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ORIGINAL ARTICLE

Synergistic activity of nilotinib and established chemotherapeutic drugs in imatinib-sensitive and -resistant *BCR-ABL*-positive cells

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Abstract We investigated various combination treatment regimens employing nilotinib with established chemotherapeutic agents (daunorubicin, mitoxantrone, etoposide and cytarabine) in imatinib-sensitive and -resistant *BCR-ABL*-positive cells. Mitoxantrone or cytarabine showed synergism (CI < 1) in combination with nilotinib in imatinib-sensitive LAMA84 cells, whereas in imatinib-resistant LAMA84-R cells synergistic effects could be assessed for daunorubicin, mitoxantrone and etoposide when combined with nilotinib. In both imatinib-sensitive and -resistant K562 cells daunorubicin, mitoxantrone and etoposide demonstrated synergism in combination with nilotinib. Moreover, both daunorubicin and mitoxantrone led to synergistic antiproliferative effects when combined with nilotinib in imatinib-resistant Ba/F3 cells carrying point mutations in the ABL TK domain (E255K, E255V and T315I). Annexin V/propidium iodide staining revealed a significant enhancement of nilotinib-induced apoptosis in imatinib-resistant Ba/F3T315I and LAMA84-R cells upon combination with daunorubicin and mitoxantrone, respectively. Our results demonstrate the efficacy of combination treatment regimens employing nilotinib and established chemotherapeutic agents in improving antileukemic effects in imatinib-sensitive and imatinib-resistant cells. This may be the foundation for further study on the potential of the applied combinations in a clinical setting.

Keywords BCR-ABL · Nilotinib · Combination treatment · Imatinib resistance · Synergism

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Introduction

Chronic myelogenous leukemia (CML) is a malignant myeloproliferative disease characterized by the chromosomal translocation t(9;22)(q34;q11) known as the Philadelphia (Ph^1) chromosome [1–3]. On the molecular level this aberration leads to the expression of the *BCR-ABL* fusion proto-oncogene with transforming potency due to a deregulated ABL tyrosine kinase (TK) activity [3, 4]. The development and application of imatinib targeting the ABL TK has revolutionized the treatment of CML making imatinib the therapeutic gold standard for this disease [5, 6]. However, resistance to imatinib can evolve. Up to now various molecular mechanisms of resistance have been described, most notably mutations in the BCR-ABL TK domain and an increased BCR-ABL activity resulting from gene amplification, up-regulation, or multiple Ph chromosomes [7–10]. Consequently, the effort to overcome imatinib resistance within current treatment strategies lead to the development of second-generation TK inhibitors (TKI).

Nilotinib is a potent, second-generation aminopyrimidine ATP-competitive inhibitor of BCR-ABL. It has been shown not only to be at least 20-fold more potent than imatinib in killing cells with non-mutated BCR-ABL in vitro, but also to inhibit the activity of the majority of mutant forms of BCR-ABL occurring in imatinib-resistant patients [11, 12]. Furthermore, recent clinical trials found nilotinib effective and safe in patients with CML post-imatinib

failure or intolerance [13, 14]. However, the efficacy of nilotinib and other newer TK inhibitors is best in the chronic phase of CML and decreases with disease evolution to further stages. In particular, the blast crisis remains a serious clinical challenge [15, 16]. With either conventional intensive chemotherapy or the newer targeted therapies applied as single treatment, long-term survival in myeloid blast crisis is rare reaching a plateau below 10% if the patients are not offered subsequent allogeneic stem cell transplantation upon achievement of response. On the other hand, an induction therapy leading to remission of blast crisis or to restoration of chronic phase was shown to be associated with improved outcome in patients receiving an allogeneic transplant [17].

A number of preclinical studies by several investigators including our group could comprehensively show that combination treatment regimens, employing a TK inhibitor (imatinib) and substances with a differing mode of action, lead to synergistic antiproliferative effects presenting a feasible strategy to increase the antileukemic efficacy and to circumvent imatinib resistance [18–27]. In order to elucidate whether these observations can be translated to nilotinib, in the present study, we investigated several combinations applying nilotinib and established chemotherapeutic agents (daunorubicin, mitoxantrone, etoposide and cytarabine) in different imatinib-sensitive and in particular imatinib-resistant BCR-ABL-positive leukemia cell lines.

Materials and methods

Cell lines and culture conditions

K562 and LAMA84 cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and were all sensitive to submicromolar concentrations of imatinib [21, 22]. The imatinib-resistant cell lines LAMA84-R and K562-R were provided by Professor Junia Melo (London, UK) and were characterized in detail previously [21]. BCR-ABL-positive wild-type (wt) Ba/F3$_{wt}$ cells expressing p185$^{BCR-ABL}$ and the corresponding imatinib-resistant lines carrying mutations in the ABL TK domain (E255K, E255V and T315I) were established in the lab of Professor Justus Duyster (Munich, Germany) [28].

All cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) at standard conditions (37°C, fully humidified atmosphere of 95% air and 5% CO2). In addition, LAMA84-R and K562-R cells were cultured in medium containing 1 μM imatinib.

Drugs

Nilotinib was kindly provided by Novartis Pharma (Basel, Switzerland). Daunorubicin (DNR; Sigma, Steinheim, Germany), mitoxantrone (Sigma), etoposide (Bristol Arzneimittel, Munich) and cytarabine (Ara-C; Cell Pharm, Hannover, Germany) were purchased from the manufacturers. Nilotinib was initially dissolved in sterile 10% dimethyl sulfoxide solution (DMSO; Merck, Darmstadt, Germany). Other applied cytostatic drugs were dissolved in phosphate-buffered saline (Dulbecco's PBS; Invitrogen). Stock solutions at a concentration of 10 mM of each drug were prepared and stored at −20°C until use. For experiments, serial working dilutions were prepared.

Cell proliferation assay and analysis of drug effects and interactions

MTT assays were used for the assessment of cell proliferation with minor modifications as described previously [18, 21]. After 48 h of incubation with tested drugs, means of four replicate wells for each drug dilution and the control (cells grown in absence of the drug) were used to calculate the extent of relative cell proliferation and growth inhibition (defined as fraction affected (Fa) [18]). Each cell line was treated with five increasing concentrations (doubling with each increment) of nilotinib and chemotherapeutic drug alone or their combination, respectively. Since nilotinib, in contrast to the other agents, was dissolved in 10% DMSO the serial drug dilutions and controls were prepared to finally achieve 0.05% DMSO in all wells (control, single agent and drug combinations; total volume 100 μl). At this concentration no antiproliferative effects of the applied DMSO solution could be observed in the investigated cell lines (data not shown).

Dose–effect relationships and drug interactions upon combination of nilotinib and established drugs were analyzed according to the median-effect method of Chou and Talalay [29] employing the CalcuSyn Software (Biosoft, Cambridge, UK). For single-agent treatment, mean IC50 values for each drug and cell line were calculated; for combinations mean combination index (CI) values based on equitoxic drug ratios were assessed with $CI < 1$, $CI = 1$ or $CI > 1$ representing synergistic, additive or antagonistic effects of the substances, respectively, and combination data were depicted as CI versus Fa plots [18, 20]. In cell lines where no antiproliferative activity upon single-agent treatment was assessable, an analysis in terms of sensitization (potentiation) or inhibition of nilotinib activity was performed instead with relative $IC50 > 1$ indicating inhibition and relative $IC50 < 1$ indicating potentiation of nilotinib activity, respectively (for details see Topaly et al. [20] and Radujkovic et al. [22]).

Apoptosis assay

Assessment of apoptosis rates was done by annexin V/propidium iodide (PI) staining using the annexin V-FITC Kit (Immunotech, Marseille, France) 48 h after start of incubation as described previously [21, 22]. Data collection and analysis were performed with a FACScalibur flow cytometer (Becton–Dickinson, Heidelberg, Germany) applying the CellQuest Software (Becton–Dickinson).

Statistics

Three to five independent experiments were performed and the results were depicted as mean value ± standard deviation (SD). Statistical significant differences of the data were calculated using the Student *t* test with significance levels of $p < 0.05$ and $p < 0.01$.

Results

Single-agent treatment

Imatinib-sensitive LAMA84 and K562 cells were all sensitive to nanomolar concentrations of single-agent nilotinib (Table 1). In the imatinib-resistant counterparts LAMA84-R and K562-R substantially higher nilotinib concentrations for corresponding antiproliferative effects were required. Imatinib-resistant K562-R cells displayed a remarkable resistance towards nilotinib with an IC50 value of 21,972 nM, i.e., approximately 600-fold higher than in the respective imatinib-sensitive K562 cells (34 nM); in the LAMA84-R line the IC50 value (124 nM) was 10-fold higher as compared to the parental line (11 nM). In imatinib-resistant Ba/F3 cells exhibiting point mutations in the BCR-ABL TK domain $Ba/F3_{E255K}$, $Ba/F3_{E255V}$ and $Ba/F3_{T315I}$, the IC50 values of nilotinib were 365, 791 and 23,582 nM, respectively, i.e., about 16-, 34- and 1,000-fold higher than in the corresponding parental imatinib-sensitive $Ba/F3_{wt}$ cell line (Table 1).

Daunorubicin, mitoxantrone and etoposide displayed a concentration-dependent inhibition of proliferation after a 48-h treatment. Both intercalating drugs, the anthracycline daunorubicin and the anthracendione mitoxantrone, showed similar antiproliferative effects in LAMA84 cells (Table 1). In imatinib-resistant LAMA84-R cells considerably higher concentrations of both agents were necessary for growth inhibition as compared to the imatinib-sensitive LAMA84 line with mitoxantrone and daunorubicin IC50 values being increased by 10- and 20-fold, respectively, implying cross-resistance towards both compounds (Table 1). Regarding imatinib sensitivity, no significant differences in the cytotoxicity of the substances could be observed in K562 cells.

Table 1 Effects of single-agent nilotinib and established chemotherapeutic drugs on the cell growth of different BCR-ABL-positive imatinib-sensitive and -resistant cell lines after 48-h treatment

Cell line	IC_{50} (nM), mean ± SD (concentration range tested)[a]				
	Nilotinib	Daunorubicin	Mitoxantrone	Etoposide	Cytarabine
LAMA84	11 ± 2 (2–32)	344 ± 23 (62.5–1,000)	365 ± 107 (62.5–1,000)	3,178 ± 1,054 (625–10,000)	175 ± 23 (32–512)
LAMA84-R	124 ± 31 (20–320)	7,219 ± 1,118 (1,250–20,000)	3,783 ± 1,040 (625–10,000)	4,017 ± 432 (625–10,000)	n/e (5,000–80,000)
K562	34 ± 9 (6.25–100)	1,634 ± 524 (312.5–5,000)	6,065 ± 1,186 (1,250–20,000)	22,745 ± 2,914 (4,000–64,000)	n/e (5,000–80,000)
K562-R	21,972 ± 1,598 (6,250–100,000)	2,363 ± 177 (625–10,000)	6,969 ± 1,311 (2,000–32,000)	30,012 ± 5,718 (8,000–128,000)	n/e (5,000–80,000)
Ba/F3wt	23 ± 6 (2.5–40)	106 ± 26 (12.5–200)	4 ± 1 (0.4–6.4)	ND[c]	ND
Ba/F3E255K	365 ± 41 (62.5–1,000)	88 ± 6 (15.625–250)	3 ± 1 (0.5–8)	ND	ND
Ba/F3E255V	791 ± 189 (100–1,600)	99 ± 6 (12.5–200)	3 ± 1 (0.4–6.4)	ND	ND
Ba/F3T315I	23,582 ± 1,923 (6,250–100,000)	92 ± 20 (25–400)	2 ± 1 (0.5–8)	ND	ND

Mean values (nM) ± 1SD are given, $n = 5$ each

ND Not determined, *n/e* not evaluable, insufficient dose–response correlation in the median-effect plot ($r < 0.95$)

[a] Concentrations were doubled with each increment

The activity of etoposide was substantially higher in the LAMA84 cells as compared to K562 with both imatinib-resistant cell lines (LAMA84-R and K562-R) showing a reduced sensitivity (not significant) towards etoposide (Table 1). For cytarabine, only in imatinib-sensitive LAMA84 cells an IC50 value of 175 nM was calculable; in LAMA84-R cells and in the K562 pair no cytarabine activity could be detected. Daunorubicin displayed similar effects on the growth of all investigated Ba/F3 lines; the same applies to mitoxantrone (Table 1).

Combination treatment

In the imatinib-sensitive LAMA84 cell line combination treatment with nilotinib plus daunorubicin and nilotinib plus etoposide led to antagonistic (CI > 1; Fig. 1a) and

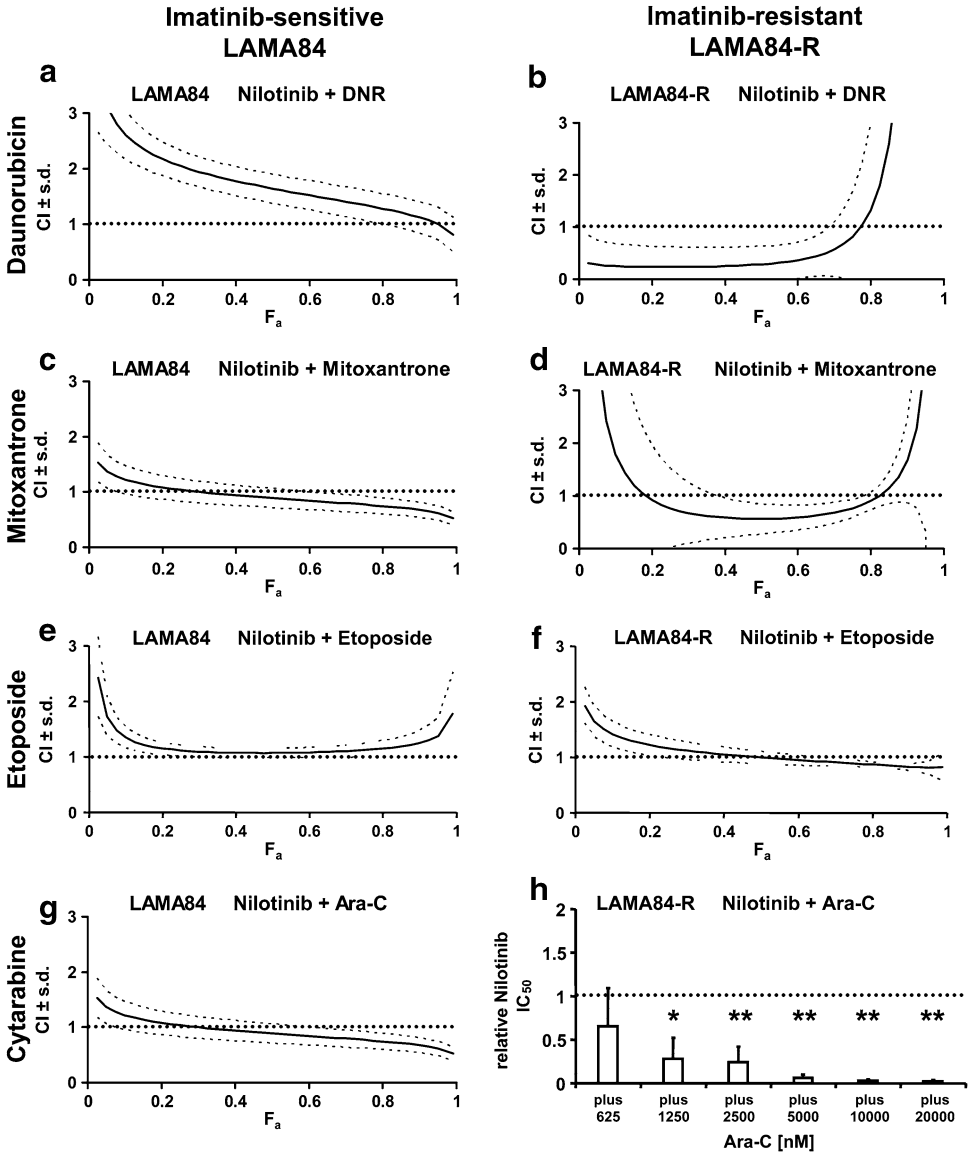

Fig. 1 Analysis of drug interactions in imatinib-sensitive and -resistant LAMA84 cells. **a** Imatinib-sensitive LAMA84 cells displayed antagonistic (CI > 1) effects upon combination treatment with nilotinib and daunorubicin. **c**, **g** Treatment of imatinib-sensitive LAMA84 cells with combinations of nilotinib plus mitoxantrone and nilotinib plus cytarabine showed synergistic effects (CI < 1) above affected fractions approximately >0.6. **e** For the combination nilotinib plus etoposide additive (CI ~ 1) antiproliferative effects were discernible in LAMA84 cells. **b**, **d**, **f** In imatinib-resistant LAMA84-R cells synergistic effects were observable for the combination nilotinib plus daunorubicin (Fa ≤ 0.7), nilotinib plus mitoxantrone (0.4 ≤ Fa < 0.8) and nilotinib plus etoposide (Fa ≥ 0.65). **h** Non-cytotoxic doses of cytarabine potentiated nilotinib activity in LAMA84-R cells (cf. "Materials and methods") *CI* combination index, *Fa* fraction affected. The *dotted line* at CI = 1 in Fig. 1a–g represents additive, >1 represents antagonistic, <1 represents synergistic effects of the applied drug combinations. The *dashed line* in **a**–**g** represents SD. The *dotted line* at 1 in **h** represents the relative nilotinib IC50 value; >1 indicates inhibition, <1 indicates potentiation of nilotinib activity. The *error bars* in **h** represent SD. $n = 5$ for each experiment. $^{*}p < 0.05$, $^{**}p < 0.01$

additive (CI ~ 1; Fig. 1e) effects, respectively. In contrast, the combinations employing mitoxantrone and cytarabine were both clearly synergistic denoted by CI values < 1 above the growth inhibition level >60% (Fa > 0.6) (Fig. 1c, g). Combination treatment of imatinib-resistant LAMA84-R cells resulted in enhanced antiproliferative effects for every regimen. Analysis of combined effects revealed synergism with CI values of 0.36 ± 0.35, 0.59 ± 0.24 and 0.91 ± 0.07 for nilotinib plus daunorubicin, nilotinib plus mitoxantrone and nilotinib plus etoposide, respectively, at the 60% growth inhibition level (Fa = 0.6) (Fig. 1b, d, f). Since cytarabine was inactive in LAMA84-R cells (Table 1), the median-effect method was not applicable for investigating combination effects. However, non-cytotoxic doses of cytarabine (≥1,250 nM) led to a significant reduction of nilotinib IC50 values (relative IC50 < 1) signifying potentiation of nilotinib activity (Fig. 1h).

In imatinib-sensitive K562 cells daunorubicin and mitoxantrone showed synergistic effects when combined with nilotinib at Fa ≥ 0.4 and Fa > 0.3, respectively, (Fig. 2a, c); nilotinib plus etoposide acted synergistically at Fa < 0.8 (Fig. 2e). In the imatinib-resistant K562-R line, combination treatment employing daunorubicin and etoposide displayed synergism (Fig. 2b, f), whereas the combination nilotinib plus mitoxantrone was at best additive at Fa = 0.6 (Fig. 2d). In both imatinib-sensitive and -resistant K562 cells no significant potentiation of nilotinib activity could be observed by cytarabine (Fig. 2g, h).

Combination treatment of imatinib-sensitive $Ba/F3_{wt}$ cells with nilotinib plus daunorubicin and nilotinib plus mitoxantrone resulted in improved antiproliferative effects as compared to the respective monotherapies with CI values indicating synergism above affected fractions ≥0.45 and Fa > 0.3, respectively (Fig. 3a, b). Depending on the

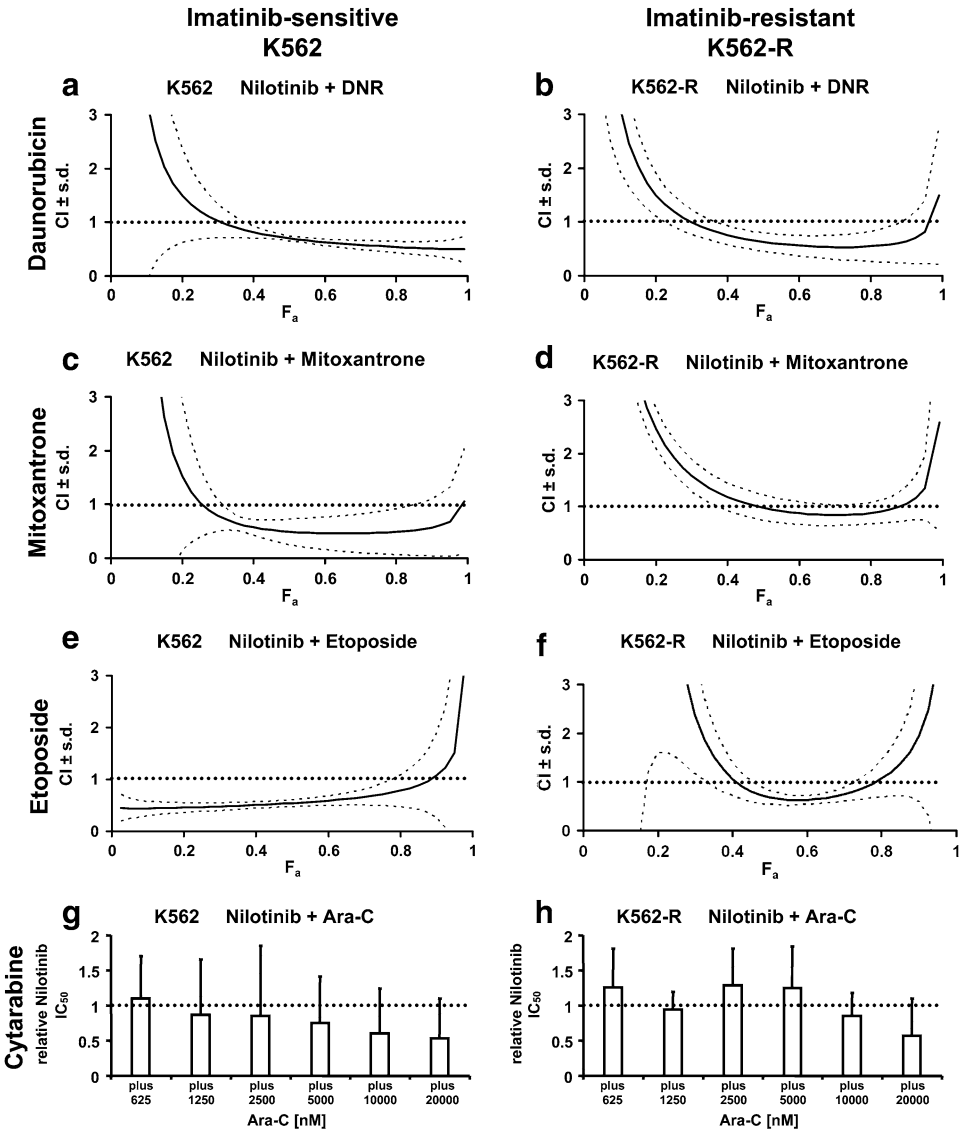

Fig. 2 Analysis of drug interactions in imatinib-sensitive and -resistant K562 cells. **a**, **c**, **e** In imatinib-sensitive K562 cells synergistic effects were detectable for the combination nilotinib plus daunorubicin (Fa ≥ 0.4), nilotinib plus mitoxantrone (Fa > 0.3) and nilotinib plus etoposide (Fa < 0.8). **b**, **f** The combinations nilotinib plus daunorubicin and nilotinib plus etoposide acted synergistically in imatinib-resistant K562 cells at 0.35 < Fa < 0.9 and 0.45 < Fa < 0.75, respectively. **d** Treatment of K562-R cells with nilotinib plus mitoxantrone displayed at best additive effects (CI ~ 1 at 0.4 < Fa < 0.9). **g**, **h** Cytarabine showed no significant potentiation of nilotinib activity in both imatinib-sensitive and -resistant K562 cells. *CI* combination index, *Fa* fraction affected. The *dotted line* at CI = 1 in Fig. 1a–g represents additive, >1 represents antagonistic, <1 represents synergistic effects of the applied drug combinations. The *dashed line* in **a**–**f** represents SD. The *dotted line* at 1 in **g** and **h** represents the relative nilotinib IC50 value; >1 indicates inhibition, <1 indicates potentiation of nilotinib activity. The *error bars* in **g** and **h** represent SD. *n* = 5 for each experiment

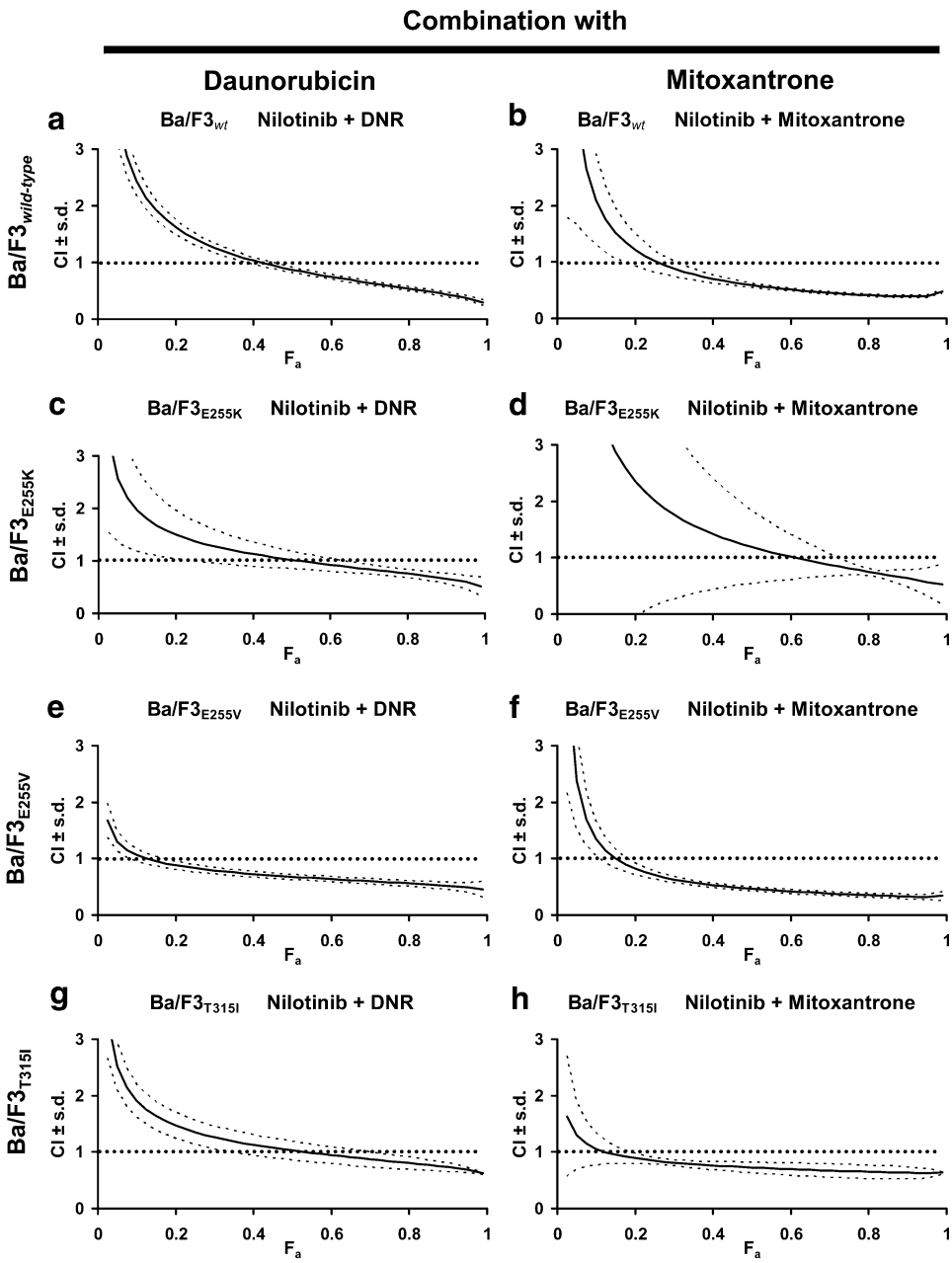

Fig. 3 Analysis of drug interactions in imatinib-sensitive Ba/F3$_{wt}$ and imatinib-resistant Ba/F3$_{E255K}$, Ba/F3$_{E255V}$ and Ba/F3$_{T315I}$ cell lines. **a**, **b** Both investigated combinations nilotinib plus daunorubicin and nilotinib plus mitoxantrone showed synergism in Ba/F3$_{wt}$ above affected fractions ≥0.45 and >0.3, respectively. **c**, **e**, **g** Co-treatment with nilotinib and daunorubicin resulted in synergistic effects in all investigated imatinib-resistant Ba/F3$_{E255K}$, Ba/F3$_{E255V}$ and Ba/F3$_{T315I}$ cell lines above Fa ≥ 0.65, Fa > 0.15 and Fa > 0.7, respectively. **d**, **f**, **h** Synergism could also be observed for the combination nilotinib plus mitoxantrone above affected fractions >0.7 and ≥0.2 in Ba/F3$_{E255K}$ and both Ba/F3$_{E255V}$ and Ba/F3$_{T315I}$ cells, respectively. *CI* combination index, *Fa* fraction affected. The *dotted line* at CI = 1 represents additive, >1 represents antagonistic, <1 represents synergistic effects of the applied drug combinations. The *dashed line* represents SD. *n* = 5 for each experiment

level of growth inhibition, the combination nilotinib plus daunorubicin showed synergistic cytotoxic effects also in the imatinib-resistant Ba/F3$_{E255K}$ (Fa ≥ 0.65), Ba/F3$_{E255V}$ (Fa > 0.15) and Ba/F3$_{T315I}$ (Fa > 0.7) cell lines (Fig. 3c, e, g). For the combination nilotinib plus mitoxantrone, synergistic effects were discernible with CI values < 1 above Fa > 0.7 in Ba/F3$_{E255K}$ and above Fa ≥ 0.2 in both Ba/F3$_{E255V}$ and Ba/F3$_{T315I}$ cells (Fig. 3d, f, h).

Induction of apoptosis

Monotherapy of the imatinib-resistant cell lines K562-R and LAMA84-R with low-dose nilotinib, daunorubicin or mitoxantrone increased the early apoptotic cell fractions (annexin V+/PI− cells) as compared to untreated controls. For 1,000 nM daunorubicin this effect was more obvious with apoptotic fractions of 18% (K562-R) and 15% (LAMA84-R) as compared to nilotinib- (13 and 11%) and mitoxantrone-induced apoptosis (10% and 8%) in the respective lines. Compared to the corresponding monotherapies, drug combinations led to enhanced apoptosis levels (16–23%) with a significant increase for nilotinib plus mitoxantrone in the LAMA84-R cell line (Fig. 4a).

In the investigated imatinib-resistant Ba/F3$_{E255K}$ and Ba/F3$_{T315I}$ cells, respectively, only a marginal increase in the proportion of apoptotic cells upon administration of low-dose

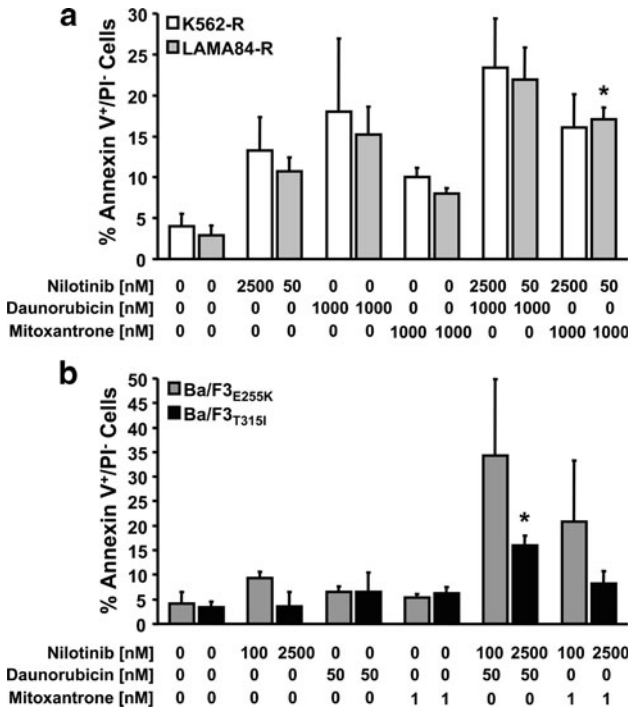

Fig. 4 Induction of apoptosis in imatinib-resistant cell lines after treatment with different concentrations of single-agent nilotinib, daunorubicin and mitoxantrone or with combinations. **a** Combination treatment of imatinib-resistant K562-R and LAMA84-R cell lines increased early apoptotic cell fractions (annexin V+/PI− cells) as compared to the respective monotherapies. A significant increase of the early apoptotic cell fraction could be demonstrated for the combination nilotinib plus mitoxantrone in the LAMA84-R cell line. **b** In imatinib-resistant Ba/$F3_{E255K}$ and Ba/$F3_{T315I}$ cells similar effects were discernible. Significant enhancement of apoptosis could only be observed in the Ba/$F3_{T315I}$ cell line upon treatment with the combination nilotinib plus daunorubicin. (*$p < 0.05$) The *error bars* represent SD. $n = 3$ for each experiment

single-agent nilotinib (9 and 4%), daunorubicin (6% for both lines) and mitoxantrone (5 and 6%) could be observed as compared to untreated controls (4 and 3%; Fig. 4b). Co-treatment of Ba/$F3_{E255K}$ cells increased the early apoptotic cell fractions for both combinations (34% for nilotinib plus daunorubicin and 21% for nilotinib plus mitoxantrone). However, only in the Ba/$F3_{T315I}$ cell line a significant enhancement of apoptosis could be observed after treatment with the combination nilotinib plus daunorubicin versus monotherapy (Fig. 4b).

Discussion

Our preclinical studies with combinations of imatinib and classic chemotherapeutic drugs demonstrated significant synergistic action that translated in a better efficacy in a clinical setting in myeloid blast crisis of CML [30]. To present, the majority of patients who progress to a blast crisis of CML have been pre-exposed to imatinib treatment at earlier disease stages and harbor a cell clone resistant to this drug. Several studies demonstrated that BCR-ABL signaling remains one of the principal mechanisms of disease pathogenesis in imatinib resistance. In cases of BCR-ABL TK mutations that confer imatinib resistance or in cases of up-regulated TK activity as compared to wild-type BCR-ABL, the newer more potent ABL kinase inhibitors are the rational treatment following imatinib failure.

However, in the course of disease evolution additional pathophysiological mechanisms may gain an increasing influence on cell proliferation, thus diminishing the efficacy of single-agent TK inhibitors in blast crisis. The logical action to overcome drug resistance is the use of drug combinations with different mechanisms of action. In the present study we investigated the effects of classical chemotherapeutic drugs cytarabine, daunorubicin, etoposide, and mitoxantrone that are typically used in the treatment of acute leukemia. The drugs were used as single agents and as combination partners for nilotinib in imatinib-sensitive CML cells and in BCR-ABL-positive leukemia cells with defined BCR-ABL TK mutations or other mechanisms of imatinib resistance.

In our previous published reports the effects of imatinib treatment as a single agent and in combination with various compounds were studied extensively both in imatinib-sensitive and -resistant cells [18, 19, 21, 22]. Compared to imatinib treatment, the single-agent nilotinib showed an improved efficacy in the investigated imatinib-sensitive cell lines, reflected by IC50 values in the nanomolar range (Table 1). In the imatinib-resistant LAMA84-R cell line nilotinib was also more effective than imatinib underlining nilotinib's high potency [11, 12, 21] (Table 1). Only the K562-R cell line, in which several different resistance mechanisms are probably involved [7, 21, 31] showed a high resistance to both imatinib and nilotinib (IC50 values > 20,000 nM) (Table 1). With the exception of Ba/$F3_{T315I}$, single-agent nilotinib was also active in the highly imatinib-resistant Ba/$F3_{E255K}$ and Ba/$F3_{E255V}$ cell lines, which is consistent with previous findings [11, 28]. Monotherapy with established chemotherapeutic agents resulted in variable antiproliferative effects in the investigated cell lines. Interestingly, in both the K562 and LAMA84 pair higher concentrations of the applied intercalating drugs and etoposide were required for growth inhibition of the imatinib-resistant cells implying cross-resistance (Table 1). Since daunorubicin, etoposide and mitoxantrone are substrates for the P-glycoprotein (Pgp) mediated drug efflux [32–34], the known increased Pgp activity of these imatinib-resistant cell lines is one possible explanation [7, 21]. Cytarabine which displayed no antiproliferative effects in the tested dose range in imatinib-resistant LAMA84-R and K562-R cells (Table 1), however, has not been demonstrated to be a Pgp substrate [35].

Combination therapy employing nilotinib and classical chemotherapeutic drugs led to improved antileukemic effects. In imatinib-resistant LAMA84-R cells these effects resulted either from synergism (CI values < 1) or potentiation of nilotinib activity (Fig. 1). In the imatinib-resistant K562-R cells, synergistic interactions could be observed to a lesser extent as compared to the imatinib-sensitive counterpart (Fig. 2). In addition, the applied drug combinations acted synergistically also in Ba/F3 cells expressing defined imatinib-resistant BCR-ABL mutants. However, in T315I carrying cells, conferring complete resistance to imatinib [11], high doses of nilotinib were required to assess the synergistic interaction (>25,000 nM) limiting the clinical significance (Fig. 3).

The antiproliferative effects of nilotinib are associated with induction of apoptosis [11, 36]. In our study, we investigated the pro-apoptotic effects in imatinib-resistant cells. Since clinically isolated BCR-ABL kinase domain mutants show a varying incidence and resistance to imatinib [6, 11, 37], we gave preference to the imatinib-resistant Ba/F3$_{T315I}$ and Ba/F3$_{E255K}$ cell lines harboring the more common BCR-ABL mutants with different levels of imatinib resistance in our experiments. Regarding combination treatment, the apoptosis results in Fig. 4 appear to be inconsistent with the synergistic effects observed in the cell proliferation assays (Figs. 1, 2, 3). In our experiments, the extent of apoptosis was defined as the proportion of early apoptotic cells (annexin V+/PI−) which in contrast to the MTT assays shows no direct correlation to the viable cell number [38]. The corresponding fractions of dead (necrotic) cells (annexin V+/PI+) were considerably higher and displayed a stronger correlation to the cell proliferation experiments (data not shown). In our experiments, a significant increase in the number of early apoptotic cells as compared to the respective monotherapies could only be observed in imatinib-resistant Ba/F3$_{T315I}$ and LAMA84-R cells for the combinations nilotinib plus daunorubicin and nilotinib plus mitoxantrone, respectively, signifying the potential of the applied intercalating drugs to enhance nilotinib-induced apoptosis (Fig. 4).

The underlying molecular mechanisms of the synergistic antiproliferative and pro-apoptotic effects of tyrosine kinase inhibitors in combination with classic antineoplastic agents are poorly investigated. Chow et al. [39] could show that the pro-apoptotic activity of nilotinib is not restricted to BCR-ABL, c-Kit, or PDGFR-positive cells. Nilotinib-induced apoptosis in cell lines of lymphatic origin was shown to critically rely on activation of caspase-6 and caspase-9 and differential expression of Src-kinases [39]. With regard to our results in Ba/F3$_{T315I}$ cells, originally derived from a murine pro B cell line [40] and showing no or only negligible expression of c-Kit or PDGFR, one could speculate that nilotinib is able to exert BCR-ABL independent pro-apoptotic effects which are only evident after sensitization by daunorubicin. Furthermore, the possibility of a physically altered interaction between nilotinib and the kinase domain of BCR-ABL after sensitization by standard chemotherapy allowing nilotinib to bind to the mutated T315I target cannot be ruled out either. In a recent report, Tiwari et al. [41] could demonstrate that nilotinib is able to reverse multidrug resistance by inhibiting the activity of ABCB1/Pgp and ABCG2/BCRP/MXR transporters. In one of our previous studies comprising functional rhodamine 123 efflux assays, we could show that imatinib-sensitive LAMA84 cells displayed a lower Pgp activity than imatinib-resistant LAMA84-R cells, whereas K562 exhibited negligible and K562-R cells low Pgp activity [21]. Consequently, in these cell lines the observed synergy between nilotinib and the investigated agents, which with the exception of cytarabine are substrates for the Pgp mediated drug efflux [32–35], may be attributed to the inhibition of Pgp by nilotinib.

The drug concentrations applied in our in vitro synergy experiments were primarily selected to enable a reliable analysis according to the Chou and Talalay method, i.e., around the IC50 in vitro. Of course, it is of great relevance, whether these concentrations are also achievable in vivo and the respective synergistic effects can be expected in a clinical setting. Zhou et al. [42] reported a steady-state C_{max} of 2,160.7 ng/mL (corresponding to 4,081 nM) in CML patients treated with the standard dose of nilotinib 400 mg twice daily. For daunorubicin, depending on the drug formulation varying plasma concentration can be observed. Feldman et al. [43] demonstrated a C_{max} of 30,185 ± 6,198 ng/mL (57,221 ± 11,749 nM) in patients treated with a liposomal daunorubicin formulation. In the work of Canal et al. [44], the mean maximum plasma concentration of mitoxantrone reached 6,429 ± 2,427 µg/L (14,464 ± 5,460 nM) at maximum tolerated dose (90 mg/m^2) versus 789 ± 158 µg/L (1,775 ± 355 nM) after administration of 15 mg/m^2. In 14 patients, who received the standard dose of 100 mg/m^2 etoposide, peak and trough serum concentrations of 18.5 µg/mL (31,433 nM) and 0.2 µg/mL (340 nM), respectively, could be observed [45]. In patients with relapsed AML or a blast crisis of CML steady-state plasma levels of cytarabine ranged between 7,000 and 24,000 nM after infusion at a dose rate of 250 mg/m^2 per hour for 36–72 h [46]. Thus, with the exception of K562-R and Ba/F3$_{T315I}$ cells, the concentrations used in our in vitro studies are achievable in a clinical setting (Table 1).

Nilotinib is approved for imatinib-resistant and -intolerant CML patients in the chronic and accelerated phase of the disease [13, 14]. The newer studies report hematologic and cytogenetic responses to nilotinib in blast crisis, although the response rates are as expected lower [15, 47]. With nilotinib, as a representative of second-generation

TKI, displaying synergistic or additive drug interactions with a number of established chemotherapeutics in CML cells, the preclinical data reported in this work support combination treatment regimens for clinical testing. Based on our previous clinical experience with imatinib-based combination treatment in myeloid blast crisis patients [30], we propose a modern treatment concept for this patient group beginning with an induction treatment with a well-tolerated chemotherapy regimen, e.g., mitoxantrone/etoposide (NOVE regimen) given along with nilotinib. Upon response to induction treatment, eligible patients should be assigned to a consolidation with allogeneic stem cell transplantation. Patients not eligible for allogeneic transplantation but fit enough to receive an intermediate-intensive chemotherapy treatment can be offered an alternative consolidation with, e.g., intermediate-dose cytarabine combined with nilotinib. After completion of consolidation, a life-long maintenance treatment with nilotinib can be expected to be advantageous for patients not receiving an allogeneic transplant and therefore still harboring a residual CML clone. Regarding the low incidence of CML blast crisis under efficient imatinib treatment, nowadays a concerted multicenter effort is needed to implement such a treatment concept in order to yield patient recruitment sufficient for further analysis.

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