Pharmacological agents with inherent anti-autophagic activity improve the cytotoxicity of imatinib

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Abstract. Resistance to tyrosine kinase inhibitors (TKIs) remains a limitation to the treatment of chronic myeloid leukaemia (CML), due in part, to the induction of autophagy. We examined whether disruption of autophagy with the pharmacological agents, brefeldin A, vincristine and chloroquine, improves the cytotoxicity of imatinib. In K562 CML cells, all drugs tested, in combination with imatinib impaired the expression or cellular distribution of LC3 and Beclin 1 (autophagy markers) and reduced the recovery of cells following drug withdrawal. The combination of imatinib and an agent that impedes autophagy demonstrates impressive potential as a more curative regime for CML.

Introduction

The Philadelphia chromosome, a hallmark of chronic myeloid leukaemia (CML), generates the fusion oncoprotein, Bcr-Abl, and a constitutively active tyrosine kinase (1,2). Chronic phase CML is well managed in most patients with tyrosine kinase inhibitors (TKIs), most notably imatinib, whereas the advanced stage of CML known as blast crisis is a highly aggressive drug-resistant disease and is analogous to acute leukaemia (3).

Despite the success of imatinib for the treatment of CML, treatment failures due to resistance and relapse are still common. Bcr-Abl amplification, mutations or the acquisition of Bcr-Abl-independent mechanisms have been shown to play a role in the development of resistance (4). In addition, relapse is common when imatinib is withdrawn, even in patients who achieve a complete molecular response and this can re-emerge as the same imatinib susceptible disease (5). This highlights the need for potentially life-long treatment and the possibility of resistant disease emerging later on.

We and others have shown that the induction of autophagy in response to treatment with imatinib is associated with persistence and recovery of a subpopulation of leukaemia cells (6,7). Autophagy is a survival mechanism initiated in response to cellular stress or starvation. It removes damaged organelles and aggregated proteins and can recycle cytoplasmic content (8). Inhibition of autophagy can significantly reduce the recovery of CML cells following imatinib treatment (7). This is one of the most promising strategies for improving the curative ability of imatinib.

Currently, there are no specific autophagy inhibitors. There are, however, a limited number of pharmacological agents that have been reported to disrupt processes or organelles required for effective autophagy. Combining such agents with imatinib may therefore compromise the autophagic recovery employed by leukaemic cells, potentially improving treatment regimens for CML. We evaluated three agents in combination with imatinib: chloroquine, vincristine and brefeldin A. Chloroquine, a lysosomotropic agent, damages lysosomal integrity leading to the inhibition of autophagosome turnover (9) and is currently in clinical trials in combination with imatinib for treatment of CML (10). The second agent in the present study, vincristine, is a microtubulin de-polymerising agent which arrests cells in the G2/M phase. Evidence suggests that cells undergoing mitosis are resistant to autophagic induction (11) and therefore, G2/M inhibitors may have anti-autophagic activity in combination regimes. Vincristine has also been reported to impede autophagy by disrupting the microtubulin network and inhibiting the trafficking required for autophagosome and lysosomal fusion (12). The third agent in this study, brefeldin A disrupts protein transport in the Golgi compartment (13). While this may lead to endoplasmic reticulum (ER) stress and potentially increase autophagy, brefeldin A has been reported to block an alternative autophagic pathway (14). This may be due to involvement of the Golgi region in the initiation of certain types of autophagy.

In the present study, we investigated the autophagic response of K562 CML cells following imatinib treatment alone and combination treatment with potential autophagy-disrupting agents. Our data indicate that the early implementation of imatinib combined with autophagy-disrupting agents, reduces cell recovery and may potentially improve the curative ability of imatinib in CML patients.

Materials and methods

Cell culture/reagents. K562 cells (blast crisis CML) were obtained from Deutsche Sammlung von Mikroorganismen...
und Zellkulturen GmbH (DSMZ) and were maintained in RPMI-1640 medium, HEPEs modification, supplemented with 10% foetal calf serum, 1% penicillin and streptomycin (Sigma-Aldrich, Ireland Ltd., Dublin, Ireland) and 2 mmol/l L-glutamine (Gibco-BRL, Paisley, UK) cultured at 37°C in 5% CO₂. Chloroquine, vincristine and brefeldin A were all from Sigma-Aldrich Ireland Ltd. Imatinib was obtained from Novartis Pharma AG (Basel, Switzerland).

**Cell viability and cell cycle analysis.** Cells were resuspended in PBS with 50 µg/ml propidium iodide (PI; Sigma-Aldrich Ireland). FL-2 fluorescence was measured using a FACS Calibur® flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). Statistical significance was determined using the Student's t-test. P-values of <0.05 were considered statistically significant. For the prolonged viability analysis, following the initial 24-h treatment, the drug was removed and cells were resuspended in fresh media. Viability was assessed over the subsequent 5 days. For cell cycle analysis, cells were fixed in 70% ethanol prior to PI staining.

**Cell morphology and immunofluorescence.** For cell morphology, cytospun cells were stained using Pro-Diff (Braidwood Laboratories, Ireland). For immunofluorescence staining, the cytospun cells were fixed in 4% parafomaldehyde for 20 min and washed in PBS. Permeabilisation and blocking were carried out using 0.005% Saponin/PBS and 0.2% BSA/PBS, respectively. For antibody staining, LC3 (Abgent, San Diego, CA, USA) and Beclin 1 (Cell Signaling Technology, UK) were incubated (1:200) for 1 h at room temperature. After incubation with the appropriate secondary Alexa Fluor antibody, samples were mounted with Gold Plus with DAPI antifade reagent (Invitrogen, Dublin, Ireland). Images were captured using a DP70 digital microscope camera and Olympus DP-Soft823 version 3.2 software. All images are representative of at least three separate experiments.

**Results**

**Distribution of LC3 and Beclin 1 following treatment with chloroquine, vincristine and brefeldin A with and without imatinib.** We previously demonstrated that induction of autophagy in K562 CML cells limits the cytotoxic effect of imatinib and facilitates the recovery of treated cells (6). Viability and morphologic data for K562 cells are shown in Fig. 1A to enable comparison with the subsequent combination treatments. In the present study, we examined whether combinations of imatinib with potential autophagy-disrupting agents, chloroquine, vincristine and brefeldin A, alter expression of LC3 and Beclin 1 (autophagy markers).

**Chloroquine.** K562 cells were treated with 5 µM imatinib, 25 µM chloroquine or a combination of both for 24 h. Cells treated with chloroquine alone displayed increased LC3 staining when compared to that in the control K562 cells (Fig. 1B-ii, upper panel) which was likely due to the inhibition of autophagosome turnover. K562 cells treated with chloroquine and imatinib together also showed increased LC3 expression in some cells when compared to the controls. This expression, however, was significantly reduced when compared to that in cells treated with either agent alone and was localised to a peri-nuclear region (Fig. 1B-ii, lower panel), suggesting impairment at either initiation or trafficking in addition to compromised turnover. Beclin 1 expression was also increased in cells treated with imatinib, exhibiting a more peri-nuclear localised staining pattern (Fig. 1C-i, lower panel). Cells treated with chloroquine alone or the combination with imatinib displayed diminished staining of Beclin 1, again suggesting that initiation of autophagy may be impaired in addition to turnover (Fig. 1C-ii). The distribution of both markers suggests that autophagy was impeded in the chloroquine-treated cells.

**Vincristine.** We investigated whether vincristine disrupts imatinib-induced autophagy. Cells were treated for 24 h with 10 nM vincristine, 5 µM imatinib or a combination of both. K562 cells treated with vincristine alone demonstrated a mixed LC3 staining pattern. Cells displaying mitotic disturbances [NB irregular DAPI-stained nuclei (white arrows)] did not express LC3. In contrast, cells with normal nuclei exhibited LC3 staining analogous to that in the control cells or was slightly elevated (Fig. 1B-iii, upper panel). A similar pattern was observed in the combination-treated cells (Fig. 1B-iii, lower panel). Beclin 1 was also absent or significantly reduced in the majority of vincristine-treated cells displaying nuclear mitotic abnormalities (Fig. 1C-iii, upper panel). Cells treated with the combination of imatinib and vincristine demonstrated reduced overall Beclin 1 expression when compared to expression in the cells treated with imatinib alone (Fig. 1C-iii, lower panel). This reduced/absent Beclin 1 staining, particularly in the G₂/M-arrested cells, indicated a reduced capacity to initiate autophagy.

**Brefeldin A.** K562 cells were treated with 1 µg/ml brefeldin A (BFA), 5 µM imatinib or a combination of both for 24 h. Cells treated with BFA alone showed reduced expression of LC3 when compared to that in the control cells (Fig. 1B-iv, upper panel). In cells treated with the combination of imatinib and BFA, LC3 staining was dramatically reduced compared to staining in cells treated with imatinib alone (Fig. 1B-iv, lower panel). Therefore, BFA impeded autophagy initiation in cells treated with imatinib. Notably, BFA treatment alone induced dramatic condensation of Beclin 1 to single peri-nuclear foci (Fig. 1C-iv, upper panel). Cell treated with the combination treatment exhibited a mixture of single discrete Beclin 1 foci and less condensed peri-nuclear localisation (Fig. 1C-iv, lower panel) suggesting that autophagy initiation was impaired.

**Effect of chloroquine and imatinib treatment on cell viability and recovery.** K562 cells were treated with 5 µM imatinib, 25 µM chloroquine or a combination of both for 24 h and cell viability was assessed over 5 days (Fig. 2A). K562 viability was unaffected by treatment with chloroquine alone at any time point. Forty-eight hours post treatment, the viability of K562 cultures treated with imatinib alone was 23.7±0.3%. However, following the combination therapy, cell viability was much lower at 4.7±0.1%, indicating a marked improvement when combining chloroquine and imatinib, which was not observed following the 24-h treatment. This combination effect was sustained as indicated by the absence of any recovery at day 5 (Fig. 2A).
Cells treated with chloroquine alone began to display vesicle accumulation in a small percentage of cells at 24 h (Fig. 2B-i, upper panel). At 48 h and 5 days post chloroquine treatment alone, cells showed an extensive build-up of vacuoles in their cytoplasm (Fig. 2B-i, middle and lower panel), consistent with previous reports (9). Notably, this did not significantly affect the viability of the culture.

Cells treated with the combination of imatinib and chloroquine for 24 h showed limited cytoplasmic vacuoles (Fig. 2B-ii, upper panel) in contrast to the high level of vacuoles observed in cells treated with chloroquine alone.
ules in cultures treated with imatinib alone (Fig. 1A-ii). At 48 h post imatinib and chloroquine combination treatment, cells exhibited a mixed morphology, with fragmented or intact nuclei, small dark shrunken cells and/or necrotic cells. At 5 days post treatment, only cell fragments remained (Fig. 2B-ii, middle and lower panel). The combination of chloroquine and imatinib was clearly a superior treatment when compared to either therapy alone, improving cytotoxicity and reducing the potential for cells to recover following treatment.

**Vincristine and imatinib treatment.** As vincristine limited the autophagy induced by imatinib (Fig. 1B and C), we investigated whether this agent improves cytotoxicity. Cells were treated for 24 h with 10 nM vincristine, 5 µM imatinib or a combination of both. Five days post drug removal, the viability of the cells treated with the combination of vincristine and imatinib was reduced to 3.5±0.1%. This was statistically significant (P=0.0015) when compared to imatinib treatment alone (Fig. 3A).

Morphological analysis of vincristine-treated cells indicated that the majority of cells were arrested in mitosis at 24 h (Fig. 3B-i, left upper panel). In the cell treated with the combination of vincristine and imatinib, this morphology dominated and there was an absence of cytoplasmic vesicularisation (Fig. 3B-ii, right upper panel). At 48 h post vincristine treatment some cells exhibited tight clustering of cytoplasmic vacuoles (red arrows) and numerous multi-nucleated cells were evident (Fig. 3B-i, middle panel). This was also consistent with earlier LC3 staining in a limited number of cells, which appeared confined to a peri-nuclear area (Fig. 1B). The combination treatment at 48 h showed fewer cells with vesicles and many fragmented cells (Fig. 3B-ii, middle panel). Five days post treatment, cells treated with vincristine alone showed accumulation of cytoplasmic vesicles in the remaining intact cells, similar to cells
treated with imatinib, indicative of cells attempting recovery; some vesicular clustering was still evident (Fig. 3B-i, lower panel). The combination treatment showed only cell fragments and demonstrated a clear advantage over the single agents in reducing culture viability (Fig. 3B-ii, lower panel). Cell cycle analysis confirmed that a 24-h treatment with vincristine with
or without imatinib, arrested cells in the G_{2}/M phase of the cell cycle (Fig. 3C).

**Brefeldin A and imatinib treatment.** Immunofluorescence of LC3 and Beclin 1 indicated that BFA inhibited autophagy
The arrested cells in the vincristine-treated populations also reduced the recovery of K562 cells following drug withdrawal. Vincristine was more effective than either therapy alone and clinical trials for solid tumours treated with various agents. Chloroquine is considered to be less toxic. Treatment regimens such as malaria or for rheumatoid arthritis where hydroxyl-leukemic cells is aided by autophagy and by inhibiting the autophagic response we can improve the cytotoxic impact of imatinib (6,7). We, therefore, assessed whether disruption of other processes important for autophagy, reduces the recovery following imatinib withdrawal. The viability and recovery of CML-derived K562 cells were significantly reduced following the combination therapies.

The long-term viability of K562 cells treated with chloroquine and imatinib was significantly reduced when compared to long-term viability in cells treated with imatinib alone. Our analysis is consistent with another study of CML cell lines and CML patient samples which showed an improvement in imatinib-induced cytotoxicity with chloroquine (7). It is currently unclear, however, whether inhibition of late vesicular/lysosomal fusion is the sole reason for this enhancement. Our analysis of LC3 and Beclin 1 distribution in treated cells (at 24 h prior to cell death) showed reduced LC3 accumulation and substantially decreased Beclin 1 expression in the combination-treated cells. Reduced vesicular content was also evident. It is possible that this was a consequence of a number of chemotherapeutic regimens. Vincristine has been incorporated in treatment regimens for acute leukaemias and blast crisis CMLs. It has also been combined with other non-targeted therapies such as cisplatin or etoposide for treatment of small-cell lung cancer (18). It is possible that these types of agents have dual activity: disruption of cell division and impairment of autophagy.

Treatment of K562 cells with BFA had the most dramatic effects on distribution of autophagy markers. Cytotoxicity was accelerated in cells treated with a combination of BFA and imatinib; however, BFA was also highly effective alone 5 days after drug withdrawal. BFA has been reported to be effective against pancreatic, glioblastoma and prostate cancer (19-21). A generation of new derivatives such as breflate (22-24) is overcoming problems associated with the low water solubility of BFA, making it an interesting candidate for future clinical trials.

We previously showed that specific autophagy knockdown (siRNA approach) reduced the recovery of imatinib-treated cells (6). However, in the combinations presented here we cannot conclusively attribute the enhanced cytotoxicity of the combined treatments with imatinib to autophagy inhibition. These agents have other activities. The aim of the present study was to indicate that certain pharmacological agents can also impede autophagy as part of their activity and this may be an important factor in the design of combination treatment regimes where the efficacy of one of the agents is limited by autophagy.

Newer TKIs such as nilotinib (Bcr-Abl inhibitor) or dasatinib and INNO-406 (src family kinase inhibitors) developed to improve treatment of imatinib-intolerant or -resistant leukaemia have also been reported to be limited by autophagy (7,25). We and others have also shown that the induction of autophagy is not limited to the treatment with TKIs but also with other nontargeted therapies such as VP16 or SAHA (6,26). Autophagy has also been reported to be a barrier in achieving successful treatment of other cancer types, including breast, prostate and oesophageal cancer treated with tamoxifen, sorafenib or 5-fluorouracil (27-29).

An important part of the rationale for using autophagy inhibitors in cancer is the potential for the eradication of the more resistant residual cells and importantly the transformed stem cells which can lead to re-population of disease. Hydroxylchloroquine has been reported to be effective at targeting primitive CML cells including colony forming cells and long-term culture-initiating cells (7). Notably, autophagy has been reported to be important for normal stem cell longevity (30). This suggests that normal cells may also be affected by prolonged treatment with autophagy inhibitors. These studies highlight the need for the development of more selective inhibitors and a better understanding of their effects on normal cells for effective clinical management.

The present study suggests that the early implementation of regimes which incorporate an autophagy-deregulating agent in combination with TKIs may reduce the residual disease present in CML. Importantly, this will remain largely targeted if the autophagy inhibitor has limited toxicity on its
own. Regimes designed to reduce autophagy may limit the development of TKI resistance and the toxicity associated with dose escalation or less targeted treatments.

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