NADPH oxidases as therapeutic targets in chronic myeloid leukemia

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Running Title: NADPH oxidase inhibitors and BCR-ABL inhibitors synergize
ABSTRACT

Purpose: Cancer cells show higher levels of reactive oxygen species (ROS) than normal cells and increasing intracellular ROS levels is becoming a recognized strategy against tumor cells. Thus, diminishing ROS levels could be also detrimental to cancer cells. We surmise that avoiding ROS generation would be a better option than quenching ROS with antioxidants. Chronic myeloid leukemia (CML) is triggered by the expression of BCR-ABL kinase, whose activity leads to increased ROS production, partly through NADPH oxidases. Here we assessed NADPH oxidases as therapeutic targets in CML.

Experimental design: We have analyzed the effect of different NADPH oxidase inhibitors, either alone or in combination with BCR-ABL inhibitors, in CML cells and in two different animal models for CML.

Results: NADPH oxidase inhibition dramatically impaired the proliferation and viability of BCR-ABL-expressing cells due to the attenuation of BCR-ABL signaling and a pronounced cell-cycle arrest. Moreover, the combination of NADPH oxidase inhibitors with BCR-ABL inhibitors was highly synergistic. Two different animal models underscore the effectiveness of NADPH oxidase inhibitors and their combination with BCR-ABL inhibitors for CML targeting in vivo.

Conclusion: Our results offer further therapeutic opportunities for CML, by targeting NADPH oxidases. In the future it would be worthwhile conducting further experiments to ascertain the feasibility of translating such therapies to clinical practice.

Key words: NADPH oxidase, Reactive oxygen species (ROS), BCR-ABL inhibitors
TRANSLATIONAL RELEVANCE

BCR-ABL inhibitors are indicated for the front line treatment of CML leading to excellent therapeutic results. However, primary or secondary resistances to these treatments might occur. Therefore alternative or complementary therapeutic approaches are desirable. Inhibition of NADPH oxidases hampered BCR-ABL signaling and induced a pronounced cell-cycle arrest. Moreover, the combination NADPH oxidase inhibitors with BCR-ABL inhibitors was seen to be highly synergistic. Therefore, our results show that targeting NADPH oxidases offers novel therapeutic opportunities against CML, either alone or in combination with traditional BCR-ABL inhibitors. This could be a good strategy for either preventing the appearance of resistances to BCR-ABL inhibitors, or for consideration as an alternative in the event of such resistances already existing. Moreover, we provide evidence showing that this strategy could also be applied to other kinase-driven cancers such as Acute Myeloid Leukemia (AML).
INTRODUCTION

Chronic myeloid leukemia (CML) is characterized by the t(9;22) q(34; q11) translocation encoding the BCR-ABL oncoprotein, which triggers the chronic phase of the disease (1). BCR-ABL inhibitors are indicated for the front line treatment of CML and lead to excellent therapeutic results (2). However, primary or secondary resistances to these treatments arising from drug-resistance often resulting from the emergence of mutant forms of BCR-ABL, increasing a risk of progression to blast crisis (3, 4).

Excessive production of reactive oxygen species (ROS) has traditionally been associated with aging and degenerative diseases (5), but it is now becoming accepted that a moderate degree of ROS production, the so-called “redox signaling”, is physiologically important (6). Cancer cells have higher levels of ROS than healthy cells (7, 8), suggesting that ROS could be important for the initiation and progression of cancer (9, 10), including leukemia (11). BCR-ABL-expressing cells also show high levels of ROS, which may contribute to cell transformation (12), cell growth (13), resistance to apoptosis (14) or increased DNA damage (15-17). The excess of ROS in BCR-ABL-expressing cells could come from mitochondria (12), enhanced glucose uptake (18), or the activity of NADPH oxidases (14). With the exception of mitochondria, NADPH oxidases are the main source of cellular ROS (19). This family consists of seven members (Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2). Each NADPH oxidase seems to have a particular pattern of expression, and most cells express different members of this family (20). The importance of these enzymes in cancer is beginning to be recognized (21) and, interestingly, it has recently been shown that NADPH oxidase inhibitors are efficient at preventing the growth of colon cancer xenografts (22). This suggests that NADPH oxidases could be exploited as therapeutic targets in the cancer treatment setting.

With the foregoing in mind, we were interested in evaluating the potential of NADPH oxidases as therapeutic targets in CML, either alone or in combination with BCR-ABL inhibitors. We show that the inhibition of NADPH oxidases dramatically impairs the proliferation and viability of BCR-
ABL-expressing cells. Moreover, we observed a strong synergistic effect when NADPH oxidases and BCR-ABL inhibitors were combined. Two different animal models were studied and were seen to reflect the effectiveness of NADPH oxidase inhibitors and their combination with BCR-ABL inhibitors for CML targeting *in vivo*. Similar results were found in AML, and hence we suggest that this strategy could also be applied to other kinase-driven cancers.
METHODS

Cell cultures

Bone marrow mononuclear cells (BM-MNC) and CD34⁺ cells were isolated from patients and healthy donors as described (23). CD33⁺ and CD15⁺ cells were purified with a FACSAria III cell sorter. Cell purity was above 93% (Supplementary Figure 6B). K562, a human cell line derived from a patient with chronic myeloid leukemia was obtained from the ATCC (CCL 243). Boff 210 cells express p210 BCR-ABL constitutively and derive from the BaF/3murine hematopoietic cell line, as previously described (25). Authentication of BM-MNC was performed analyzing BCR-ABL expression by PCR, or BCR-ABL protein expression in K562 and Boff 210 cells by immunoblotting, every 3-4 months. Cell lines were grown in 10% FBS-supplemented medium plus 100 U/mL penicillin, 100 U/ml streptomycin and 2 mM L-glutamine. K562 and MOLM-13 cells were grown in RPMI, Boff 210 cells in DMEM and MV4-11 cells in IMDM. Proliferation was analyzed by the MTT assay as described (24). The colony-forming ability of K562 cells and patient BM cells was analyzed by culturing in HSC-CFU methylcellulose medium. 1000 K562 cells or 5000 patient BM-MNC were seeded per well in 0.5 ml of medium. Cells were grown at 37°C and 5% CO₂ in an incubator and colonies were counted 14 days later.

Analysis of drug interactions

Drug interactions were analyzed according to the Chou and Talalay median-effect method (26), using CalcuSyn Software (Biosoft, Cambridge, UK). The combination index (CI) < 1, CI = 1 or CI > 1 represent synergistic, additive or antagonistic effects respectively.

Intracellular ROS levels

ROS were measured using 2’,7’-dichlorofluorescein diacetate (DCFDA) as before (24).

Immunoblotting
Proteins cell extracts and immunoblotting was performed as reported previously (24). Quantification of bands was performed by densitometry with a CS-9000 Shimadzu equipment. Quantification is shown below the corresponding blots.

**Cell Cycle analysis and viability**

Unsynchronised K562 cells were fixed in 70% ethanol and incubated with propidium iodide (PI) (50μg/mL) in the presence of RNase (50μg/mL). Cells were acquired with a Becton-Dickinson, FACSCalibur cytometer using CellQuest software (BD Biosciences). Cell viability was determined by staining with annexin V-PE/7-AAD, as described previously (24).

**Animals**

Female CB17-SCID mice were purchased from Charles River Laboratory (Barcelona, Spain). Transgenic mice expressing p210 BCR-ABL in HSCs (BCR-ABL<sup>tg/tg</sup>) (27) were obtained from the RIKEN Bioresource Center, Japan. Animals were kept in specific pathogen-free animal facilities and monitored daily. The K562 cell xenograft tumor model in CB17-SCID mice was established by subcutaneous injection of 10x10<sup>6</sup> cells in 100 μL of RPMI-1640 medium and 100 μL of Matrigel. When the tumors became palpable, the mice were randomized to the control group (receiving the vehicle alone) or the different treatment groups and administered for 22 days with either, 1mg/Kg DPI (i.p.), 100 mg/Kg imatinib (orally) or combinations of these drugs. The same treatment was applied over two weeks for young male or female BCR-ABL<sup>tg/tg</sup> transgenic mice with high levels of neutrophils (50%). The effectiveness of the treatment was followed by analyzing the percentage of granulocytes (Gr1<sup>+</sup>) in peripheral blood (PB). Animal experiments were approved by the Bioethics Committee of the University of Salamanca, according to EU and national laws.

**Quantitative RT-PCR and RNA interference**

NADPH oxidases expression levels were analyzed by quantitative RT-PCR (RT-qPCR). Oligonucleotides sequences and technique details are available in Supplementary Information. Nox2, Nox5 and Duox2 expression was downregulated in K562 cells with specific shRNA
(sequences available in Supplementary Information) by lentiviral infection as described (24). An oligonucleotide against firefly luciferase was used as a control (28).

**Statistical analysis**

Data are shown as means ± standard error of the mean. Student's *t*-test, or the Mann-Whitney U test, in the case of the animal models, were used to analyze statistical significance: ### *p* < 0.001, ## *p* < 0.01, # *p* < 0.05 when compared to control cells; *** *p* < 0.001, ** *p* < 0.01, * *p* < 0.05 when compared to kinase inhibitor-treated cells, and +++ *p* < 0.001, + *p* < 0.01, + *p* < 0.05 when compared with NADPH oxidase inhibitor-treated cells.
RESULTS

The combination of NADPH oxidases inhibitors and BCR-ABL inhibitors hampers the proliferation of CML cells in a synergistic manner

Bearing in mind that NADPH oxidases contribute to BCR-ABL-mediated ROS production (14), we were prompted to evaluate these enzymes as molecular targets for CML treatment. DPI and apocynin, two different NADPH oxidases inhibitors, hampered the proliferation of two different CML cell lines in a dose-dependent manner (Figure 1 and Supplementary Figure 1). Moreover, the combination of the BCR-ABL inhibitor imatinib with DPI was significantly more effective than either individual treatment (Figure 1A, 1B and 1D).

Analysis of these combinations by the Chou-Talalay method (26) revealed a combination index (CI) lower than 1, reflecting high synergism (Figure 1C and 1E). The same synergy was found when different inhibitors for BCR-ABL (nilotinib) or NADPH oxidases (apocynin) were used (Supplementary Figure 1C and 1D). Although DPI and apocynin are commonly used to target NADPH oxidases, they are not specific. DPI is a on-competitive inhibitor of flavoproteins and therefore at high concentrations targets other flavoproteins. Apocynin prevents the assembly of Nox2 complex, although it may exert other effects (31). Accordingly, we tested a novel inhibitor, VAS3947, that is apparently specific towards NADPH oxidases (32). The combination of VAS3947 with imatinib was also synergistic (Supplementary Figure 1E), suggesting that the effects observed when using DPI or apocynin were indeed due to the inhibition of NADPH oxidases.

When we applied the same strategy to BM-MNC isolated from newly diagnosed CML patients who had not yet started treatment, the results were very similar to those described for CML cell lines, the combined treatment proved to be significantly more effective than the individual treatments (Figure 2A, 2B and 2E). Moreover, the combination index was lower than 1 (Figure 2C and 2D),
especially with the nilotinib combinations, also indicating a synergistic effect. Interestingly, we did not observe synergy when BM-MNC from healthy donors were used. (Supplementary Figure 2).

Furthermore, we analyzed the response of hematopoietic progenitors to these treatments. After 24h of incubation, the number of CD34$^+$ cells isolated from CML patients was significantly reduced by DPI or the combined treatment. Interestingly, CD34$^+$ cells isolated from healthy donors were unaffected by the same treatments (Figure 2F).

Inhibition of NADPH oxidases is effective for the in vivo treatment of CML

To evaluate the possible use of NADPH oxidase inhibitors for in vivo CML treatment, we initially used a K562 cell xenograft model in CB17-SCID mice. As expected, imatinib treatment slowed tumor growth significantly (Figure 3A). Moreover, DPI treatment showed a similar effect to that described for imatinib (Figure 3A), suggesting that in vivo treatment with NADPH oxidase inhibitors could be a good strategy for CML treatment. Furthermore, the combined treatment showed a clear trend towards greater effectiveness than the single treatments, in agreement with the synergy observed in vitro.

We also used a transgenic mouse line, in which BCR-ABL is specifically expressed in HSCs (27), that elicits a myeloproliferative syndrome very similar to human CML (1). Individual imatinib or DPI treatments reduced the number of granulocytes in PB, while their combination was significantly more effective (Figure 3C).

We did not find significant differences in the animals’ weights between the different experimental groups (Figure 3B and 3D), suggesting that the treatments were well tolerated. Thus, in keeping with our in vitro observations two different in vivo animal models show that NADPH oxidase inhibitors are effective for CML treatment, and that these inhibitors can enhance the activity of standard BCR-ABL inhibitors.
NADPH oxidase and BCR-ABL inhibitors reduce intracellular ROS levels

Bearing in mind the strong synergism observed when NADPH oxidase and BCR-ABL inhibitors were combined, we sought to understand the mechanism of these effects. Individual treatments led to a slight reduction in the content of intracellular ROS levels, while combination therapy caused a further decrease in such levels, both in K562 cells (Figure 4A) and in BM-MNC from patients (Figure 4B). This decrease could be related to the mechanism of action of these drugs. Indeed, two different antioxidants, N-acetyl cysteine (NAC) and quercetin, inhibited K562 cell proliferation (Supplementary Figure 3A and 3B). Moreover, rotenone, an inhibitor of mitochondrial complex I, hampered cell proliferation (Supplementary Figure 3C) and reduced the intracellular ROS content (Supplementary Figure 3D). These results suggest that CML cell proliferation would be correlated with the levels of intracellular ROS, and support the notion that decreasing the ROS content could be a good strategy for inhibiting CML cell proliferation.

NADPH oxidase activity contributes to maintaining the activation of the BCR-ABL signaling

Because ROS can regulate cellular signaling (6) and the cell cycle (33), we analyzed both aspects. As expected, imatinib produced a strong inhibition of BCR-ABL phosphorylation in K562 cells (Figure 4C). Interestingly, DPI alone also produced a noticeable decrease in BCR-ABL phosphorylation and, more importantly, the combination induced a further decrease in BCR-ABL activation (Figure 4C), explaining, at least in part, the strong inhibition of cell proliferation and the clonogenic capacity reported above.

Interestingly, DPI treatment reduced BCR-ABL protein levels, without affecting the mRNA (Figure 4C and Supplementary Figure 3E to 3G), suggesting that NADPH oxidases would regulate BCR-ABL protein stability.

We also looked at downstream effectors of BCR-ABL. The activation status of CRKL and STAT5 was also affected by single treatments and, in agreement with what we observed for BCR-ABL phosphorylation, the combined treatment produced a significantly stronger inhibition (Figure 4D).
In the case of ERK1/2, the combination of imatinib plus DPI did not seem to produce a stronger inhibition than imatinib alone (Figure 4D). However, when we combined nilotinib and DPI, ERK1/2 phosphorylation was significantly reduced in comparison with single treatments (Figure 4E).

These results suggest that NADPH oxidase-driven ROS production is necessary to maintain the activation of the BCR-ABL signaling pathway, perhaps through the inactivation of protein tyrosine phosphatases (PTPs), given the sensitivity these enzymes to oxidation (6). In this regard, it is interesting to note that a role for SHP1 as a BCR-ABL tumor suppressor was proposed some time ago (34). SHP1 specific activity was enhanced by imatinib, DPI or their combination (Supplementary Figure 4A), suggesting that the increase in SHP1 activity, due to a lower level of oxidative stress, could be related to BCR-ABL signaling attenuation. This effect seems to be specific to SHP1, since the activity of the closely related SHP2 was not affected by DPI treatment (Supplementary Figure 4B), and moreover, the inhibition of SHP2 did not alter cell proliferation (Supplementary Figure 4C).

It has recently been reported that DPI inhibits the expression of multidrug resistance-1 gene (MDR1) in hepatocellular carcinoma cells (35), which can alter drug pharmacodynamics. However, DPI did not altered MDR1 messenger levels in K562 cells (Supplementary Figure 4D). Since Rac GTPase seems to be a suitable therapeutic target for CML (36), and since some NADPH oxidase complexes depend on Rac, we analyzed the GTPase activity upon DPI treatment, but found no differences with the control (Supplementary Figure 4E).

**NADPH oxidase inhibition blocks cell cycle progression and affects cell viability**

Cell cycle analysis showed that DPI induced a pronounced G₂/M phase arrest (Figure 5A and 5B). The increase in phospho-histone H3 (S10) (37) and the decrease in wee1 (38) (Figure 5C) are consistent with an arrest in mitosis. An increase in the double-strand DNA break marker, γH2AX, was also observed (Figure 5D). There was a substantial increase in cell numbers in the sub-G₁
phase (Figure 5A and Supplementary Figure 5A), especially with the combined treatment. This suggests that the cells cannot overcome this cell cycle arrest, and therefore that they are destined for apoptosis, which could also explain the synergistic effect of the combined treatments. Indeed, all treatments increased cell death, but the combination showed a tendency towards a higher percentage of early apoptotic cells (Annexin V+/7AAD− cells) (Figure 5E), in agreement with the higher number of cells in the sub-G1 phase after the combined treatment.

**CML cells express different NADPH oxidase isoforms**

Most cells express several NADPH oxidases (39) and hence an intriguing issue is whether there is functional specificity between them or whether, instead, there is redundancy. It would be interesting to settle this issue for CML cells in order to evaluate the appropriateness of seeking novel pan-inhibitors or specific inhibitors against particular isozymes. Our analysis shows that K562 cells express Nox2, Nox5 and Duox2 (Figure 6A), Nox2 being the predominant isoform.

To test whether BCR-ABL signaling was affected by any particular NADPH oxidase we generated stable cell lines whose expression was down-regulated (Figure 6B). The clonogenic capacity of all these cell lines was significantly reduced with respect to control cells (Figure 6C). Moreover, BCR-ABL signaling was also attenuated when any of the NADPH oxidases was down-regulated, since we detected a decrease in the activation and levels of BCR-ABL (Figure 6D), and in the activation status of, ERK1/2 and STAT5 (Figure 6E). These results are in agreement with a redundancy of function of the three NADPH oxidases expressed in K562 cells, at least regarding BCR-ABL signaling and clonogenic capacity. We generated stable cell lines in which pairwise combinations or the three Nox were downregulated at the same time (Supplementary Figure 7A). The activation of BCR-ABL signaling was more compromised in these cell lines than in the individual knockdowns (Supplementary Figure 7B and 7C). These results suggest that BCR-ABL would not rely on a unique Nox isoform to maintain its signaling activated. This also means that it would be
more effective to target all Nox isoforms rather than a particular one in order to hamper BCR-ABL signaling.

We next analyzed the pattern of expression in BM-MNC from 4 different patients (Figure 6F). Nox2 and Nox5 were present in all the patients analyzed. Duox1 or Duox2 were expressed in 3 out of 4 patients, and only one of the patients expressed Nox3.

We also analyzed the NADPH oxidase pattern of expression at different stages of myeloid differentiation in healthy donors. Interestingly, immature CD34+ progenitors expressed all NADPH oxidases except Nox1 and Nox4 (Figure 6G). This pattern of expression differs from that reported by Piccoli et al (40). In their study the authors used CD34+ cells isolated from peripheral blood after mobilization with G-CSF, but failed to perform an analysis of the relative expression of the different Nox isoforms, whereas we purified CD34+ cells from BM-MNC. CD33+ myeloid progenitors expressed mainly Nox2 and Nox5, while mature CD15+ myeloid cells expressed Nox2, Nox5, Duox1 and Duox2 (Figure 6G), an expression profile very similar to that of CML patients BM-MNC (Figure 6F). It is worth mentioning that Nox2 was always the most abundant isoform, and that Nox5 was present in all the types of hematopoietic cells analyzed.

**NADPH oxidases as therapeutic targets in acute myeloid leukemia**

Our results support the notion that NADPH oxidases are necessary to maintain the activation of BCR-ABL signaling, which would explain the synergistic effect of the combined treatment. Other oncogenic kinases seem to increase the production of ROS through NADPH oxidases (30). Therefore, we speculated that the same strategy as that followed here could be applied to other kinase-driven cancers. We attempted to check this in acute myeloid leukemia (AML) cells expressing the activating mutation of FMS-like tyrosine kinase-3 (FLT3-ITD), because it has been reported that NADPH oxidase ROS production promotes AML cells proliferation (41), and moreover FLT3-ITD activates ROS production through NADPH oxidases (11). We tested two different FLT3-ITD inhibitors (Midostaurin and Sorafenib) combinaned with DPI in two FLT3-
ITD-expressing cell lines (MOLM-13 and MV411). As occurred for CML cells, DPI also inhibited the proliferation of both the AML cell lines, and the combined treatments proved to be synergistic (Figure 6H and Supplementary Figure 8). Therefore, our results show that combining kinase inhibitors with NADPH oxidase inhibitors would offer a therapeutic strategy to be considered in kinase-driven cancers.

**DISCUSSION**

Cancer cells have higher levels of intracellular ROS than healthy cells (7, 8), which may somehow contribute to cell transformation (9, 10). The idea of exploiting this difference in the therapeutic arena was proposed a few years ago (42), and interest has now been rekindled (6, 29). ROS-enhancing treatments would induce cancer cells to cross an oxidative stress threshold incompatible with cell viability, whereas healthy cells would be spared, because they would not reach that lethal threshold. The number of contributions describing treatments based on this strategy is expanding rapidly, including a recent report showing the feasibility of using this strategy in BCR-ABL-positive cells too (14).

Given that elevated intracellular levels of ROS seem to contribute to cancer cell proliferation and survival, we reasoned that reducing ROS levels might be also a good strategy to combat cancer cells. In fact, antioxidant intake to prevent stress-related process such as ageing or cancer is an old and very popular idea (43). However, if we knew the source of ROS in cancer cells, we could prevent their occurrence, which strikes us as being a better strategy than the simple use of ROS scavengers to reduce oxidative stress indiscriminately. Accordingly, we analyzed the possible use of NADPH oxidases as therapeutic targets in CML. The reason for this choice is that it has long been known that BCR-ABL triggers ROS production (12). More recently, it has been suggested that NADPH oxidases contribute to BCR-ABL-driven ROS production (14), and ROS seem to be important for the regulation of the growth of BCR-ABL-expressing cells (18). However, the possible use of this family of enzymes as therapeutic targets in CML has not yet been addressed.
NADPH oxidase inhibitors effectively hamper the proliferation and clonogenic capacity of CML cells, also inducing a pronounced cell cycle arrest in mitosis. NADPH oxidase inhibitors were also seen to be effective in vivo in two different animal models, supporting the possible future use of this strategy in the treatment of CML. Moreover, the combinatorial use of BCR-ABL and NADPH oxidase inhibitors proved to be highly synergistic, opening the possibility of using this strategy in the future for the improvement of CML treatment.

Intracellular ROS levels were significantly reduced upon treatment with NADPH oxidases and BCR-ABL inhibitors. Antioxidants or rotenone treatment also impaired CML cell proliferation. And hence we propose that the capacity of NADPH oxidase and BCR-ABL inhibitors to hamper CML cell proliferation would be linked to their ability to reduce intracellular ROS levels.

NADPH oxidases inhibitors prevented the full activation of BCR-ABL signaling. Accordingly, BCR-ABL activation of NADPH oxidase-driven ROS production would represent a feed-forward mechanism aimed at sustaining the activation of the signaling pathway. A feasible way of attaining this would be through the oxidation and inhibition of PTPs, such as SHP1.

Downregulating the different Nox isoforms expressed in K562 cells caused effects similar to those elicited by the use of chemical inhibitors. We did not find any specificity among the different NADPH oxidases, therefore, the strategy for the future would be to search for novel NADPH oxidase pan-inhibitors rather than looking for molecules specific towards a particular NADPH oxidase.

Finally, we show that inhibition of NADPH oxidases hampered the proliferation of AML cells, and the combination of NADPH oxidase inhibitors with FLT3-ITD inhibitors was also synergistic. NADPH oxidases may be important for sustaining the signaling activated by oncogenic kinases. Therefore we propose that NADPH oxidases could be interesting therapeutic targets not only for CML or AML, but also for any other kinase-driven cancer.

In sum, our results demonstrate that targeting NADPH oxidases could be an appropriate strategy for CML treatment because of the effectiveness of their inhibition in vitro and in vivo, and also
because of the high degree of synergism observed when they are combined with BCR-ABL inhibitors. This could improve traditional therapy for this disease and could be a good strategy for either preventing the appearance of resistances to BCR-ABL inhibitors, or for consideration as an alternative in the event of such resistances already existing.

**Disclosure of Potential Conflicts of Interest**

The authors declare that they have no conflict of interest.

**Authors' Contributions**

BSS performed experiments, analyzed data, did the figures and edited the paper. SGH performed some of the mice *in vivo* experiments. GLR, RPR and MRG performed experiments. AP and CG helped to design *in vivo* experiments. JFSM, FSG and CC provided samples. MLL, AP, CG, JFSM, FSG and CC edited the paper. AHH designed experiments, performed experiments and wrote the paper.

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**Supplementary information is available at Clinical Cancer Research's website.**
References


FIGURE LEGENDS

Figure 1. Inhibitors of NADPH oxidases and BCR-ABL synergize to avoid the proliferation of CML cell lines. K562 and Boff 210 cells were treated with DPI, imatinib or a combination thereof for 48h and 24h respectively. A) K562 cell proliferation. B) K562 cell colony-forming assays. C) Drug interaction in K562 cells. A representative experiment is shown, and the CIs (means ± SE) of 8 different experiments are depicted. D) Boff 210 cell proliferation. E) Drug interaction in Boff 210 cells. A representative experiment is shown, and the CIs (means ± SE) of 4 different experiments are depicted.

Figure 2. Inhibitors of NADPH oxidases and BCR-ABL synergize to prevent the proliferation of BM-MNC from CML patients. Proliferation of BM-MNC treated 48h with imatinib, DPI, or their combination (A), or with nilotinib, DPI, or their combination (B). Analysis of the interaction of DPI with imatinib (C) or with nilotinib (D). A representative experiment and the CIs of different patients are shown. E) Colony-forming assays upon treatment with imatinib, DPI or their combination. F) CD34+ cells isolated from CML patients or from healthy donors were treated with imatinib, DPI or a combination thereof. The number of cells was counted 24h later and normalized to untreated control cells.

Figure 3. NADPH oxidase inhibitors are effective for in vivo CML treatment. The in vivo effectiveness of NADPH oxidase inhibitors was tested in K562 xenografts in CB17-SCID mice and in a transgenic mouse line expressing p210 BCR-ABL in HSCs. Mice were treated with imatinib (100 mg/Kg), DPI (1 mg/Kg) or their combination. A) Tumor volumes in the K562 xenograft model. B) Mouse body weight was monitored during the treatment. C) Percentage of granulocytes in BCR-ABL transgenic mice. Data are normalized with respect to the percentage at day 0 of...
treatment, which was considered to be 100. Note the increase in granulocytes in the control mice.

D) Mouse body weight was monitored during the treatment.

Figure 4. NADPH oxidase-driven ROS production contributes to maintaining the activation of the BCR-ABL signaling pathway. Intracellular ROS levels upon 2h treatment with imatinib, DPI, or their combination, in K562 cells (A) or BM-MNC isolated from three different CML patients (B). K562 cells treated with imatinib or nilotinib, DPI, or their combinations for 16h were used to analyze the activation of BCR-ABL (C), or some of its downstream targets (D and E).

Figure 5. NADPH oxidase inhibitors block the cell cycle. Unsynchronized K562 cells treated with 0.5 μM imatinib, 5 μM DPI or their combination were labeled with PI for cell cycle analysis. A) A representative experiment and the quantification of the sub-G1 phase of 5 experiments are shown. B) Cell cycle distributions of living cells at different time points (means ± SE of 5 different experiments). C) Levels of Wee1 kinase and phospho-Histone H3 (S10). D) The levels of γH2AX were analyzed by flow cytometry (means ± SE of 4 different experiments). E) Cell viability after 48h of treatment. A representative experiment is shown.

Figure 6. CML and myeloid cells express several NADPH oxidases. NADPH oxidase downregulation in K562 cells decreases proliferation and clonogenic capacity and hampers BCR-ABL signaling. The pattern of expression of NADPH oxidases was analyzed by RT-qPCR. The reaction was repeated at least 3 times. A) Relative expression of NADPH oxidases in K562 cells. B) Nox2, Nox5 or Duox2 were downregulated by RNAi in K562 cells. Relative expression of each isoform in the corresponding downregulated cell line, considering expression in control cells as 1. C) Colony assays in Nox downregulated cell lines. D) BCR-ABL protein and phosphorylation levels in Nox downregulated cell lines. E) ERK1/2 and STAT5 activation in Nox downregulated cell lines. F) Relative expression of NADPH oxidases in BM-MNC from CML patients. G)
Relative expression of NADPH oxidases in CD34⁺, CD33⁺ and CD15⁺ cells from healthy donors.

H) Proliferation of MV4-11 cells treated with DPI, FLT3-ITD inhibitors (midostaurin and sorafenib), or combinations thereof for 48h. Interaction between DPI and midostaurin or DPI and Sorafenib. A representative experiment for each combination, and the CIs (means ± SE) of 3 or 5 different experiments for Sorafenib and Midostaurin respectively, are depicted.
Figure 1

A

% Proliferation

n = 17  n = 17  n = 17

DPI 0.2 µM  DPI 1 µM  DPI 5 µM

B

CFUs / 1000 cells

n = 3  n = 3  n = 3

Control  IM 0.5 µM  DPI 1 µM  Combination

C

Cl +/− 1.96 s.d.

IM/DPI K562

D

% Proliferation

n = 4  n = 4  n = 4

DPI 0.2 µM  DPI 1 µM  DPI 5 µM

E

Cl +/− 1.96 s.d.

IM/DPI BOFF210

Cl +/− 1.96 s.d.

IM (µM)  0.1  0.5  1  5  0.1  0.5  1  5  0.1  0.5  1  5  0.1  0.5  1  5

DPI (µM)  0.05  0.5  2.5  5  0.05  0.5  2.5  5  0.05  0.5  2.5  5
Figure 2

A  % Proliferation

B  % Proliferation

C  CI

D  CI

E  CFUs / 5000 Cells

F  % cells

Control  IM 2.5 µM  DPI 0.2 µM  Combination

NILO 1 µM  DPI 0.2 µM  Combination

IM (µM)  0.5  0.5  0.5  1  1  1  2.5  2.5  2.5  5  5  5  10  10  10

DPI (µM)  0.2  1  5  0.2  1  5  0.2  1  5  0.2  1  5  5  5  5

NILO (µM)  0.01  0.05  0.1  1  5  0.01  0.05  0.1  1  5  0.01  0.05  0.1  1  5

DPI (µM)  0.2  0.2  0.2  0.2  0.2  1  1  1  1  1  5  5  5  5  5

Control  Imatinib 0.5 µM  DPI 1 µM  Combination

CD34+ CML  CD34+ Healthy donors

n=9  n=9  n=9  n=9  n=7  n=7  n=7

+  #  ***
Figure 3

A

Mean tumor volume (mm³)

Days of treatment

Control
DPI 1mg/Kg
Imatinib 100 mg/Kg
DPI 1mg/Kg + Imatinib 100 mg/Kg

p = 0.027
p = 0.014
p = 0.05
p = 0.05
p = 0.05
p = 0.014
n = 4
n = 4
n = 4
n = 4
n = 4

B

Mean weight (g)

Days of treatment

Control
DPI 1mg/Kg
Imatinib 100 mg/Kg
DPI 1mg/Kg + Imatinib 100 mg/Kg

p = 0.05
p = 0.014
p = 0.05
p = 0.05
p = 0.05
p = 0.014
n = 4
n = 4
n = 4
n = 4
n = 4

C

% Granulocytes (Normalized values)

Weeks of treatment

Control
IM (100 mg/Kg)
DPI 1mg/Kg
Combination

n = 4
n = 7
n = 3
n = 5
n = 3
n = 4
n = 4
n = 4
n = 4

D

Mean weight (g)

Days of treatment

IM 100 mg/Kg
DPI 1mg/Kg
Combination
Control

n = 4
n = 3
n = 4
n = 4
n = 4
n = 4
n = 4
n = 4

* p < 0.05
** p < 0.01
## p < 0.001
Figure 4

A. Bar graph showing % Intracellular ROS for Control, IM 0.5 µM, DPI 0.2 µM, and Combination treatments. The data points are labeled with n-values (n = 6, n = 5, n = 4, n = 6) and statistical symbols (n = 6 # # #, n = 5 ** ** **, n = 4 []).

B. Bar graph showing % Intracellular ROS for P.4, P.6, and P.8. The graphs compare Control, IM 0.5 µM, DPI 5 µM, and Combination treatments.

C. Western blot images for pBCR-ABL and BCR-ABL with corresponding quantification for TUBULIN. The percentages are labeled with 1 and 2.

D. Western blot images for pCRKL, CRKL, pSTAT5, STAT5, pERK 1/2, and ERK 1/2 with quantification for TUBULIN. The percentages are labeled with 1 and 2.

E. Western blot images for p-ERK 1/2 with quantification for ERK 1/2. The percentages are labeled with 1 and 2.
Figure 5

A

Control 48h

Imatinib 48h

DPI 48h

Combination 48h

B

Control

Imatinib

DPI

Combination

C

24h

32h

48h

D

E

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**Figure 6**

- **A**
  - mRNA Relative Expression
  - NOX2: n=4
  - NOX5: n=3
  - DUOX2: n=5

- **B**
  - mRNA relative expression
  - Nox2 RNAi: n=12
  - Nox5 RNAi: n=7
  - Duox2 RNAi: n=9

- **C**
  - CFUs / 1000 cells
  - LUC RNAi: n=8
  - Nox2 RNAi: n=6
  - Nox5 RNAi: n=6
  - Duox2 RNAi: n=6

- **D**
  - mRNA Relative Expression
  - NOX2 RNAi: 0.01
  - NOX5 RNAi: 0.41
  - DUOX2 RNAi: 0.51

- **E**
  - Protein expression
  - pBCR-ABL / TUBULIN
  - BCR-ABL / TUBULIN
  - TUBULIN

- **F**
  - mRNA Relative Expression (log)
  - Nox2: n=9
  - Nox5: n=12
  - Duox2: n=7

- **G**
  - mRNA Relative Expression (log)
  - CD34+
  - Sample 1: Nox2
  - Sample 2: Nox2

- **H**
  - CI + 1.96 s.d.
  - Fractional Effect
  - MID/DPI MV4-11
  - SOR/DPI MV4-11

- **Additional**
  - LUC RNAi
  - Nox2 RNAi
  - Nox5 RNAi
  - Duox2 RNAi

- **Analysis**
  - CD34+
  - CD33+
  - CD15+
  - Sample 1: Nox2
  - Sample 2: Nox2
  - Sample 3: Nox2

- **Research**
  - January 1, 2018
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NADPH oxidases as therapeutic targets in chronic myeloid leukemia

Beatriz Sanchez-Sanchez, Sara Gutierrez-Herrero, Guillermo Lopez-Ruano, et al.

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