spain

Abstract. *Aim: The aim of the present study was to determine the relationship between the tyrosine kinase inhibitors, STI571 and dasatinib effects and protein kinase C (PKC) status in HMC-1^{560} and HMC-1^{560,816} cell lines. Material and Methods: Viability results were obtained by two different methods: MTT and a flow cytometry with Annexin V-FITC/PI double-staining protocol. The lipid-based transfection method was used to silence PKC. Results: Long-term PKC activation induces apoptosis in both HMC-1 cell lines. Moreover, PKC activation potentiates STI571 and dasatinib cytotoxic effects in HMC-1^{560} and HMC-1^{560,816} cells, respectively, by increasing necrotic populations. To investigate this PKC effect, the role of PKCδ, an isoform intimately related with apoptotic cell death, was studied. The results obtained evidence that either STI571 or dasatinib apoptotic cell death are PKCδ-dependent. Particularly, STI571 showed less dependence to PKCδ than dasatinib. Conclusion: PKCδ modulation is essential and determines mastocytosis treatment effectiveness, since STI571 and dasatinib effects are PKCδ-dependent.*

Allergic responses are characterized by a cascade of events that lead to an immediate hypersensitivity reaction in which mast cells (MCs) have a crucial role. MCs are derived from CD34$^+$, CD117$^+$ (c-kit$^+$) and CD13$^+$ bone marrow progenitors and release several inflammatory mediators to the bloodstream after their activation (1). MCs produce several mediators and express the high-affinity IgE receptor (FCεRI). Two different subtypes, MC_T and MC_{TC}, were described after immunohistochemical studies in human tissues, depending on the presence of tryptase (MC_T) or tryptase+chymase (MC_{TC}) simultaneously. However, this classification is controversial since recent studies have demonstrated that all MCs are able to produce chymase. Out of all the inflammatory mediators produced by MCs, histamine, prostaglandin D2 and platelet-activating factor (PAF) contribute to induce symptoms after MC activation (MCA). MCA syndrome (MCAS) is a term applied when one or various of these three criteria are present: 1) chronic or recurrent clinical signs, 2) MCs are present and 3) the symptoms respond to MC-stabilizing agents therapy or drugs against MC mediators are used (2). In this context, MCAS are sub-divided into three variants: 1) primary MCAS, with c-kit D816V$^+$-mutated clonal MCs, 2) secondary MCAS, in patients with allergy or atopic disorder without clonal MCs, 3) idiopathic MCAS, patients that fulfill the three criteria but do not have allergen-specific IgE and clonal MCs (3). Along with MCAS, two other MC disorders have been described, MC hyperplasia and mastocytosis. Firstly, MC hyperplasia is defined as an increase of tissue MCs as a consequence of different disorders like chronic infections or cancer. Secondly, (mono)clonal MCs increase is named mastocytosis and can be sub-divided into cutaneous mastocytosis (CM), systemic mastocytosis (SM) and localized MCs tumors (4).

The c-kit ligand stem cell factor (SCF), also named MC growth factor, steel factor or kit ligand, activates MCs. Nevertheless, the human mast cell line (HMC)-1 is SCF-independent. Two different HMC-1 sublines have been described: HMC-1^{560} and HMC-1^{560,816}. HMC-1 cell lines do not have FCεRI receptors in the cellular surface, unlike human tissue MCs. Either HMC-1^{560} or HMC-1^{560,816} sublines have the Val560 >Gly mutation at codon 560 in the juxtamembrane position of c-kit. This mutation implies that c-kit receptor is constitutively phosphorylated and therefore does not need SCF for its activation (5). On the other hand, the second subline has another c-kit mutation; Asp816 >Val at codon 816. Mastocytosis is characterized by c-kit mutant MCs abnormal growth and expansion (*e.g.* Asp-816 to Tyr or Asp-816 to Phe). Specifically, Asp-816 to Val$^+$ cells are present in 80% of the patients with SM. This Asp816 >Val mutation was firstly described in adult patients with SM and was defined as rare in pediatric cases. However, the presence of Asp816 >Val$^+$

Abbreviations: PKC: Protein kinase C, HMC-1: human mast cell line, TyrK: tyrosine kinase.

Correspondence to: Luis M. Botana, Dept. Farmacología, Fac. Veterinaria, 27002 Lugo, Spain. Tel/Fax: +34 982822233, e-mail: Luis.Botana@usc.es

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cells in the 42% of pediatric patients was more recently described (6). C-kit mutations are not only present in matocytosis, since are also described in Ewing's tumors, thymic and ovarian cancers, neuroblastoma or adenoid cystic carcinoma. For SM treatment, several drugs have been used, such as interferon-α (IFN-α), the nucleoside analog 2-chlorodeoxyadenosine cladribine and the group of tyrosine kinase (TyrK) inhibitors. Chronic myeloid leukemia (CML) is a disorder present in patients with SM. SM with associated clonal haematological non-mast cell lineage disease (SM-AHNMD) is the name of this SM type (7). CML was the first cancer associated with an oncogene marker, the Philadelphia chromosome, characterized by a chromosome translocation which leads to Breakpoint Cluster Region-Abelson Leukaemia (Bcr-Abl) oncogene formation. TyrK inhibitors have been widely used in SM-AHNMD treatment, since most of them inhibit other Src family kinases as well as c-kit receptor (8). Imatinib (Signal Tranduction Inhibitor (STI) 571 or Gleevec®) was the first TyrK inhibitor used. This drug has activity against several oncogenic TyrKs: Bcr-Abl, c-kit, platelet-derived growth factor receptor, discoidin domain receptor and colony-stimulating factor receptor-1. Imatinib resistances emergence and its inefficiency against HMC-$1^{560,816}$ cell line gave birth to the second generation of TyrK inhibitors, including compounds such as dasatinib (BMS354825), nilotinib (AMNI07), midostaurin (PKC412) and bosutinib (SKI-606) (9). The multikinase inhibitor dasatinib is able to inhibit Bcr-Abl, c-kit, PDGRF and ephrin receptor kinase (10). Interestingly, it is a good choice to induce cell death in HMC-$1^{560,816}$ cells, unlike imatinib. Also, dasatinib+midostaurin combination shows a synergistic effect indicating that different TyrK inhibitors co-treatment is an usable tool for SM and CML treatment (11).

PKC plays a primordial role on MCs activation and degranulation, therefore, the PKC effect in mastocytosis has been described in several studies (12,13). PKC is a family of serine/threonine kinases with different isoforms divided into three classes depending on their sensitivity to Ca^{2+} and phorbol esters: (1) Ca^{2+}-dependent isozymes (or cPKCs); α, β_1, β_2 and γ that are activated by diacylglicerol (DAG) or 12-O-tetradecanoylphorbol-13-acetate (PMA); (2) Ca^{2+}-independent isozymes (or novel PKCs); δ, ε, η, μ and θ, activated by PMA; and (3) atypical isozymes (ζ and ι/λ), which are PMA and Ca^{2+}-independent. PKC activation has been described as an essential signal for MC exocytosis (14). Src TyrKs family activation is considered as a proximal event for MC activation and its relationship with PKC isoforms has been widely studied. Src belongs to a 11-member family, including Lyn. Specifically, Syk, Btk, Lyn and Fyn are proteins related with exocytosis granules (15). Hence, in order to determine receptor-proximal TyrKs role on MCs secretory granules, gene-inactivated mice were used. Out of all, Lyn is the only protein that is not essential for MC exocytosis, whereas exocytosis is abrogated in Fyn, Syk and Btk-deficient MCs. Interestingly, PKCα and PKCβ II activation is increased upon FcεRI stimulation in $lyn^{-/-}$ MCs (16). Also, cell degranulation is restored by PKCβ and Ca^{2+} in RBL-2H3 cells, together with PKCβ membrane translocation. In addition, IL-6 production and degranulation are inhibited in PKCβ-deficient MCs (17). PKCδ is another important isoform related with MC exocytosis regulation. Specifically, SHIP/Shc/PKCδ complex is essential to regulate MC exocytosis, therefore, possible molecules that interact with SHIP, like PKCδ, might modulate MC degranulation (18).

As it has been shown, either PKC or TyrK inhibitors take part on intracellular pathways in which Src proteins are involved. PKC and TyrK proteins relationship has been described in several studies since PKC and TyrK inhibition is an useful tool against aberrant MCs (19). In this sense, TyrK and PKC inhibitors combination is a highly effective therapy in mastocytosis patients. Among all TyrK inhibitors that have cytotoxic effect against HMC-1 cell line, STI571 and dasatinib are two of the most potent studied (20, 21). Moreover, long-term PKC activation (PMA treatment) induces cell death in several cell lines (22, 23). Also, PKC potentiates TyrK inhibitors dasatinib and nilotinib effect in HMC-1 cell line (19). Therefore, since PKC inhibition effect over TyrK inhibitors cytotoxicities has been already described, the purpose of this study is to clarify the consequences of PKC modulation, this time through its activation.

Materials and Methods

Chemicals. STI571 was provided by Dr. Luis Escribano Mora (Centro de Estudios de Mastocitosis de Castilla la Mancha, Hospital Virgen del Carmen, Toledo, Spain). Dasatinib (sc-358114), negative siRNA control (sc-37007) and PKCδ siRNA (sc-36253) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PMA and bovine serum albumin (BSA) were from Sigma-Aldrich (Madrid, Spain). Phosphate buffered saline (PBS) was from Invitrogen (Barcelona, Spain). Anti Mouse IgG was purchased from GE Healthcare (Barcelona, Spain). Anti β-actin and polyvinylidene flouride (PVDF) membrane were from Millipore (Temecula, CA, USA). Anti PKCδ was from BD Biosciences (Madrid, Spain). Cell Lab ApoScreen™ Annexin V and DNA Prep™ Stain were from Beckman Coulter (Fullerton, CA, USA). Polyacrylamide gels and molecular weight marker Precision Plus Protein™ Standards Kaleidoscope™ were from BioRad (Barcelona, Spain). GeneSilencer® was from Genlantis (San Diego, CA, USA).

Cell cultures. HMC-1^{560} cells were kindly provided by Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and HMC-$1^{560,816}$ cells were kindly provided by Dr. Luis Escribano Mora with permission from Dr. J. Butterfield. They were maintained at 37˚C in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Invitrogen, Spain) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Spain) and 100 IU/ml penicillin + 100 μg/ml streptomycin (Gibco, Invitrogen, Spain) in an atmosphere containing 5% CO_2. The medium was re-newed once a week.

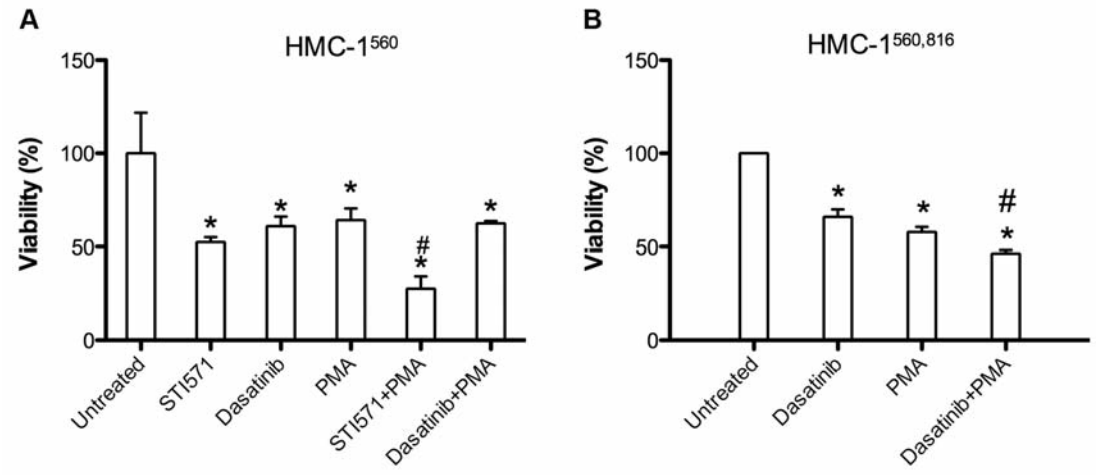

Figure 1. *Effect of tyrosine kinase inhibitors, STI571 and dasatinib, and PKC activation on cell viability of the HMC-1 cell line. (A) HMC-1^{560} cells and (B) HMC-1^{560,816} cells. Cells were incubated for 48 h with 25 nM STI571, 0.012 μM (HMC-1^{560}) and 0.6 μM (HMC-1^{560,816}) dasatinib and 100 ng/ml PMA at 37˚C. Mean±SEM of three experiments. *Significant differences between untreated and treated cells. (A) #significant differences between STI571- and STI571+PMA-treated cells. (B) #Significant differences between dasatinib- and dasatinib+PMA-treated cells.*

MTT assay. HMC-1^{560} and HMC-1^{560,816} cell lines were incubated with STI571, dasatinib and PMA for 48 h at 37˚C. Then cells were washed with saline solution and incubated with MTT (250 μg/ml) for 30 min. After washing with saline solution cells were re-suspended in 200 μl of water and sonicated for 1 min. Absorbance was determined in a Bio-Tek Synergy 4 plate reader at wave length of 595 nm.

Apoptotic and necrotic cell death determination by flow cytometry. Apoptosis was detected by Annexin-V-FITC/PI staining using the Cell Lab ApoScreen™ Annexin V kit. HMC-1^{560} and HMC-1^{560,816} cells were incubated with STI571, dasatinib and PMA for 48 h (37˚C). Then cells were prepared exactly following the manufacturer's instructions and an Amnis Corporation IS-100 flow cytometer was used.

Transfection by lipid-based method. GeneSilencer® (Genlantis) was composed by the transfection reagent and the diluent. The manufacturer's instructions were followed carefully. On the day of transfection two solutions were prepared. Solution A, composed by diluent, FBS/antibiotic –free IMDM medium and PKCδ siRNA. Control siRNA (sc-37007) was used as negative control for evaluating RNAi off-target effects. Solution B was composed by transfection reagent diluted in FBS/antibiotic-free IMDM medium. Solutions A and B were mixed and incubated for 5 min at room temperature. HMC-1^{560} cells were incubated in a total volume of 500 μl in a FBS/antibiotic-free IMDM medium. Cellular concentration was 2×10^6/ml. 500 μl of IMDM (supplemented with 20% FBS and Penicillin/Streptomicyn 2×) were added to HMC-1^{560} cells after 5 h transfection. 19 h later cells were incubated with STI571, dasatinib and PMA. HMC-1 cells were incubated with the different compounds for 48 h at 37˚C.

Western blotting. Cells were re-suspended in 80 μl lysis buffer with the follow composition: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1%Triton X-100, 1× complete protease inhibitor (Roche, Madrid (Madrid) Spain) and 1x phosphatase inhibitor cocktail (Roche, Madrid (Madrid) Spain). Protein concentration determination was carried out by using Bradford assay and BSA as protein standard. For separating proteins according to their molecular weight sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure was used. Proteins were transferred to a PVDF membrane which was blocked with 0.5% BSA and then it was incubated for 10 min with anti PKCδ (1:1,000). After two washes with washing buffer (PBS+0.1%Tween), the membrane was incubated for 10 min with the secondary antibody anti-Mouse IgG conjugated with horseradish peroxidase. A chemiluminescence detection kit (SuperSignal West Femto; Pierce, Rockford, IL, USA) was used to determine protein expression levels. Relative protein expression was calculated in relation to β-actin (0.3:1,000).

Statistical analysis. Results were analyzed using the Student's *t*-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean±SEM.

Results

PKC activation effect over STI571 and dasatinib cytoxicities in HMC-1^{560} y HMC-1^{560,816} cell lines. As was previously described 25 nM STI571 induces 50% cell death in HMC-1^{560} cell line whereas the IC_{50} for HMC-1^{560} and HMC-1^{560,816} are 0.012 μM and 0.6 μM dasatinib, respectively. Cells were also incubated with PMA and STI571 or dasatinib simultaneously in order to determine the long-time PKC activation effect over STI571 and dasatinib cytotoxicities. First, in HMC-1^{560} cells, a decrease of 50% and 40% on cell viability is induced by 25 nM STI571 and 0.012 μM dasatinib, respectively (Figure 1A). Also, PKC activation significantly decreased cell viability (36%), whereas PMA and STI571 co-incubation caused a higher decrease (73%) than that observed with STI571-alone. However, PMA does not potentiate the effect of dasatinib alone. In HMC-1^{560,816} cells, 0.6 μM dasatinib and PKC

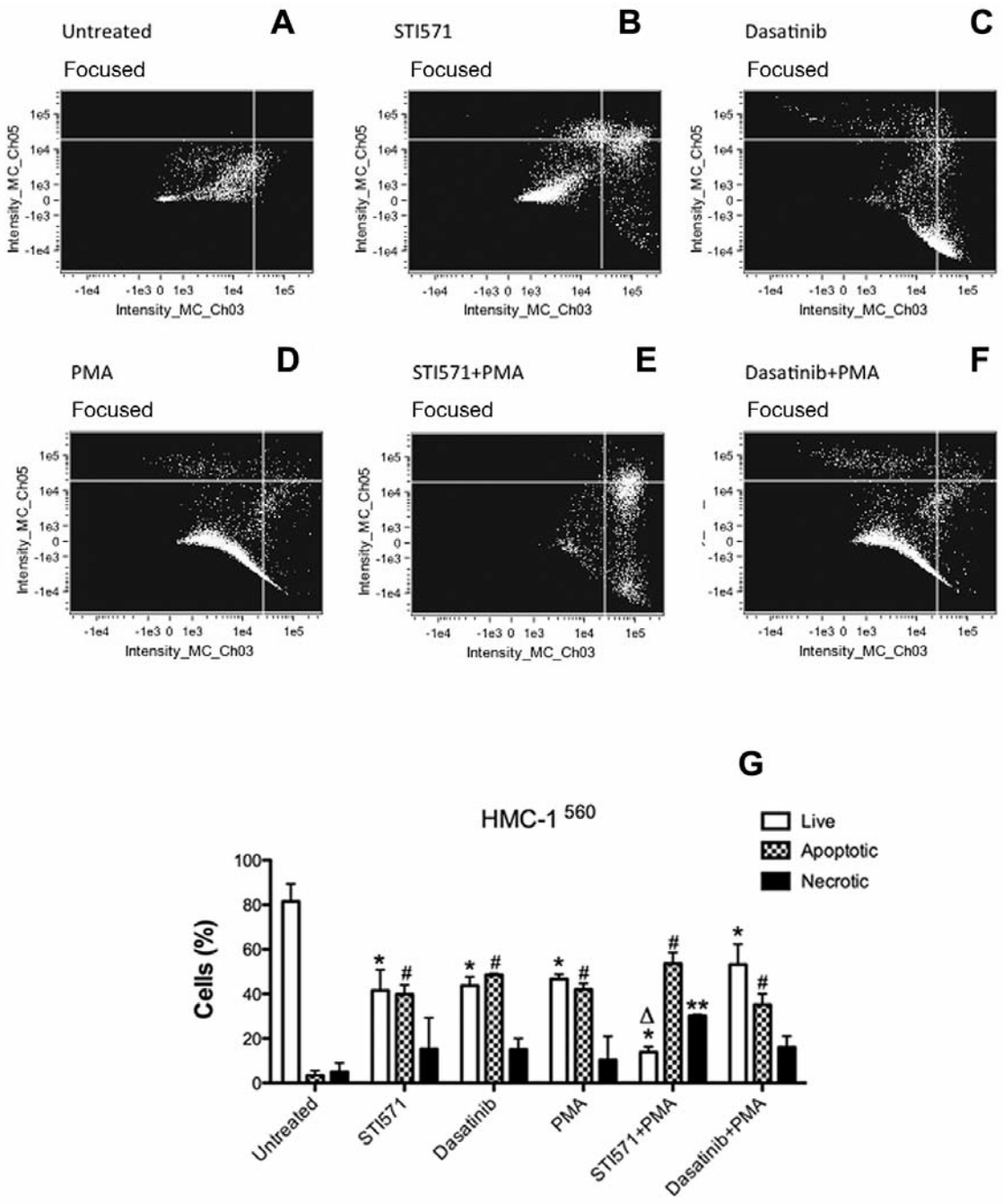

Figure 2. *Effect of STI571, dasatinib and PMA treatments on population distribution (live, apoptotic and necrotic) in the HMC-1^{560} cell line. HMC-1^{560} cells were incubated for 48 h with of 25 nM STI571, 0.012 μM dasatinib and 100 ng/ml PMA at 37˚C. Live (botton left panel), apoptotic (botton right panel) and late apoptotic/necrotic cells (upper panel) were detected by Annexin V-FITC/PI staining. (A), (B), (C), (D), (E) and (F) represent one representative experiment of untreated, STI571, dasatinib, PMA, STI571+PMA and dasatinib+PMA treatments, respectively. (G) mean±SEM of the three experiments. *significant differences in live populations between the treatments. #significant differences in apoptotic populations between the treatments. **significant differences in necrotic populations between the treatments. Δsignificant differences in live population between STI571- and STI571+PMA-treated cells.*

activation cause a similar decrease on cell viability, 40 and 43%, respectively. Moreover, a higher decrease (54%) is observed after dasatinib+PMA simultaneous addition.

PKC activation-induced cell death in HMC-1 cell lines. Results presented in Figures 1A and 1B demonstrate that PMA has a potent cytotoxic effect against both MC lines,

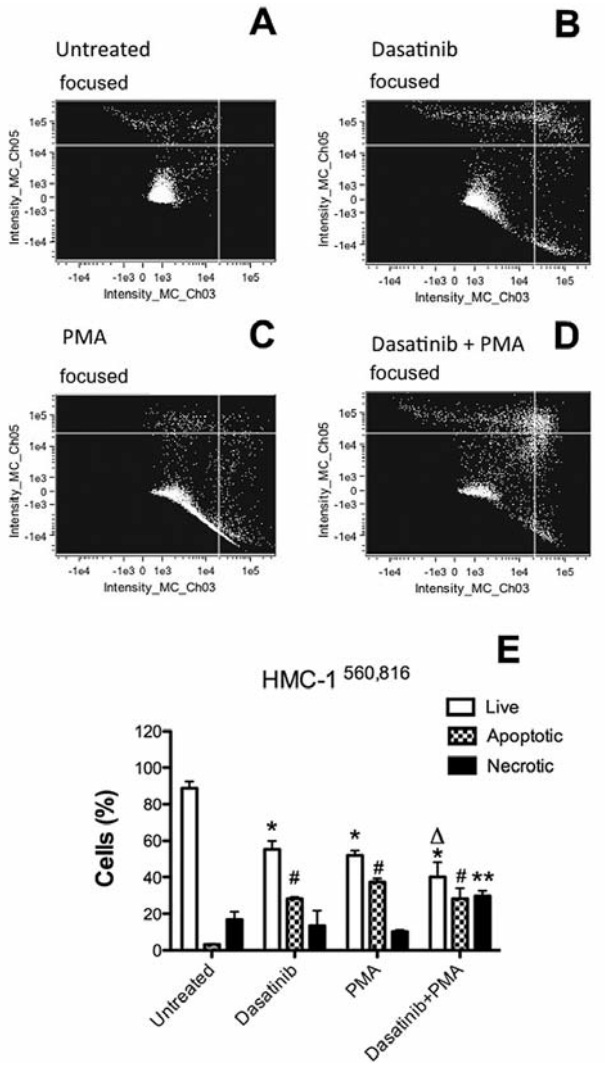

Figure 3. *Effect of dasatinib and PMA treatments on population distribution (live, apoptotic and necrotic) in HMC-1^{560,816} cell line. HMC-1^{560,816} cells were incubated for 48 h in the presence 0.6 μM dasatinib and 100 ng/ml PMA at 37˚C. Live (botton left panel), apoptotic (botton right panel) and late apoptotic/necrotic cells (upper panel) were detected by Annexin V-FITC/PI staining. (A), (B), (C) and (D) represent one representative experiment of untreated, dasatinib, PMA and dasatinib+PMA treatments, respectively. (E) mean±SEM of the three experiments. *significant differences in live populations between the treatments. #significant differences in apoptotic populations between the treatments. **significant differences in necrotic populations between the treatments. Δsignificant differences in live population between dasatinib- and dasatinib+PMA-treated cells.*

however, the pathway followed by this kinase, apoptotic or necrotic, remains undefined. In this sense, Annexin V-FITC /PI technique was next employed to determine the distribution of the three cellular populations: live, apoptotic and necrotic in both HMC-1 cell lines. For HMC-1^{560} cell line, Figures 2A to 2F represent the intensity of Annexin V-FITC (X axis) and PI (Y axis) at different conditions and Figure 2G shows the results of three experiments. The percentage of live cells is 81% in untreated cells, whereas apoptotic and necrotic populations represent 5 and 14%, respectively. A significant decrease (from 81 to 54%) on live population is observed after STI571 treatment, accompanied with an increase on apoptotic population (from 5 to 36%). Nevertheless, the necrotic population is not modified. Moreover, a significant decrease on live population (81 to 43%) after dasatinib treatment is perceived. Dasatinib also increases the percentage of apoptotic cells (48%) whereas the necrotic population slightly increases from 5 to 15%. Besides, long-term PKC activation has a similar effect than the one induced by STI571 and dasatinib. PMA induces apoptotic cell death (41% of apoptotic cells) and does not modify necrotic population. Drug combinations tested, STI571+PMA and dasatinib+PMA, decrease cell viability inducing apoptosis (53% and 32% of apoptotic cells for STI571+PMA and dasatinib+PMA treatments, respectively). Moreover, STI571+PMA induces an increase on necrotic cell death (30%), not observed after dasatinib+PMA treatment. In the HMC-1^{560,816} cell line, the distribution of different populations is represented in Figures 3A (untreated), 3B (dasatinib), 3C (PMA) and 3D (dasatinib+PMA), while Figure 3E shows the mean of three experiments. As Figure 3E shows, dasatinib and PKC activation cause an increase of apoptotic cells (25% and 34%, respectively) without modifying necrotic population. Surprisingly, dasatinib+PMA combination induces an acute decrease on live cells, matched by a significant increase of apoptotic and necrotic cells.

PKC activation effect over STI571 and dasatinib effectiveness in PKCδ-silenced HMC- 1 cells. The results described confirm that PKC activation potentiates TyrK inhibitors effect on cell viability either in HMC-1^{560} or HMC-1^{560,816} cell line. It was reported that PKC is related with apoptosis in several cell lines. Specifically, Ca^{2+}-independent isoform PKCδ is associated with apoptotic pathway. Therefore, live, apoptotic and necrotic populations determination was next carried-out in PKCδ-silenced HMC-1 cells. The effectiveness of lipid-based transfection method is determined in both MC lines (Figure 4). In HMC-1^{560} cells, cytosolic PKCδ levels significantly diminish after 24 h (25% of decrease), 36 h (37%) and 48 h (50%) (Figures 4A and 4C). A similar result is obtained in HMC-1^{560,816} cells, since cytosolic PKCδ expression decreases 29%, 55% and 62% after 24, 36 and 48 h respectively (Figures 4B and 4D). PKC activation effect over STI571 and dasatinib mechanisms of action was next evaluated (Figure 5). As Figure 5A shows, PKCδ silencing partially blocks STI571 cytotoxic effect since cell viability rises from 52 to 70%, even though STI571-induced viability decrease is still significant in HMC-1^{560} cells. This effect only

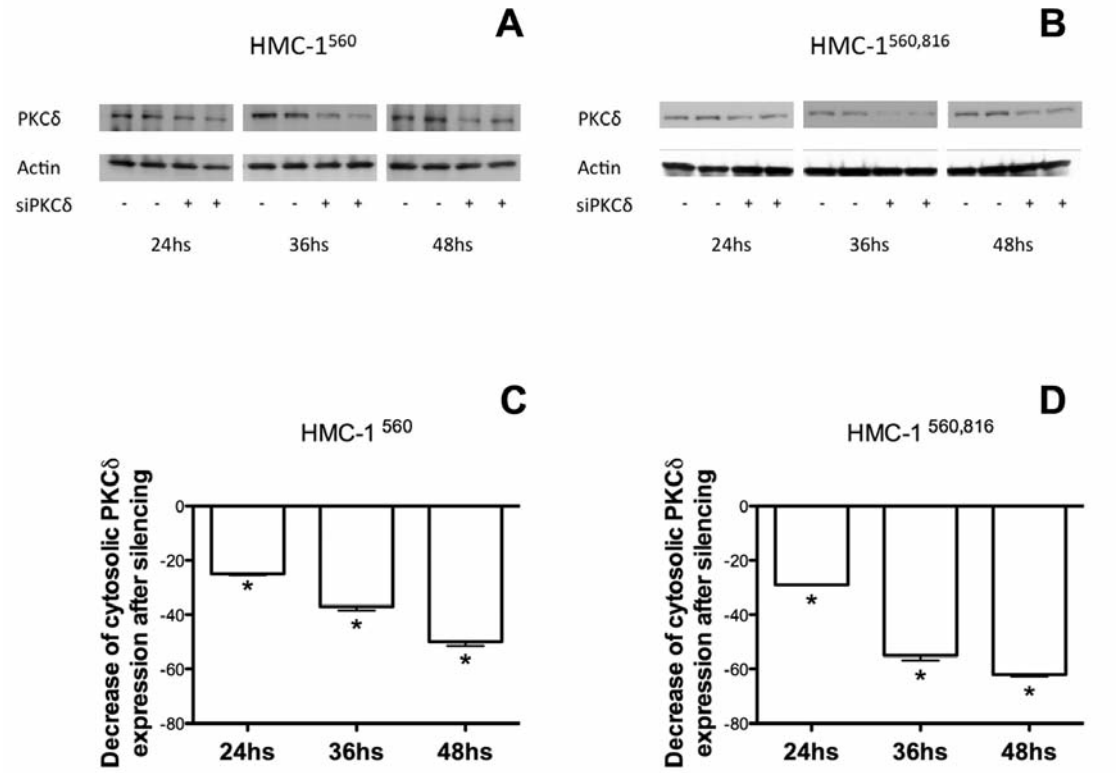

Figure 4. *Determination of PKCδ silencing efficiency by western blott analysis in HMC-1 mast cell lines. PKCδ siRNA was incubated for 24, 36 and 48 h in HMC-1^{560} and HMC-1^{560,816} cell lines. (A) and (B) show a representative image of each condition in HMC-1^{560} and HMC-1^{560,816} cell line, respectively. (C) and (D) is the mean±SEM of three experiments in HMC-1^{560} and HMC-1^{560,816} cell line, respectively. Cytosolic PKCδ values were calculated respect to actin band intensity. *significant differences between non-silenced and PKCδ-silenced cells.*

happens after STI571 treatment, since cell viability is not modified under the remaining treatments. In HMC-1^{560,816} cells, PKCδ-silencing avoids dasatinib, PMA and dasatinib+PMA-induced cytotoxicities (Figure 5B). Once observed that STI571, dasatinib and PMA cytotoxic effects are PKCδ-dependent. Next, the determination of live, apoptotic and necrotic populations in HMC-1-PKCδ silenced cells is carried-out in order to clarify this PKCδ reliance. In HMC-1^{560} cells, PKCδ silencing completely blocks cytotoxic effects of all treatments tested (Figures 6C, 6D, 6E and 6F) except for STI571 treatment (Figure 6B), since apoptotic population suffers a slight increase despite PKCδ silencing (Figure 6G). For HMC-1^{560,816} cells, no modification is observed after dasatinib (Figure 7B), PMA (Figure 7C) and dasatinib+PMA (Figure 7D) treatments. Hence, the increase of necrotic cells previously observed in dasatinb+PMA-treated cells (Figure 3E) is abolished with PKCδ silencing.

Therefore, in addition to the apoptotic effect observed after STI571 and dasatinib in HMC-1^{560} and HMC-1^{560,816} cells, respectively, it can be concluded that PKCδ activation stimulates necrosis when TyrKs are inhibited, increasing TyrK inhibitors cytotoxicities.

Discussion

One of the most important groups utilised in SM treatment is the TyrK inhibitors. Their effectiveness against HMC-1 cell line and bone marrow cells has been widely described (9). Either STI571 or dasatinib are characterized by their cytotoxic effect against cells that have the bcr-abl oncoprotein in a constitutively activated state, but other TyrK receptors, such as c-kit, are also an important target for both compounds. The presence of Asp-816 to Val mutation avoids STI571 cytotoxic effect by interfering with drug binding in HMC-1^{560,816} cells, however, this blockage does not take place with Val-560 to Gly activating mutation (24). On the other hand, Asp-816 >Val mutation does not affect dasatinib mechanism of action and consequently cell death is observed after dasatinib treatment. Results shown herein indicate that TyrK inhibitor STI571 induces apoptotic cell death in HMC-1^{560} cells. These results are in accordance with those obtained in other cell lines (human colon adenocarcinoma cells and CML cell lines bcr/abl positive), in which STI571 increases caspase-3 activity, an indicator of apoptosis activation. Moreover, results presented indicate that, as for STI571, dasatinib also induces apoptosis

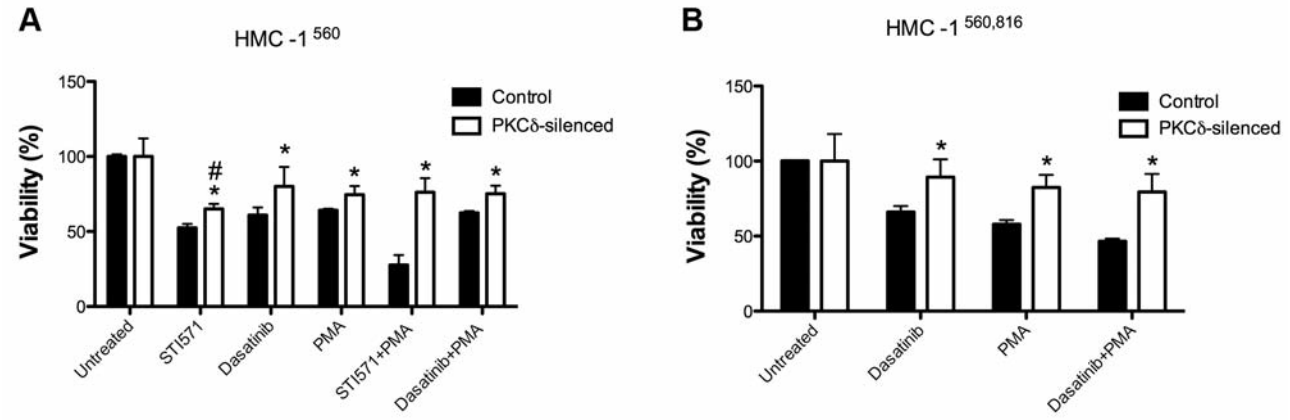

Figure 5. *Effect of tyrosine kinase inhibitors, STI571 and dasatinib, and PKC activation on cell viability in PKCδ-silenced HMC-1^{560} and HMC-1^{560,816} cell lines. Cells were incubated for 48 h with STI571, dasatinib and PMA at 37˚C. (A) and (B) results obtained in HMC-1^{560} and HMC-1^{560,816} cell lines, respectively. Mean±SEM of three experiments. *Significant differences between no silenced and PKCδ-silenced cells for the different treatments. #Significant differences between untreated and STI571 PKCδ-silenced cells.*

in HMC-1^{560} and HMC-1^{560,816} cell lines, in agreement with the results previously obtained for HMC-1^{560,816} cells (25). STI571 and dasatinib apoptotic activities are PKCδ-dependent, since PKCδ cytosolic levels decrease leads to TyrK inhibitors effect disappearance. It is important to note that dasatinib presents a higher PKCδ dependence than STI571. In this sense, in spite of having silenced the PKCδ isoform, an increase of apoptotic population is observed after STI571 treatment in HMC-1^{560} cells, unlike to that observed after dasatinib treatment. This fact might be related with dasatinib targets (Btk and Lyn proteins), since Lyn is a protein related to PKCδ isoform and both take part in degranulation pathway regulated by Src homology 2 domain-containing inositol-5'-phophatase (SHIP). In this regard, PKCδ and Lyn relationship has been widely described in antigen-induced MC degranulation (26). Therefore, considering the strong dependence of dasatinib mechanism of action by PKCδ, this isoform may be also considered an important target for this compound.

Results obtained demonstrate that apoptotic cell death induced by PKC activation is completely PKCδ-dependent in HMC-1^{560} and HMC-1^{560,816} cells. Phorbol esters role over cell death is controversial, since their effect is usually described as cytoprotective after a short incubation (27), since inhibit apoptosis in thymocytes, T lymphocytes and chronic lymphocytic leukemia B-cells (28), Jurkat leukemic T-cells (29) and nerve cells. In this case, phorbol esters neuroprotection occurs through an extensive phosphorylation pathway in which are involved extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK) and finally PKCδ (30). On the other hand, phorbol esters can also have an opposite role in several cell lines, such as human bronchial epithelial cells. Specifically, PKCδ is related with cell death caused by the carcinogen asbestos in these cells. Also, asbestos-induced cell death is described as PKCδ–dependent and nuclear PKCδ translocation takes place after its activation (23). Hence, this means that PKC activation can protect cells against cytotoxic agents or conversely to induce or potentiate cell death. In our case, PKC activation has an evident apoptotic effect over both HMC-1 cell lines. It is important to note that PKCδ activity is not exclusively related with apoptotic cell death, as it was widely described in the present study, but also with necrotic pathway (31). In fact, this PKCδ dual role has been previously described as dependent on the localization of the protein and the presence of pro- or antiapoptotic mediators (32).

PKC is a protein intimately related with MC activation, adhesion and migration (14, 33). MCs activation starts after antigen aggregation to FCεRI and is enhanced by SCF-c-kit binding. Also, Btk is the responsible of up regulating MC activation through FCεRI pathway. Specifically, Btk and Lyn have been described as the most important targets for dasatinib in neoplastic MCs and TyrK inhibitors were defined as potent modulators of MC degranulation (34, 35). Dasatinib inhibits human basophils IgE-dependent histamine release (35), while STI571 induces a decrease on histamine release in both HMC-1^{560} and HMC-1^{560,816} cell lines (36, 37). Therefore, either TyrK inhibition or PKC activation may regulate MC activation. Surprisingly, the effect obtained after TyrK and PKC pathways modulation is completely different, depending on the compound (STI571 or dasatinib). PKC activation induces a enhancer STI571 cytotoxic effect that implies a higher necrotic effect on HMC-1^{560} cells. However, long-term PMA incubation does not provoke any modification after dasatinib treatment, thus, both compounds might be sharing a similar pathway,

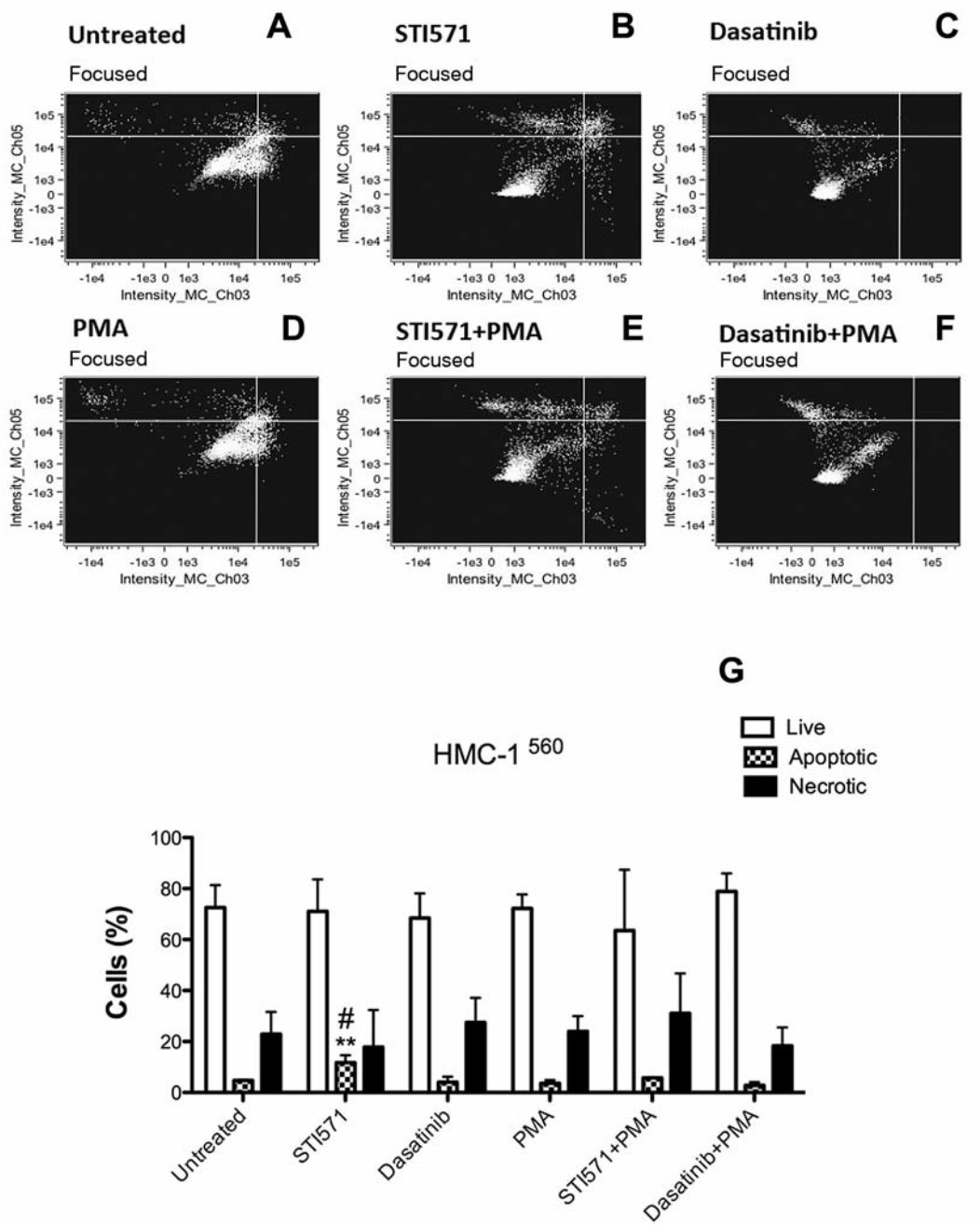

Figure 6. *Effect of 25 nM STI571, 0.012 μM dasatinib and 100 ng/ml PMA treatment in PKCδ–silenced HMC-1^{560} cells viability. HMC-1^{560} cells were incubated for 48 h at 37˚C with the different compounds. Live (botton left panel), apoptotic (botton right panel) and late apoptotic/necrotic cells (upper panel) were detected by Annexin V-FITC/PI staining. (A), (B), (C), (D), (E) and (F) represent one representative experiment of untreated, STI571, dasatinib, PMA, STI571+PMA and dasatinib+PMA treatments, respectively. (G) mean±SEM of the three experiments. **Significant differences between untreated and STI571-treated cells on apoptotic population.*

probably through PKCδ. In HMC-1^{560,816} cells, PKC activation and dasatinib cytotoxicities are additive, and as for STI571 in HMC-1^{560} cells, this fact is accompanied by a necrotic population increase. Interestingly, previous results obtained in our laboratory demonstrate that STI571 (in HMC-1^{560}) and dasatinib (in HMC-1^{560,816}) treatments lead to nuclear PKCδ translocation. Therefore, this PKCδ translocation might be related with a final extreme damage ending in necrotic cell death (present at 25% of the cells). In addition to PKC activation, the inhibition of this protein has

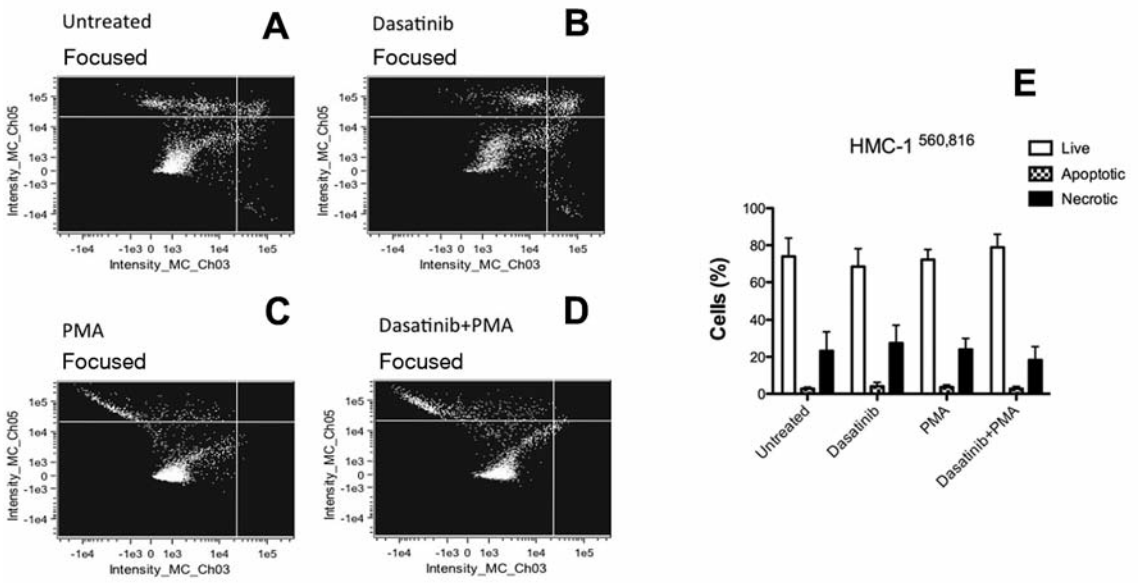

Figure 7. *Effect of 0.012 μM dasatinib and 100 ng/ml PMA treatments in PKCδ–silenced HMC-1^{560,816} cells viability. HMC-1^{560,816} cells were incubated for 48 h at 37˚C with the different compounds. Live (botton left panel), apoptotic (botton right panel) and late apoptotic/necrotic cells (upper panel) were detected by Annexin V-FITC/PI staining. (A), (B), (C) and (D) represent one representative experiment of untreated, dasatinib, PMA and dasatinib+PMA treatments, respectively. (E) mean±SEM of the three experiments.*

been also described as a potential target to induce cell death in multiple myeloma cells. In this regard, it is necessary to clarify that PKC inhibitors were previously described as an anti-cancer drugs. Specifically, midostaurin, which inhibits Ca^{2+}-dependent PKCs as well as acts as a TyrK inhibitor, induces an effectively HMC-1 cell death (19). Interestingly, a synergistic effect of midostaurin and dasatinib has been found in HMC-1 cell line (11), therefore a crosstalk between PKC regulation and TyrK inhibition might be taking place. In summary, results obtained in this study demonstrate that long-time PKC activation can be used as a potential tool in SM treatment, alone or in combination with TyrK inhibition.

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