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Priority Report

Cancer Research

Antifungal Therapy with Itraconazole Impairs the Anti-Lymphoma Effects of Rituximab by Inhibiting Recruitment of CD20 to Cell Surface Lipid Rafts

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Abstract

Immunotherapy with rituximab alone or in conjunction with chemotherapy has significantly improved the treatment outcome of B-cell lymphoma patients. Nevertheless, a subpopulation of patients does not respond to rituximab. The reason for treatment failure as well as the exact mechanism of action is still uncertain. The function of rituximab has long been associated with the partitioning of CD20 molecules to membrane microdomains. Here, we show that concomitant antifungal treatment with itraconazole impairs the rituximab anti-lymphoma effect both *in vitro* and *in vivo*. At the molecular level, recruitment of CD20 to lipid rafts is inhibited in the presence of itraconazole. Furthermore, calcium influx, which is crucial for rituximab-mediated cell death, was nearly completely abolished by itraconazole treatment. In contrast, the antifungal drug caspofungin did not inhibit CD20 recruitment to lipid rafts, nor did it affect calcium influx or the cytotoxic effect of rituximab. The finding that itraconazole also abolished the cytotoxic effects of other therapeutic antibodies directed against lipid raft–associated molecules (i.e., CD20 and CD52) but not those against the non–raft-associated molecule CD33 further supported our proposed mechanism of action. Our results argue that concomitant medications must be adjusted carefully to achieve optimal antitumor effects with monoclonal antibodies. *Cancer Res; 70(11); 4292–6. ©2010 AACR.*

Introduction

The monoclonal antibody (mAb) rituximab directed against the cell surface molecule CD20 of mature B cells has been proved to be successful in the treatment of a variety of B-cell malignancies (1, 2). Rituximab has been shown to induce cell killing via antibody-dependent cell-mediated cytotoxicity (3), complement-dependent cytotoxicity (CDC; ref. 4), and the induction of apoptosis (5). On the molecular level, membrane microdomains have been shown to be associated with the function of rituximab. Binding of rituximab causes a rapid redistribution of CD20 molecules to detergent-resistant membranes termed lipid rafts (6). These specialized microdomains of the plasma membrane are highly enriched in sphingolipids and cholesterol (7).

It is well documented that patients with hematologic malignancies have a high risk of developing invasive fungal infections. The higher risk is attributed to host defense impairment due to intensive cytotoxic and immunosuppressive therapies. *Candida* spp. have been the main cause of invasive fungal infections. As early treatment initiation in patients with invasive fungal infections has a profound effect on mortality rates, different antifungal treatment strategies like prophylaxis and empirical and preemptive treatment have been developed. Azoles are the most broadly used antifungal drugs inhibiting the sterol 14α-demethylase activity and blocking sterol biosynthesis, which is lethal in unicellular organisms (8). In animals, it has been shown that azoles lower endogenous cholesterol synthesis (9). As lipid rafts consist of sphingolipids and cholesterol and lipid rafts have been shown to be crucial for rituximab-induced cell death, we asked whether rituximab exhibits its full anti-lymphoma effect in the presence of azoles. Indeed, here we show that rituximab-mediated cell death is impaired in the presence of itraconazole both *in vitro* and *in vivo*. Furthermore, antifungal treatment with itraconazole only affects targeted treatment against raft-associated molecules (CD20 and CD52) but not against the non–raft-associated molecule CD33. This suggests the importance of the molecular event of raft recruitment for the full anti-lymphoma effect of the mAbs rituximab and alemtuzumab.

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Materials and Methods

Cell lines, reagents, and antibodies

SUDHL4, Jurkat, and Kasumi cells were obtained from DSMZ. Cholera toxin subunit B-FITC was purchased from Molecular Probes, rituximab from Roche, gemtuzumab

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from Wyeth, alemtuzumab from Bayer Schering, itraconazole from Janssen-Cilag, anti-CD20 from Novocastra (UK), and anti-CD52 and anti-CD33 mAbs from Santa Cruz Biotechnology.

Inhibition of functional rafts using methyl-β-cyclodextrine or itraconazole

Cells were incubated for 30 minutes with 10 mmol/L methyl-β-cyclodextrine (MβCD; Sigma) and for 1 hour with itraconazole or caspofungin. After addition of rituximab, cells were incubated for 20 minutes at 37°C and lysed, and then raft fractions were prepared (10).

Rituximab-mediated cytotoxicity assay

For rituximab-mediated cell death (apoptosis and CDC), 1×10^6 cells were resuspended in culture medium with 10% human serum (non–heat inactivated) with or without 100 μg/mL rituximab at 37°C for 24 hours. Dead and viable cells were discriminated by Annexin/propidium iodide (PI) staining (11).

Purification of raft fractions and Western blotting

Purification of raft fractions has been described (10).

Ca^{2+} influx

SUDHL4 cells were loaded with Fluo-3AM (4 μmol/L) and seeded in a poly-D-lysine–coated microtiter plate. After 1-hour incubation at 37°C, the cells were washed twice in assay buffer and analyzed. Rituximab was then added and, immediately, quantitative changes in the intracellular Ca^{2+} were monitored by fluorescence-activated cell sorting (12).

Xenotransplants

Six- to eight-week-old female immunodeficient mice (CD-17 scid) were obtained from Charles River. Lymphoma cells (10×10^7) suspended in sterile PBS (100 μL) were injected s.c. into the right shoulder region. Mice with similar tumor sizes (~100 mm^2) were selected and randomized to treatment groups (five mice per group). Mice were treated with 250 μg rituximab weekly i.v. or left untreated. A loading dose of itraconazole (100 μg) was administered i.v. before rituximab was injected. Then, itraconazole was given p.o. (20 mg/kg; ref. 13). Animals were monitored for general appearance and tumor volume. Mice were killed if tumors caused discomfort. All animal experiments were authorized by a regional government agency (Regierung von Oberbayern, license no. 55.2-1-54-2531-52-07).

Results

Antifungal therapy with itraconazole, but not caspofungin, impairs rituximab-mediated cell death through inhibition of CD20 raft recruitment

The mAb rituximab has been shown to effectively eradicate CD20-positive lymphoma cells both *in vitro* and *in vivo*. To test whether rituximab-mediated cell death was affected by itraconazole, we incubated the diffuse large B-cell lymphoma cell line SUDHL4 with rituximab in the presence or absence of itraconazole. Treatment with rituximab induced cell death in about 60% of the lymphoma cells. This effect was mainly due to CDC. In the presence of heat-inactivated serum, the anti-lymphoma effect of rituximab was only 20% to 30% (data not shown). The rituximab anti-lymphoma effect was almost completely abolished in the presence of itraconazole, but not by the antifungal drug caspofungin (Fig. 1A). The attenuated effect of rituximab in the presence of itraconazole was highly statistically significant (Fig. 1B). As rituximab binding results in CD20 raft recruitment necessary for its cytotoxic effect, we next asked whether the impaired response to rituximab in the presence of itraconazole was due to differences in CD20 raft recruitment. To this aim, lipid rafts were isolated by sucrose gradient centrifugation. As shown previously, rituximab treatment resulted in immediate CD20 raft recruitment (4, 11). MβCD, which inhibits the formation of functional lipid rafts by cholesterol depletion, was used as a control. CD20 raft recruitment was observed neither in the presence of MβCD nor in the presence of itraconazole (Fig. 1C). We confirmed these data using another lymphoma cell line, Nceb (data not shown).

Translocation of CD20 into lipid rafts has been shown to be crucial for calcium influx and apoptosis (12). Therefore, we next tested the rituximab-induced calcium flux in the absence and presence of itraconazole. Indeed, calcium flux was almost completely abolished in the presence of itraconazole but not caspofungin (Fig. 1D), indicating that concomitant medication with itraconazole critically interferes with the rituximab-mediated anti-lymphoma effect.

Itraconazole impairs rituximab-induced lymphoma control *in vivo*

To test whether a sufficient concentration of itraconazole that is able to impair rituximab-mediated cell death would be achieved *in vivo*, we used a xenograft mouse model. Lymphoma cells were inoculated s.c. After measurable tumors were visible, mice were left untreated or treated with rituximab or with a combination of rituximab and itraconazole. In mice treated with rituximab, no lymphoma growth for more than 2 weeks was observed. In contrast, untreated mice developed a rapid progression of the lymphoma with a 3-fold increase in size within 18 days. Most importantly, in mice that were treated with rituximab in the presence of itraconazole, rituximab failed to prevent tumor growth, indicating that the concentration was sufficient to antagonize the rituximab anti-lymphoma effect (Fig. 2). The differences in tumor growth were statistically significant (Fig. 2), showing that concomitant medication with itraconazole has to be reconsidered in conjunction with rituximab treatment. Of note, CDC induced by rituximab *in vitro* in the presence of mouse serum was significantly lower (<50%) compared with human serum (data not shown). Therefore, the *in vivo* effect in the xenograft mouse model may underestimate the effect of rituximab in humans.

Only antibody treatment against raft-associated molecules is impaired by itraconazole

The importance of lipid raft recruitment has been shown for several receptors including the antigen B- and T-cell receptors (14, 15). In patients with chronic lymphocytic leukemia, alemtuzumab, a mAb directed against CD52, induces cell death

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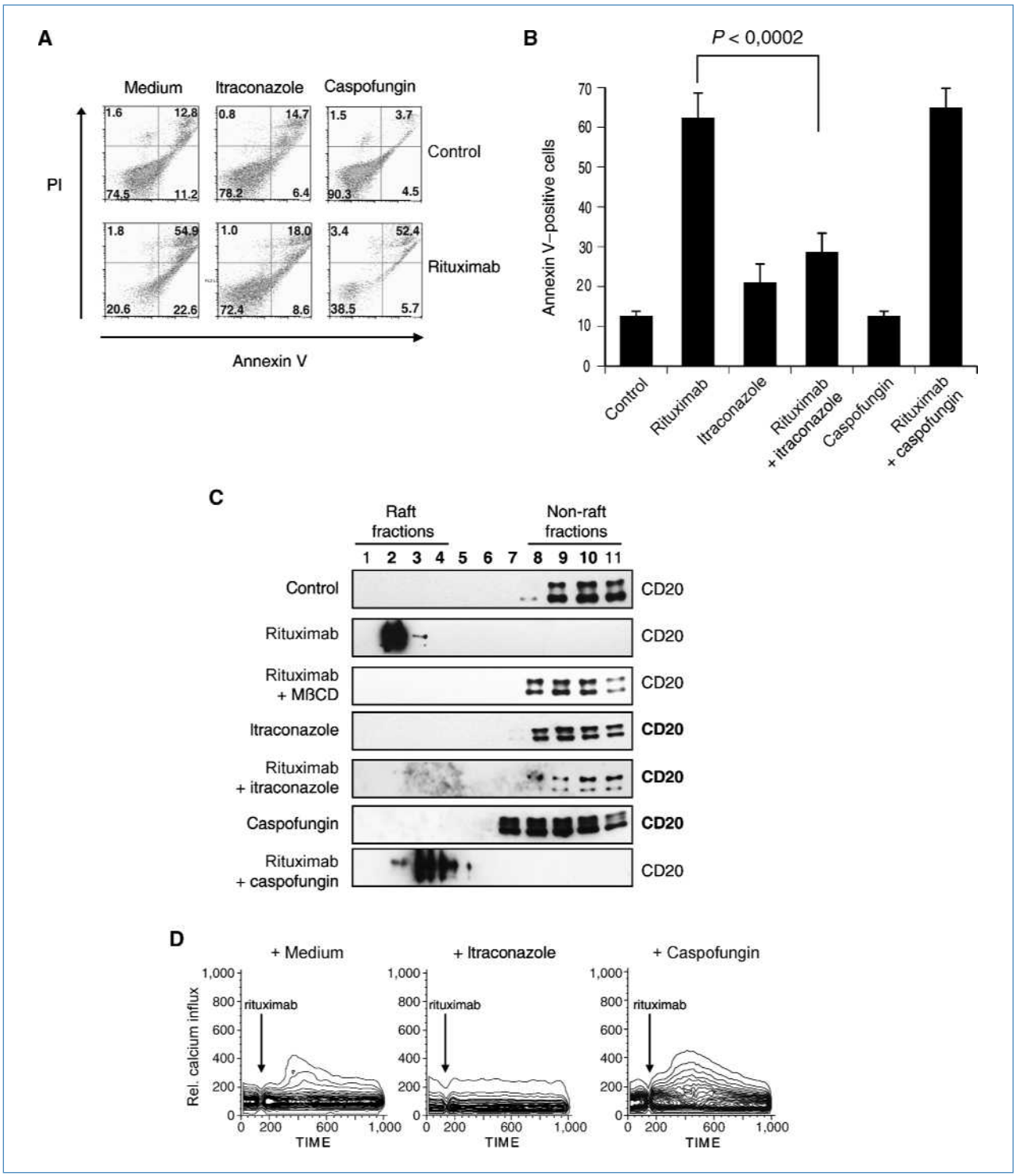

Figure 1. Antifungal therapy with itraconazole, but not caspofungin, impairs rituximab-mediated cell death through inhibition of CD20 raft recruitment. A, the diffuse large B-cell lymphoma cell line SUDHL4 was treated with 100 μg/mL rituximab for 24 h or left untreated in the presence or absence of itraconazole (10 μg/mL). Cells were analyzed by flow cytometry with Annexin/PI staining (A). Three independent experiments were analyzed, and Annexin V–positive cells are presented as mean ± SD (B). C, SUDHL4 cells were preincubated or not with 10 mmol/L MβCD, itraconazole (10 μg/mL), or caspofungin (64 μg/mL), followed by treatment with 100 μg/mL rituximab for 20 min or left untreated. Cells were lysed and sucrose gradient fractions were prepared. Raft fractions (1–4) and non-raft fractions (7–10) were separated by SDS-PAGE. Immunoblotting was done with an anti-CD20 specific mAb. D, SUDHL4 cells were incubated for 24 h with itraconazole or caspofungin, or left untreated. Then cells were incubated for 1 h at 37°C with Fluo-3AM followed by rituximab (100 μg/mL) stimulation. Rituximab induced store-operated Ca^{2+} entry in Fluo-3AM–loaded SUDHL4 cells. The kinetics of the Ca^{2+}-influx traces are shown (D).

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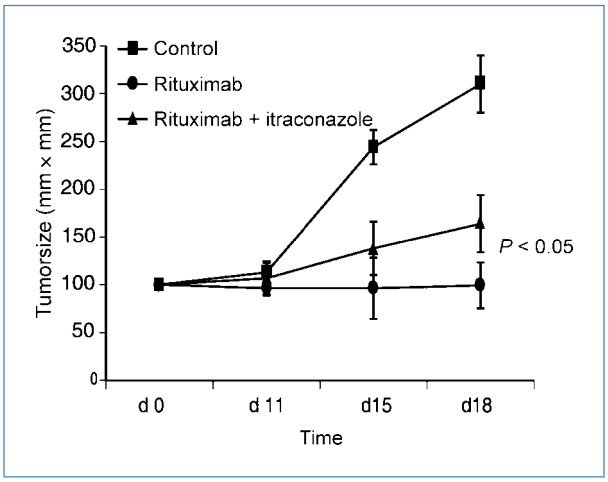

Figure 2. Itraconazole impairs rituximab-induced lymphoma control *in vivo*. Lymphoma cells (10×10^7) suspended in sterile PBS (100 μL) were injected s.c. into the right shoulder region of immunodeficient mice (CD-17 scid). Mice with similar tumor sizes (~100 mm^2) were selected and randomized to treatment groups (five mice per group). Mice were treated with 250 μg rituximab weekly i.v. or left untreated. A loading dose of itraconazole (100 μg) was administered i.v. before rituximab was injected. In addition, itraconazole was given p.o. (20 mg/kg). Animals were monitored for general appearance and tumor volume. Tumor size was measured at the indicated time points.

through a lipid raft–dependent mechanism (16). To test whether the effect of itraconazole was a general phenomenon or only affecting rituximab-mediated cell death, we treated Jurkat lymphoma cells with alemtuzumab in the presence or absence of itraconazole. Indeed, itraconazole impaired alemtuzumab-induced cell death in a dose-dependent manner (Fig. 3A). Furthermore, treatment with alemtuzumab resulted in a rapid recruitment of CD52 to lipid microdomains (Fig. 3B), indicating the importance of the molecular event.

In contrast, gemtuzumab ozogamicin is a humanized anti-CD33 mAb that is used in the therapy of patients with acute myeloid leukemia. Fungal infections are a major problem in these patients. Therefore, a possible interaction of gemtuzumab and itraconazole would be of great clinical relevance. Interestingly, in contrast to rituximab and alemtuzumab, the anti-leukemic function of gemtuzumab was not affected by itraconazole (Fig. 3A). Importantly, no CD33 recruitment to lipid rafts was observed after anti-CD33 mAb treatment (Fig. 3B). This further supports our proposed mechanism of action that itraconazole inhibits the segregation of raft-associated molecules to lipid microdomains after treatment with mAbs. However, as this anti-CD33 antibody is linked to a derivative of calicheamicin, a potent cytotoxic antibiotic, we cannot rule out that the cytotoxic effects of therapeutic antibodies directed against lipid raft–associated molecules would be less impaired by itraconazole if these antibodies were linked to toxins.

Discussion

In the present study, we show that rituximab-mediated cell death is impaired in the presence of itraconazole both *in vitro* and *in vivo*. Furthermore, not only rituximab but also alemtuzumab was affected by itraconazole. Finally, we present the underlying mechanism of how azoles interfere with anticancer drugs (i.e., mAbs). Of note, only mAbs that lead to lipid raft recruitment are affected by itraconazole. Therefore, these data have significant clinical implications for the treatment of patients with mAbs and might help in the use of these drugs without losing their efficacy by concomitant medication. Importantly, the antifungal drug caspofungin did not affect lipid raft recruitment, Ca^{2+} influx, or the rituximab anti-lymphoma effect. This finding is remarkable because another antifungal drug, nystatin, a polyene, is well known to inhibit functional lipid rafts (17). Therefore, in contrast to polyene and azole antifungal drugs,

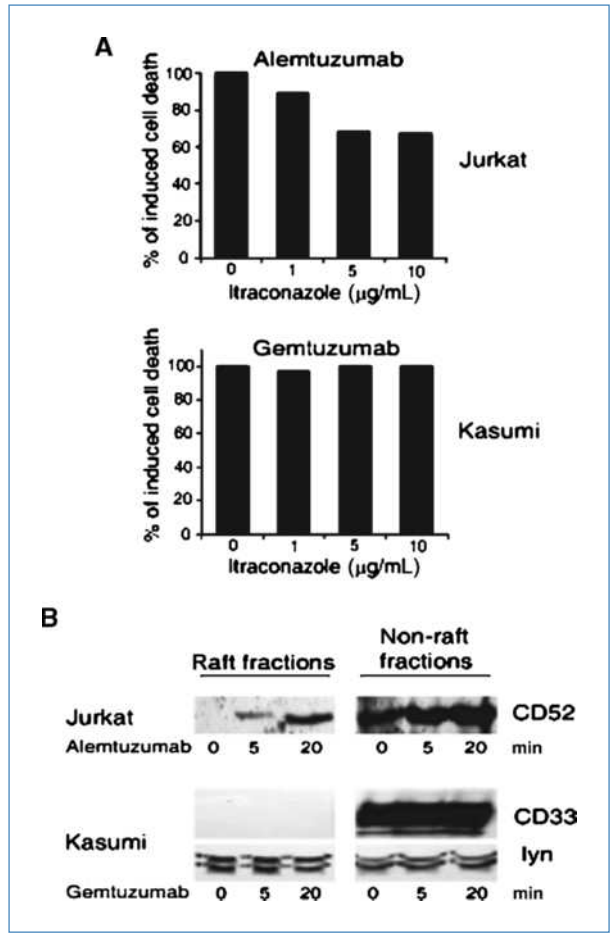

Figure 3. Only antibody treatment against raft-associated molecules is impaired by itraconazole. A, Jurkat cells or Kasumi cells were incubated with either alemtuzumab or gemtuzumab for 24 h in the presence or absence of itraconazole at the indicated concentrations. Cell death was analyzed by Annexin/PI staining. Numbers shown are the percentages of rituximab-induced cell death in the presence and absence of itraconazole compared with untreated cells. B, Jurkat cells or Kasumi cells were incubated with either alemtuzumab or gemtuzumab in the presence of itraconazole as indicated. Cells were lysed and sucrose gradient fractions were prepared. Raft fractions (1–4) and non-raft fractions (7–10) were pooled and separated by SDS-PAGE. Immunoblotting was done with an anti-CD52 or anti-CD33 specific mAb.

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the echinocandine caspofungin is the only antifungal drug that does not affect rituximab-induced cell death. Based on our *in vitro* studies, the use of caspofungin offers an easy solution to circumvent the problem of drug interference with rituximab in case a simultaneous antifungal therapy is necessary.

Of course, not every patient receiving rituximab also needs antifungal therapy. However, besides the standard R-CHOP immunochemotherapies, rituximab is also given together with chemotherapy as conditioning therapy for patients with recurrent lymphoma who receive autologous hematopoietic stem cell transplantation and for patients with CD20-positive acute lymphoblastic leukemia. For these patients, antifungal prophylaxis with azoles is recommended (18). Therefore, the scenario of a concomitant therapy of azoles and rituximab is not frequent but common.

Recently, the fact that concomitant medication may influence rituximab treatment has been shown. Statins impaired rituximab-induced anti-lymphoma effect by inducing a conformational change of CD20 (19). This led to an impaired binding of anti-CD20 mAb. However, no functional data were presented that the anti-lymphoma effect was impaired. Therefore, it is unclear from this study whether statins exhibit an anti-rituximab effect *in vivo* or whether further mAbs are affected by statins. In a recent prospective cohort analysis, no interference of statins with rituximab *in vivo* was observed (20). Therefore, the effect of this study is still unclear. We now show that itraconazole severely impairs the function of the mAb rituximab both *in vitro* and *in vivo*. Furthermore, alemtuzumab was also affected in the presence of the antifungal drug. Therefore, it seems that our finding that azoles inhibit the effect of mAbs that are directed against raft-associated molecules is a general phenomenon rather than an antibody-specific effect.

During the past few years, progress has been made in the treatment of several malignant diseases. This progress is mainly due to the development of targeted therapies with mAbs and tyrosine kinase inhibitors. The number of mAbs as well as kinase inhibitors is steadily increasing. Defining the mechanism of how mAbs are affected by concomitant medication is so important especially because not all mAbs are affected by azoles. Therefore, these data have significant clinical implications for the treatment with mAbs and might help in the use of these drugs without losing their efficacy by concomitant medication.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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