Effect of frequently used chemotherapeutic drugs on the cytotoxic activity of human natural killer cells

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Abstract

Tumors are considered to be possible targets of immunotherapy using stimulated and expanded autologous or allogeneic natural killer (NK) cells mismatched for MHC class I molecules and inhibitory NK receptors. NK cell–based immunoadjuvant therapies are carried out in combination with standard chemotherapeutic protocols. In the presented study, we characterized the effect of 28 frequently used chemotherapeutic agents on the capacity of NK cells to kill target cells. We found that treatment of NK cells with the drugs vinblastine, paclitaxel, docetaxel, cladribine, chlorambucil, bortezomib, and MG-132 effectively inhibited NK cell–mediated killing without affecting the viability of NK cells. On the other hand, the following drugs permitted efficient NK cell–mediated killing even at concentrations comparable with or higher than the maximally achieved therapeutic concentration *in vivo* in humans: asparaginase, bevacizumab, bleomycin, doxorubicin, epirubicin, etoposide, 5-fluorouracil, hydroxyurea, streptozocin, and 6-mercaptopurine. [Mol Cancer Ther 2007;6(2):644–54]

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Introduction

The goal of immune-based adjuvant therapeutic strategies is to stimulate immune effector cells to kill malignant cells, which may result in a better clinical outcome. Infiltration of solid tumors with inflammatory cells is a well-known phenomenon (1–3). Several studies showed that the infiltration of natural killer (NK) cells in malignant tumors was associated with a better clinical outcome (4, 5).

NK cells are large granular lymphocytes that are part of the innate immune system (6). Mature NK cells are mostly circulating in the peripheral blood, where they represent ~10% to 15% of all blood lymphocytes. Phenotypically, NK cells express surface antigen CD56, which has unknown function on NK cells, and lack the typical T-cell antigen CD3 (7). Physiologically, NK cells have direct cytotoxic activity against tumor targets and virus-infected cells (8). NK cells can regulate adaptive immune response by IFN-γ production and can mediate antibody-dependent cellular toxicity through membrane FcγRIII (CD16; ref. 6). These cells have the ability to directly kill target cells and produce immunoregulatory cytokines in transfused cancer patients (9–12).

The outcome of NK cell-target cell interactions is regulated by a fine integrative balance between inhibitory and activating receptors; the first elicited by the recognition of MHC class I molecules by NK inhibitory receptors (killer immunoglobulin–like receptors, CD94/NKG2A, and immunoglobulin-like transcript) and the latter by NK cell–activating (NKG2D and natural cytotoxicity receptor) receptors that can sense stress inducible glycoprotein (MHC class I chain–related proteins and UL16 binding proteins) expression on tumor- and virus-infected target cells (13). Because NK cells are able to spontaneously lyse tumor cells, considerable effort has been focused on investigating the possible use of NK cells in the treatment of human cancer. Recent understanding of NK cell development and function has permitted the investigation of novel NK cell–based immunotherapeutic modalities. A promising approach is to expand allogeneic NK cell clones *in vitro*, with typed killer immunoglobulin–like receptor expression, and to transfuse these cells into leukemic patients (14–16).

Basse et al. (17) showed that the level of accumulation of NK cells in murine tumor metastases *in vivo* is dependent on the dose and frequency of interleukin 2 (IL-2) administration. NK cells have also been used in human therapeutic regimens that involved the administration of high or low doses of IL-2, alone or in combination with IL-2–activated blood lymphocytes or purified populations of NK cells, for primary lung and renal cell carcinomas (18, 19). In other studies, extended low-dose IL-2 was used to activate NK cells in patients with solid tumors who received intensive chemotherapy or in patients with acute

myeloid leukemia (20, 21). Harker et al. (22) suggested that IL-2–induced lymphokine-activated killer cells may be useful in the treatment of chemotherapy-resistant cancers.

The chemotherapeutic regimens for malignancies restrict the effectivity of the immune system and significantly decrease the level of NK cells (23). The activity of NK cells is also affected by some cytostatic drugs (24). Utsugi et al. (25) observed enhanced NK cell–mediated cytotoxicity following treatment of the target cells with topoisomerase inhibitors. Killing by NK cells requires microtubule integrity, which may be influenced by antimicrotubule agents (26). It is important to consider the drug-induced effects when chemotherapy and NK cell–mediated immunotherapy are planned to be used in parallel.

The aim of this study was to systematically investigate the effect of the most frequently used cytostatic drugs on NK cell–mediated cytotoxicity. We carried out more than 4,000 cell survival assays on single-cell levels using automated laser confocal microscopy to establish the pattern of *in vitro* effect of 29 different cytotoxic drugs on the efficiency of NK cell–mediated killing.

Materials and Methods

Isolation and Culturing of Human Polyclonal Peripheral Blood NK Cell Cultures

Polyclonal NK cells were acquired from lymphocyte-enriched buffy coat derived from healthy donor blood (Blodbank, Karolinska Sjukhuset, Stockholm). Peripheral blood mononuclear cells were isolated by centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). NK cells were isolated from peripheral blood mononuclear cells by negative magnetic bead selection (Stemcell Technologies, Vancouver, Canada) according to the manufacturer's instructions. Purified NK cells were >95% $CD3^{-}CD56^{+}$. NK cells were cultured either overnight or for several weeks in IMDM supplemented with 10% human serum (AB, Blodbank), 2 mmol/L L-glutamine, 1× nonessential amino acids, 1 mmol/L sodium pyruvate, 50 units/mL penicillin-streptomycin, 50 μmol/L 2-mercaptoethanol (media and supplements bought from Life Technologies, Inc., Carlsbad, CA), and 100 units/mL IL-2 (Peprotech, Inc., London, United Kingdom).

Eight primary NK cell isolates were tested in the present study. Five NK cell isolates were restimulated every 3 to

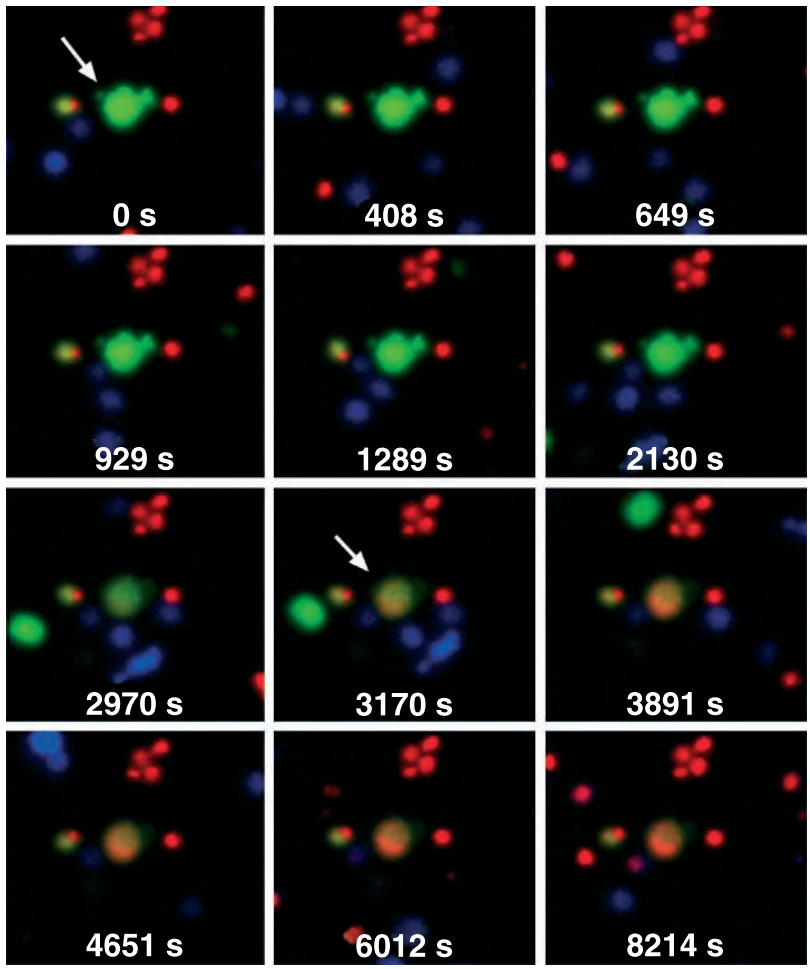

Figure 1. Time-lapse image sequence of NK cells killing a K562 target cell. Target cells were labeled with CellTracker green (CMFDA; Invitrogen). NK cells were labeled with CellTracker blue (CMAC; Invitrogen). Dead cells were labeled with ethidium bromide (*red*). Images were taken with an automated laser confocal microscope (a modified Perkin-Elmer UltraView LCI). *Arrows,* green target cells as they get killed by the surrounding NK cells and turn red. Time scale is given in seconds.

Table 1. AUC values of the used drugs and calculated RAUC values for NK cells

Drug groups		Drug name	Brand name, company	Concentrations with NK cells alone (μg/mL)
Topoisomerase inhibitor effect	Anthracyclines	Epirubicin	Epirubicin Meda, Meda	0.02–2.5
		Daunorubicin	Cerubidin®, Aventis Pharma	0.05–6.25
		Doxorubicin	Doxorubicin Teva, Teva	0.02–2.5
	Epipodophyllotoxin	Etoposide	Sigma	0.244–31.25
	Camptothecins	Topotecan	Hycamtin, GlaxoSmithKline	0.04–5
Microtubule inhibitors	Taxanes	Paclitaxel	Taxol, Orifarm	0.06–7.5
		Docetaxel	Taxotere®, Aventis Pharma	0.4–50
	Vinca alkaloids	Vincristine	Vincristine Mayne, Mayne Pharma	0.005–0.625
		Vinblastine	Velbe®, STADApharm	0.001–1.25
		Vinorelbine	Navelbine®, Pierre Fabre	0.098–12.5
	Platinum analogues	Carboplatin	Carboplatin Mayne, Mayne Pharma	0.098–12.5
		Oxaliplatin	Eloxatin, Sanofi-Synthelabo	0.05–6.25
Antimetabolites	Antifolate	Methotrexate	Methotrexate Pharmacia, Pfizer	0.244–31.25
	Purine antagonists	6-Mercaptopurine	Sigma	0.814–104.16
		Cladribine	Leustatin, Janssen-Cilag	0.01–1.25
	Pyrimidine antagonists	5-Fluorouracil	Fluorouracil Mayne, Mayne Pharma	0.488–62.5
		Cytarabine	Cytarabine Pfizer, Pfizer	0.977–125
		Gemcitabine	Gemzar, Orifarm	0.586–75
	Antitumor antibiotics	Bleomycin	Bleomycin Baxter, Baxter	0.293–37.5*
		Dactinomycin	Cosmegen®, MSD	0.005–0.625
	Protease inhibitors	Bortezomib	Velcade®, Janssen-Cilag	0.01–1.25
		MG-132	Sigma	0.098–12.5
	Alkylating agents	Cyclophosphamide	Sendoxan, Baxter	0.4–50
		Ifosphamide	Holoxan®, Baxter	0.4–50
		Chlorambucil	Sigma	0.977–125
		Streptozocin	Sigma	0.488–62.5
	Miscellaneous	Asparaginase	Asparaginase Medac, Medac	0.05–6.25*
		Hydroxyurea	Sigma	0.488–62.5
		Bevacizumab	Avastin®, Roche	0.244–31.25

NOTE: Ratio of *in vitro* and *in vivo* AUC levels (RAUC) values were formed to calculate relationship between the *in vitro* drug concentrations and the clinical doses. *In vitro* AUC values were determined using the following formula: *in vitro* used concentration (μg/mL) × 20 h.

*For bleomycin and asparaginase, the concentrations are in international units per milliliter.

†Not used in the clinical practice.

‡No data are available.

§GlaxoSmithKline Research Triangle Park N. Prescribing Information Leukeran. Available from: http://us.gsk.com/products/assets/us_leukeran.pdf.

4 days with fresh IL-2–containing media for 1 week and were periodically monitored for the $CD3^{-}CD56^{+}$ phenotype. Three NK cell isolates were stimulated with IL-2 only for 24 h.

Cell Lines and Culture Conditions

Cytotoxic NK tumor cell lines Nishi (27), NKL (28), NK92 (29), and KHYG-1 (30) were cultured in IMDM supplemented with different concentrations of IL-2 (Nishi and NK92, 100 units/mL; NKL, 200 units/mL; KHYG-1, 450 units/mL). K562 cell line was used as target in the *in vitro* killing assay. These cells were cultured in IMDM (Sigma, Dorset, United Kingdom) supplemented with 10% FCS (Sigma), 100 mmol/L L-glutamine (Sigma), and 80 μg/mL gentamicin (Sigma). Cell suspensions were grown in a humidified incubator at 37°C in an atmosphere containing 5% CO_2. Cell counts were adjusted to 1×10^6/mL and the cells were fed twice a week. The absence of *Mycoplasma* contamination was ensured by regular monitoring with Hoechst 33258 staining.

In vitro Killing Assay (Standard Chromium-51 Release Assay)

Target cells were labeled for 1 h at 37°C, 7.5% CO_2 with 100 μCi of $Na_2^{51}CrO_4$ (Amersham Biosciences, Uppsala, Sweden) added directly to the cell pellet. Radioactively labeled target cells were washed thrice with RPMI, and 15×10^3 cells were plated per well in flat-bottomed 96-well microtiter plates to replicate cell densities in the flat-bottomed microtiter plates. NK cells were subsequently added at 5:1 effector-to-target ratio per well (total volume of 200 μL/well) and incubated for 5 h at 37°C, 5% CO_2. Spontaneous release was determined by incubation of labeled target cells with media alone whereas maximal release was determined

Table 1. AUC values of the used drugs and calculated RAUC values for NK cells (Cont'd)

Concentrations with target cells together (μg/mL)	AUC *in vivo* (μg h/mL)	*In vivo* dose (AUC)	References	RAUC for NK cells
0.01–1.245	2.412	90 mg/m^2	Fogli et al. (31)	0.17–20.65
0.02–3.125	1.2786	1.5 mg/kg	Andersson et al. (32)	0.75–97.75
0.01–1.245	0.82464	50 mg/m^2	Toffoli et al. (33)	0.5–60.4
0.12–15.625	5.06	100 mg/m^2/d	Gruber et al. (34)	0.97–123.52
0.02–2.5	0.0196	1.2 mg/m^2/d	Gerrits et al. (35)	39.85–5,101
0.03–3.75	13.49	175 mg/m^2	Fogli et al. (31)	0.08–11.12
0.2–25	3.326	85 mg/m^2	Rischin et al. (36)	2.35–301
0.003–0.312	0.182	1.32 mg/m^2	Desai et al. (37)	0.5–68.67
0.005–0.625	0.218	1.7 mg/m^2	Bates et al. (38)	0.9–114.6
0.05–6.25	0.899	80 mg/m^2/wk	Freyer et al. (39)	2.17–277.94
0.05–6.25	348000	360 mg/m^2	Ghazal-Aswad et al. (40)	5.6×10^{-6}–0.0008
0.025–3.125	71.5	130 mg/m^2	Graham et al. (41)	0.02–1.75
0.12–15.625	13200000	12 g/m^2	Crews et al. (42)	3.7×10^{-7}–4.7×10^{-5}
0.4–52.08	0.2587	85 mg/m^2	Chan et al. (43)	62.8–8,052.57
0.005–0.625	0.1541	5 mg/m^2	Albertioni et al. (44)	1.26–162.17
0.244–31.245	11.59	400 mg/m^2	Casale et al. (45)	0.85–107.83
0.5–62.5	523.4	1 g/m^2	Gruber et al. (34)	0.04–4.78
0.3–37.5	9.3	1,000 mg/m^2	Fogli et al. (31)	1.25–161.3
0.15–18.75*	0.089*	8 IU/kg/d	Peng et al. (46)	65.5–8,427
0.003–0.312	300	1.5 mg/m^2	Veal et al. (47)	0.00033–0.0416
0.001–0.625	0.0438	1.45 mg/m^2	Papandreou et al. (48)	4.45–571
0.05–6.25	†			‡
0.2–25	367	50 mg/kg	Xie et al. (49)	0.02–2.72
0.2–25	1,827.7	3 g/m^2/d	Boddy et al. (50)	0.005–0.55
0.5–62.5	0.883	0.2 mg/m^2	(GlaxoSmithKline Research Triangle Park)§	22.12–2,831
0.24–31.245	‡			‡
0.025–3.125*	0.943*	30.000 IU/m^2	Ylikangas et al. (51)	1.04–132.53
0.24–31.245	82.49	15 mg/kg	Yan et al. (52)	0.12–15.15
0.122–15.625	‡			‡

by lysis of target cells in 10% SDS. Spontaneous release was <15% of the maximal release. One hundred microliters of supernatant were collected for scintillation counting on a γ irradiation counter. Each measurement was done in triplicate and the average of three wells was used for calculation of specific lysis. Specific lysis (killing effectiveness) was calculated as follows: killing effectiveness (%) = [(cpm experimental well − cpm spontaneous release) / (cpm maximal release − cpm spontaneous release)] × 100.

In vitro Killing Assay (Novel Fluorescent Labeled Killing Assay)

The effects of drugs on *in vitro* killing efficacy of different NK cell lines were assessed using microtiter plates. Twenty-nine drugs were tested, each at four different concentrations in triplicates on flat-bottomed 384-well plates. Each well was loaded with 20-μL cell suspension of NK cells containing 15,000 cells and was incubated for 20 h at 37°C in 5% of CO_2. Target (K562) cells were stained with CellTracker green CMFDA (Invitrogen, Sweden). A time-lapse image sequence of NK cell killing of target cells is illustrated in Fig. 1. As a next step, 20 μL of target cells (K562) containing 3,000 cells were seeded on the same plate and were incubated with the NK cells for 5 h (effector-to-target ratio, 5:1). Dead cells were differentially stained red with ethidium bromide. The precise numbers of green target cells and red dead cells were determined by fluorescent detection using a custom-designed automated laser confocal fluorescent microscope (a modified Perkin-Elmer UltraView LCI) at the Karolinska Institute visualization core facility. The images were captured using the computer program QuantCapture 4.0, and the living and dead target cells were identified and individually counted using the computer program CytotoxCount 3.0. The target cells were identified as dead if they were stained red and green at the same time. Both programs were developed at Karolinska Institute visualization core facility using OpenLab Automator programming environment (Improvision). On each plate, eight wells were set up as controls to determine the baseline killing activity. These wells contained NK and K562 cells in culture medium only (without drugs). As additional control, effector NK cells alone were incubated under the same conditions. To monitor the possible short-term effect on the target cells, K562 cells were incubated alone and with all the individual

drugs for 5 h. Killing effectiveness of NK cells was calculated for every well using the following formula: [number of dead target cells (both red and green signal in the same cell) / number of all (green) target cells] × 100.

To normalize the killing effect values of different NK populations, the following formula was used: (killing effectiveness in the wells with drugs / mean killing effectiveness values of the control wells) × 100%. Mean killing efficacy was determined from the average killing of all NK populations. The red-stained cells that were not green at the same time were considered as dead NK cells. The cytotoxic effect (%) of the drugs on NK cells was calculated with the following formula: (number of dead NK cells in the wells with drugs / number of dead NK cells in the control wells) × 100%.

Drugs

For the *in vitro* killing test, 29 drugs were used (summarized in Table 1). Twenty-eight of these drugs are regularly used in the treatment of human patients. All the drugs were dissolved in 50% DMSO and printed on 384-well flat-bottomed plates using a high-density metal pin array (50-nL replica volumes) in Biomek 2000 fluid dispenser robot (Beckman, Fullerton, CA). The same robot was used to generate the drug masterplates containing the triplicates of four different drug dilutions using a single-tip automatic dispenser head.

The highest drug concentration was selected as the maximum drug concentration physiochemically achievable at 600× dilution (50 nL of drug in a 30-μL assay volume). These concentrations are in a comparable range with the *in vivo* plasma concentrations in patients (31–52).[5] The starting concentrations of the dilution series (1×, 5×, 25×, 125×) for the individual drugs were initially determined based on the solubility of the different agents. The fifth to sixth rows of Table 1 represent the drug concentrations that were presented in the wells with NK cells alone and after increasing the volumes with 20-μL cell suspension of target cells.

Testing the drug plates on a large number of *in vitro* tumor cell lines and primary tumors, it was possible to find sensitive cell lines for each individual drugs, showing that all the drugs on the plate were active (data not shown).

A way to calculate a relationship between the *in vitro* drug concentrations and the *in vivo* ones is to use the area under the curve (AUC) values of the individual drugs. For this comparison, ratio of area under the curve (RAUC) values were determined by the following formula: *in vitro* used concentration (μg/mL) × 20 h/ AUC (μg h/mL) *in vivo*. A RAUC value >1 indicates that the *in vitro* used dose is higher than the one used in clinical practice. If this value is 1, it means that our *in vitro* dose corresponds to the clinical dose. *In vivo* AUC levels, clinical doses, and calculated RAUC values of the individual drugs from the literature are summarized in Table 1. RAUC values are also shown in Fig. 2.

[5] GlaxoSmithKline Research Triangle Park N. Prescribing Information Leukeran. Available from: http://us.gsk.com/products/assets/us_leukeran.pdf.

Results

Comparison of the Novel *In vitro* Killing Assay to the Standard Chromium-51 Release Assay

The two methods were compared with each other in the absence of drugs under the same conditions (incubation time, effector-to-target ratio, flat-bottomed plates instead of V-bottomed plates). The results of our single-cell fluorescence method correlate closely to the standard chromium-51 release assay on different NK cell lines. Figure 3 shows the *in vitro* killing capacity of a NK tumor cell line (NKL) and two NK cell clones tested with both methods.

Effect of Drugs on the Killing Capacity of Different NK Cells

The eight different primary NK cell polyclonal preparations from healthy donors and four NK tumor cell lines that were tested with the novel fluorescent method in the present study represent a variety of cells with different origin and *in vitro* history. The investigation included separated NK cells from healthy donors with 1 week of *in vitro* culturing together with IL-2 along with freshly separated NK cells cultured with IL-2 only for 24 h. NK tumor cell lines showed relatively low killing efficiency (≤10%) in our novel experimental model system. Microscopy evaluation of killing activity requires the use of flat-bottomed plate instead of conventional V-bottomed plates. Many of the NK tumor lines showed low spontaneous mobility and required high-level cellular crowding to be able to kill effectively. Control killing effectiveness values for all investigated NK cell lines can be seen in Table 2.

The NK cells of different origins showed a very similar killing efficacy pattern in the presence of most of the drugs. This phenomenon is illustrated in Fig. 4, summarizing the killing effectiveness of all NK cell populations in the presence of 29 drugs at four different concentrations. Each individual thin curve represents the average of three independent measurements. The thick broken line is the mean of all curves, the mean killing efficacy. The curves labeled by black spots are NK cell isolates, treated with IL-2 only for 1 day. The gray shaded area marks the SD. Figure 5 illustrates the cytotoxic effect (%) of all of the drugs on NK cells, as shown in a similar way in Fig. 4. All the mean killing efficacy values are represented in Table 3. Importantly, none of the drugs influenced the viability of the K562 target cells during the 5-h incubation (data not shown). The number of dead NK cells in the drug-treated cultures that received IL-2 only for 1 day was higher than those in the drug-treated cultures that received IL-2 for 1 week. None of the NK populations showed dose-dependent increase in the number of dead cells. Consequently, the dose-dependent decrease in the killing effect of NK cells in case of certain drugs cannot be simply explained by drug-induced NK cell death (Fig. 5).

Identification of Highly Effective and Noneffective Drugs

We found that the most effective drugs that inhibited NK cell–mediated killing were chlorambucil, MG-132, docetaxel, cladribine, paclitaxel, bortezomib, gemcitabine, and

vinblastine (Fig. 4). For MG-132, no RAUC value could be defined because this drug has not been clinically used. Oxaliplatin, dactinomycin, cytarabine, daunorubicin, vincristine, and topotecan were only marginally effective.

Most NK cell lines were not affected by bevacizumab, bleomycin, doxorubicin, epirubicin, vinorelbine, carboplatin, methotrexate, ifosphamide, etoposide, hydroxyurea, asparaginase, 5-fluorouracil, 6-mercaptopurine, streptozocin, and cyclophosphamide.

Discussion

In our experimental model, the killing effectiveness of NK cells was tested in the presence of different cytostatic drugs. Previous studies investigated NK cell–mediated cytotoxicity following treatment of target cells with certain cytotoxic drugs (25) or treatment of NK cells with a few chemotherapeutic agents before the NK cell killing assays (24, 26). No systematic study has been carried out to investigate the effect of a large number of different drugs at different concentrations.

In our experimental setup, the NK cells were preincubated alone for 20 h with the drugs to test the drug effect only on the NK cells. This was followed by coincubation with the target cells for 5 h. No drug-induced cytotoxicity was detectable on target cells during this incubation, although other effects on target cells, which influence NK cell killing, cannot be excluded.

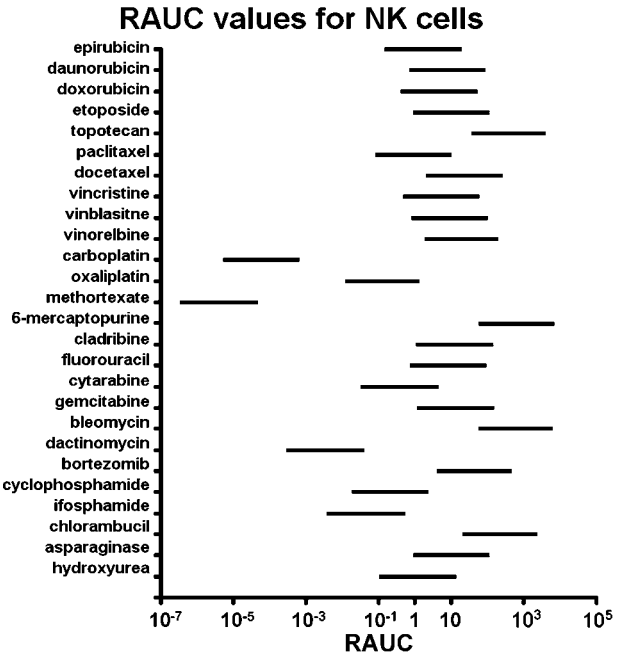

Figure 2. RAUC values for NK cells (data are also represented in Table 1). To calculate relationship between the *in vitro* used drug concentrations and the *in vivo* used values, RAUC values were determined by the following formula: *in vitro* used concentration (μg/mL) × 20 h / AUC (μg h/mL) *in vivo*. RAUC > 1, the *in vitro* dose is higher than the clinical dose. RAUC < 1, the *in vitro* dose is lower than the clinical dose. RAUC = 1, the *in vitro* dose is corresponding to the clinical dose.

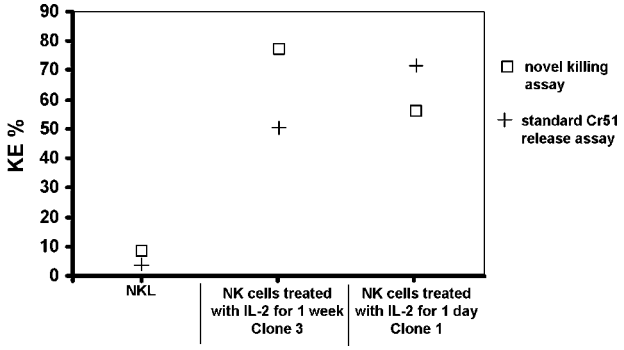

Figure 3. Comparing of the fluorescent *in vitro* killing assay to the standard chromium-51 release assay. NKL cell line and two IL-2 – activated primary NK cell polyclonal preparations were incubated together with K562 target cells for 5 h. Effector-to-target ratio was always 5:1. *KE,* killing effectiveness.

The presented data suggest that IL-2–activated NK cell lines share a common profile of cytotoxic drug sensitivity. This profile does not change with different lengths of *in vitro* culturing with IL-2.

Topotecan, 6-mercaptopurine, and bleomycin did not inhibit NK cell killing effectively even at very high AUC levels. Almost no effect could be seen in case of carboplatin, methotrexate, and dactinomycin, although this might be explained by the relatively low range of RAUC levels achieved in the microcultures.

Although chlorambucil efficiently inhibited the killing effect of NK cells, other alkylating agents like cyclophosphamide and ifosphamide did not affect NK cell killing. This might be explained by the fact that both of the latter compounds are prodrugs that have to be converted into active metabolites by the liver *in vivo*.

The effectiveness of chlorambucil may also be explained by the high *in vitro* RAUC levels (Fig. 2; Table 1). The

Table 2. Killing effectiveness of different NK cell lines

	KE (%)	SD
NK cells treated with IL-2 for 1 wk		
Clone 1	27.93	5.01
Clone 2	64.16	8.49
Clone 3	77.47	14.27
Clone 4	20.92	2.5
Clone 5	24.61	4.92
NK cells treated with IL-2 for 1 d		
Clone 1	55.99	4.67
Clone 2	41.05	3.56
Clone 3	31.83	7.01
NK tumor cell lines		
Nishi	5.9	2.53
NKL	8.55	1.54
NK92	10.05	4.48
KHYG-1	9.94	3.06

Abbreviation: KE, killing effectiveness.

in vitro doses (*in vitro* AUC levels) of the other effective drugs were in close vicinity to the clinical doses.

Unlike Reiter et al. (53) who found no inhibition of NK cell killing ability when effector cells (NK cells) alone were treated with cladribine, this agent was the only drug among the base analogues that significantly decreased the killing effect of IL-2–activated NK cells in our experimental setup.

Our data also showed that NK cells are particularly sensitive to antimicrotubule drugs and proteosome inhibitors. The dependence of NK cell–mediated killing on microtubule integrity is well known (24, 26). Here we show that different microtubule inhibitors have a variable effect on NK cell–mediated killing. Vinorelbine and vincristine had much less effect on NK cell–mediated killing than vinblastine, docetaxel, or paclitaxel.

Several studies showed that the chymotryptic activity of proteasomes in NK cells might play a role in their cell-mediated cytotoxicity (54–56). Kitson et al. (54) reported that synthetic proteasome inhibitors like CEP-1508, CEP-1612, and CEP-3117 can inhibit the rat NK proteasome in a dose-dependent manner and found a 50% inhibition of IL-2–activated NK cell–mediated cytotoxicity. Lu et al. (57) pointed out that MG-132 could reduce NK

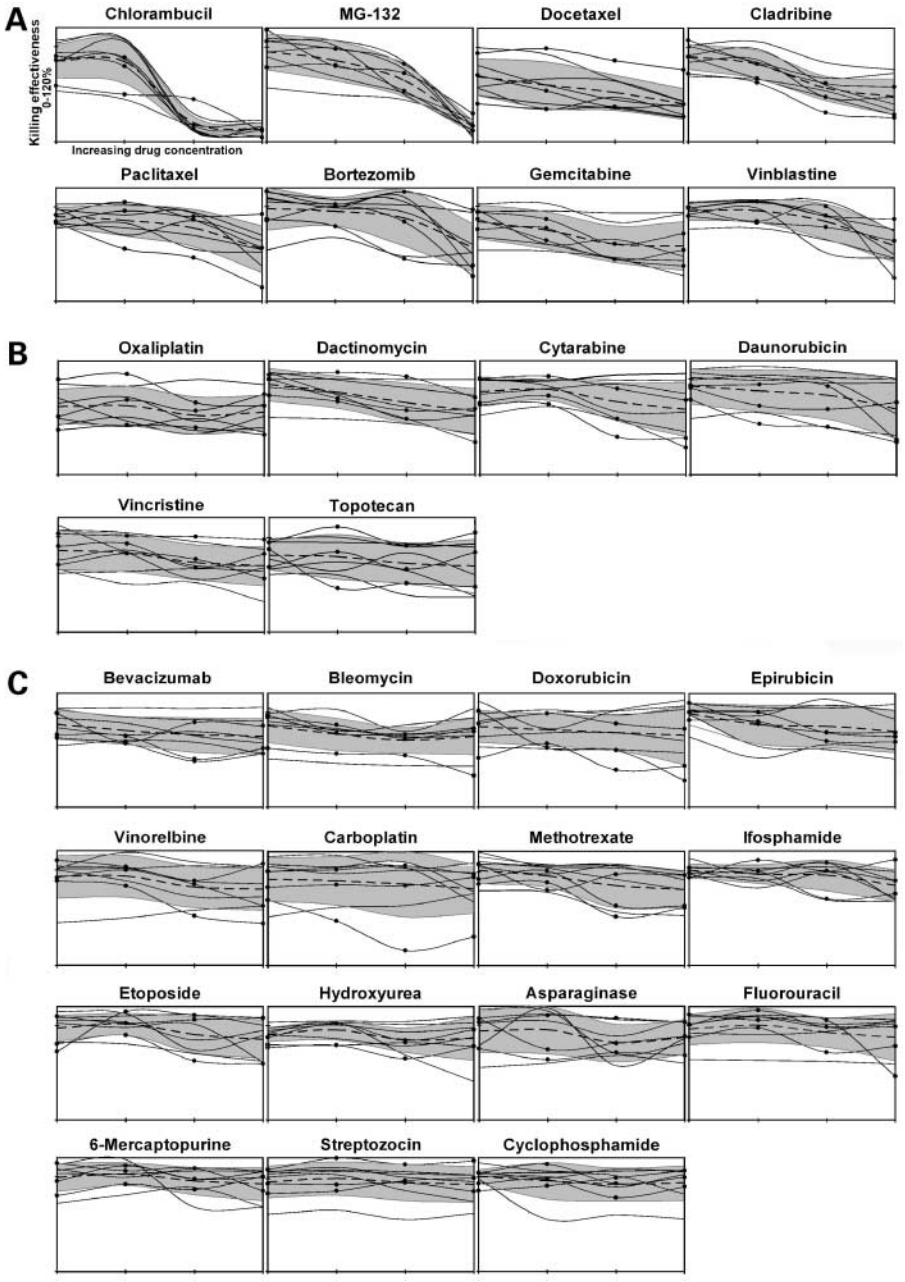

Figure 4. Killing effectiveness of NK cells tested with the fluorescent killing assay. The figure summarizes the killing effectiveness of all NK cell populations in the presence of 29 drugs at four different concentrations. *Thin curves,* average of three independent measurements. *Thick broken line,* mean of all curves, the mean killing efficacy (*MKE*). *Curves with black spots,* NK cell isolates that were treated with IL-2 only for 1 d. *Gray shaded area,* SD. **A,** effective drugs (lowest mean killing efficacy, <60%). **B,** marginally effective drugs (lowest mean killing efficacy, 60–70%). **C,** noneffective drugs (lowest mean killing efficacy, >70%). *X axis,* increasing concentrations of the drug. *Y axis,* killing effectiveness of NK cells 0% to 120%.

Table 3. Mean killing effectiveness of NK cells in the presence of different drugs

	125× dilution	25× dilution	5× dilution	Highest concentration		125× dilution	25× dilution	5× dilution	Highest concentration
Epirubicin					Paclitaxel				
MKE (%)	98.52	87.28	85.24	79.66	MKE (%)	89.43	85.03	77.36	54.97
SD	11.45	20.67	22.28	21.91	SD	9.87	16.00	19.37	24.69
Daunorubicin					Docetaxel				
MKE (%)	92.29	87.61	83.01	68.43	MKE (%)	68.49	60.11	51.97	39.85
SD	18.22	22.09	24.92	30.94	SD	19.35	24.56	18.40	16.46
Doxorubicin					Vincristine				
MKE (%)	84.77	81.47	78.30	75.29	MKE (%)	85.61	82.92	72.76	69.32
SD	16.55	17.25	18.15	30.55	SD	20.38	19.56	18.43	20.86
Etoposide					Vinblastine				
MKE (%)	97.15	101.54	90.41	85.49	MKE (%)	94.98	95.51	83.78	58.15
SD	13.66	11.38	19.18	20.52	SD	9.66	12.13	18.38	16.69
Topotecan					Vinorelbine				
MKE (%)	81.25	79.76	72.78	69.60	MKE (%)	92.91	93.43	82.14	79.76
SD	14.95	23.82	21.74	28.46	SD	22.12	21.90	21.63	21.96
Methotrexate					Carboplatin				
MKE (%)	101.79	95.65	85.15	80.89	MKE (%)	90.87	89.36	85.42	80.62
SD	14.39	11.99	23.38	18.75	SD	23.03	25.56	34.35	25.91
6-Mercaptopurine					Oxaliplatin				
MKE (%)	99.16	103.02	95.28	92.25	MKE (%)	71.79	72.72	62.47	65.78
SD	14.60	12.08	14.46	17.24	SD	18.56	20.61	19.54	18.20
Cladribine					Bleomycin				
MKE (%)	89.87	81.83	56.65	47.66	MKE (%)	85.54	76.67	70.38	74.41
SD	12.89	14.85	12.82	18.89	SD	17.85	16.97	13.54	19.01
Fluorouracil					Dactinomycin				
MKE (%)	96.24	100.34	92.61	87.67	MKE (%)	95.06	87.14	75.95	67.18
SD	15.90	18.31	15.49	25.05	SD	17.25	17.06	21.22	23.69
Cytarabine					Bortezomib				
MKE (%)	87.38	89.48	76.69	68.42	MKE (%)	97.76	94.86	88.10	56.40
SD	15.52	11.12	20.15	28.22	SD	22.20	14.83	29.71	25.26
Gemcitabine					MG-132				
MKE (%)	83.92	74.97	60.22	58.12	MKE (%)	94.95	85.47	64.43	15.79
SD	17.96	19.41	19.41	25.37	SD	18.81	20.96	17.03	6.67
Cyclophosphamide					Asparaginase				
MKE (%)	100.39	95.74	91.91	93.40	MKE (%)	93.40	94.73	81.18	87.03
SD	6.79	19.12	17.13	18.21	SD	22.02	24.74	19.73	18.42
Ifosphamide					Hydroxyurea				
MKE (%)	99.42	95.93	94.26	83.80	MKE (%)	90.27	94.93	84.75	86.42
SD	7.06	9.50	15.95	16.78	SD	7.51	10.20	12.82	22.98
Chlorambucil					Bevacizumab				
MKE (%)	84.79	83.86	19.81	13.14	MKE (%)	86.96	80.60	75.82	74.41
SD	17.64	22.51	6.37	6.67	SD	15.19	14.59	18.07	18.46
Streptozocin									
MKE (%)	96.02	99.77	95.52	93.34					
SD	16.86	17.94	19.45	19.09					

NOTE: Data were also used for preparing Fig. 4. MKE (%), mean killing effectiveness.

cell–mediated cytotoxicity of rat NK cells, but this was due to the apoptosis-inducing property of the drug. Here, we show that proteosome inhibitors severely compromise the killing efficiency of human NK cells even in the absence of apoptosis induction.

Bortezomib is a clinically used reversible inhibitor of the chymotrypsin-like activity of the 26S proteasome. A study showed that bortezomib can sensitize tumor cells to the lytic effects of dendritic cell–activated immune effector cells like NK cells or $CD8^+$ lymphocytes (58), but no data have been published on the direct inhibition effect of bortezomib on NK cell–mediated cytotoxicity.

Our results suggest that chemotherapy protocols that include proteosome inhibitors or antimicrotubule drugs may interfere with NK cell–based immunotherapy, if applied simultaneously.

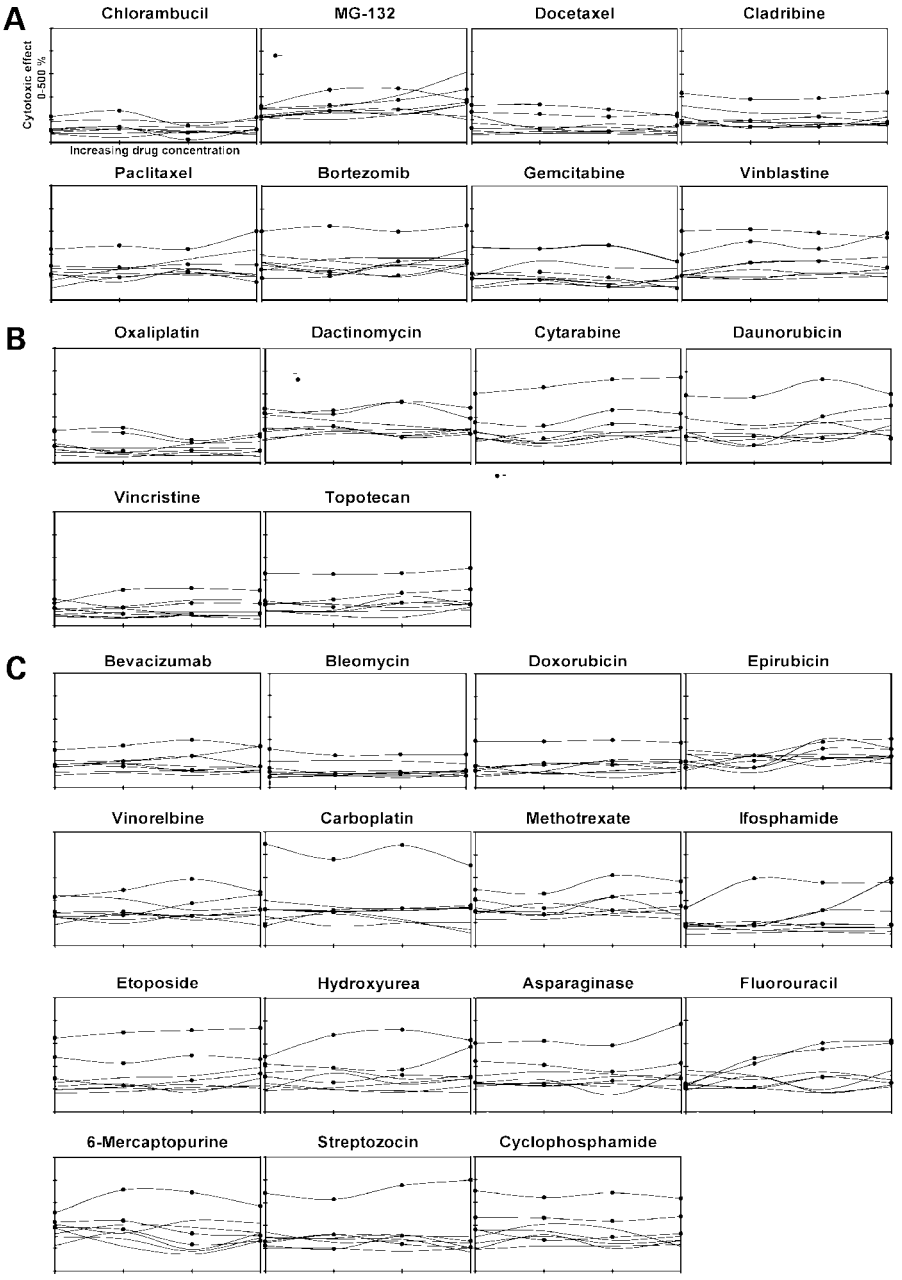

Figure 5. Cytotoxic effect on NK cells tested with the fluorescent killing assay. *Thin curves,* average of three independent measurements. *Curves with black spots,* NK cell isolates treated with IL-2 only for 1 d. The cytotoxic effect (%) of the drugs on NK cells was calculated using the following formula: (number of dead NK cells in the wells with drugs / number of dead NK cells in the control wells) × 100%. [Cytotoxic effect (%) of >100% means more dead NK cells.] **A,** effective drugs. **B,** marginally effective drugs. **C,** noneffective drugs. *X axis,* increasing concentrations of the drug. *Y axis,* cytotoxic effect of the drug compared with the control 0% to 500% (control means NK cell survival without the drug, which is 100%).

Based on the almost complete absence of inhibitory effect on NK cell–mediated killing even at high RAUC levels (concentrations that are directly comparable with maximally achieved *in vivo* therapeutic doses), we suggest that the following drugs may be effectively combined with adjuvant NK cell–based immunotherapy: asparaginase, bevacizumab, bleomycin, doxorubicin, epirubicin, etoposide, 5-fluorouracil, hydroxyurea, streptozocin, and 6-mercaptopurine.

Several anticancer drugs have been reported to induce the expression of ligands for activating NK cell receptors on the tumor cell membrane potentially increasing NK cell–mediated cytotoxicity against tumor cells. This information, in combination with the data presented here, could potentially be used in the future to design new NK cell–based adjuvant immunotherapy protocols.

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