Jpn. J. Cancer Res. 85, 659–664, June 1994

# Circumvention of Daunorubicin Resistance by a New Tamoxifen Derivative, Toremifene, in Multidrug-resistant Cell Line

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**The reversing effect of toremifene, a new tamoxifen derivative, on multidrug resistance in a K562 subline and its mechanism were studied. K562 cells were cultured in serially increasing concentrations up to 1.0 $\mu M$ daunorubicin (DNR), and were found to be 28 times more resistant to DNR in comparison to the parent cells. In the resistant cell line (K562/D1-9), intracellular accumulation of DNR was less than that of the parent cell line, and P-glycoprotein was overexpressed. The resistance was reversed by addition of toremifene in a dose-dependent manner in K562/D1-9, while toremifene had no effect in K562. DNR accumulation was also reversed by toremifene in K562/D1-9, but not in K562. However, there was no significant difference of toremifene retention between K562/D1-9 and K562, and neither verapamil nor DNR increased toremifene accumulation in K562/D1-9. Moreover, toremifene and verapamil did not show an additive effect on intracellular DNR accumulation. These results suggested that the reversing mechanism of toremifene is different from that of verapamil, and this compound could be a good candidate for overcoming multidrug resistance.**

Key words: Multidrug resistance — Daunorubicin — Toremifene

Development of drug resistance, especially multidrug resistance (MDR), is one of the most important problems in chemotherapy for acute leukemia and other cancers. Recently, overexpression of P-glycoprotein (PGP) has been identified as one of the main mechanisms of MDR.[1] In hematologic malignancies, expression of PGP has been reported to be associated with clinical resistance and early relapse during the blastic crisis of chronic myelocytic leukemia, chronic lymphocytic leukemia, multiple myeloma and acute leukemia.[2-5]

In recent years the reversal of MDR by various compounds has been reported.[6-9] However, due to their own side effects or cytotoxicity, most could not be used safely for this purpose in clinical practice. Toremifene (NK 622) is a new antiestrogenic tamoxifen derivative and is a promising agent with no serious side effects for use in breast cancer treatment.[10, 11] Tamoxifen was reported to increase daunorubicin (DNR) retention in multidrug-resistant cell lines with PGP and this effect was shown to be unrelated to its antiestrogenic properties.[7] Therefore, we examined whether toremifene could also overcome DNR resistance in a human leukemia cell line, because DNR is used mainly in acute leukemia chemotherapy rather than doxorubicin (DOX). The mechanism was also investigated.

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## MATERIALS AND METHODS

**Chemicals** Toremifene (NK622), $^3$H-toremifene and etoposide (VP-16) were kindly supplied by Nippon Kayaku Co., Tokyo. Anti-cancer agents were obtained from the following sources: DNR, from Meiji Seika Co. Tokyo; DOX, from Kyowa Hakko Co., Tokyo; vincristine (VCR), from Shionogi Seiyaku Co. Ltd., Osaka; idarubicin (IDA), from Phalmitalia Co., Milan, Italy. Monoclonal antibody for PGP, MRK16[12] was a gift from Dr. Tsuruo. All other chemicals were obtained commercially.

**Tumor cell lines** The human myelogenous leukemia cell line (K562) was obtained from Dainihon Seiyaku Co., Osaka. A DNR-resistant K562 cell line (K562/D1-9) was obtained by serial passage of wild-type K562 with incrementally increased concentrations of DNR in RPMI 1640 medium (Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with 10% fetal calf serum (FCS; Flow Laboratories, McLean, VA, USA).[13] Cells in the early log phase were used for the following experiments.

**Drug sensitivity** Drug sensitivity was tested *in vitro* by the trypan-blue dye exclusion method. The cells were incubated with various antitumor agents, DOX, VCR, DNR and IDA, for 72 h at 37°C and the $IC_{50}$ (50%-inhibitory concentration of a drug for cell growth) was determined.

**PGP phenotype** The PGP phenotype was analyzed immunocytochemically by the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique with the

monoclonal antibodies MRK16 and C219 (P-glyco-CHEK, Centcor, USA), as described elsewhere.[5] Flow cytometry (FCM, Epics Profile, Nikkaki Co., Tokyo) was also used for detection of PGP with MRK16.

**DNR accumulation** After incubation of K562 cells or K562/D1-9 cells with 1.0 $\mu M$ DNR at 37°C in RPMI 1640 with 10% FCS, cells were harvested at the designated time and washed twice with ice-cold phosphate-buffered saline (PBS), then intracellular drug accumulation was measured by FCM at 570 nm. The effect of toremifene, tamoxifen or verapamil on intracellular drug accumulation was also investigated. The concentration of verapamil was selected based on the study by Tsuruo *et al.*[6]

**DNR efflux** Cells were cultured in the presence of an adequate concentration of $^3$H-DNR. The cells were washed with a drug-free medium, resuspended in it, and cultured for a designated time. Intracellular DNR concentration was determined by previously described methods.[14] Briefly, cell suspensions were overlayered on silicon oil and centrifuged. Cells were separated from the medium containing drugs and lysed by NCS tissue solubilizer (Amersham, Arlington Heights, Canada), then the radioactivity was measured.

**Toremifene accumulation** Cells were incubated with 1.0 $\mu M$ $^3$H-toremifene in RPMI 1640 medium containing 10% FCS. Then cells were harvested after 60 min incubation. Intracellular toremifene was also determined by the silicon oil method mentioned above.

**Partition coefficients** $^3$H-Toremifene was dissolved in octanol. The same volume of pure water was added and thoroughly mixed. After centrifugation at 1000$g$ for 10 min, the levels of radioactivity of the octanolic phase and the water phase were measured. The partition coefficient was calculated as the ratio of radioactivity of the octanolic phase to that of the water phase.

## RESULTS

**Establishment of DNR resistance** A number of DOX- or vinca alkaloids-resistant cell lines have been reported and most of them have cross resistance to DNR,[1, 8, 15] but DNR-resistant cell lines appear rarely and the mechanism of DNR resistance is not known. We have established a DNR-resistant cell line K562/D1-9 by serial culture. K562/D1-9 was 28 times more resistant to DNR than the wild-type K562, on the basis of a cell growth inhibition study using the trypan-blue dye exclusion method.

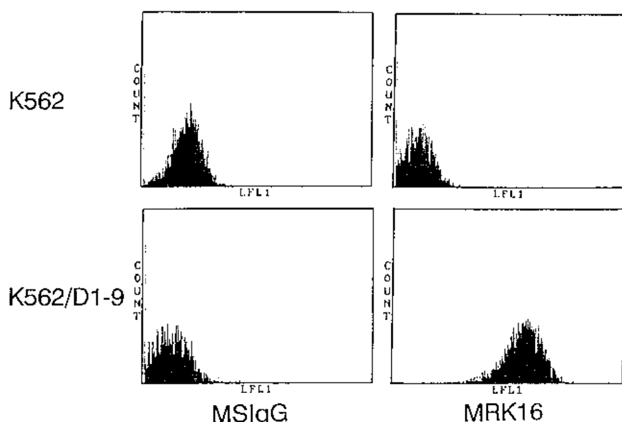


Fig. 1. Detection of PGP by FCM. Cells were incubated with MRK16 or MS-IgG (mouse IgG for negative control) at 4°C for 30 min and washed by PBS containing 0.04% $NaN_3$ and 5% FCS. The cells were reincubated with FITC-conjugated rabbit antimouse IgG for 30 min, washed twice with PBS, and then analyzed by FCM.

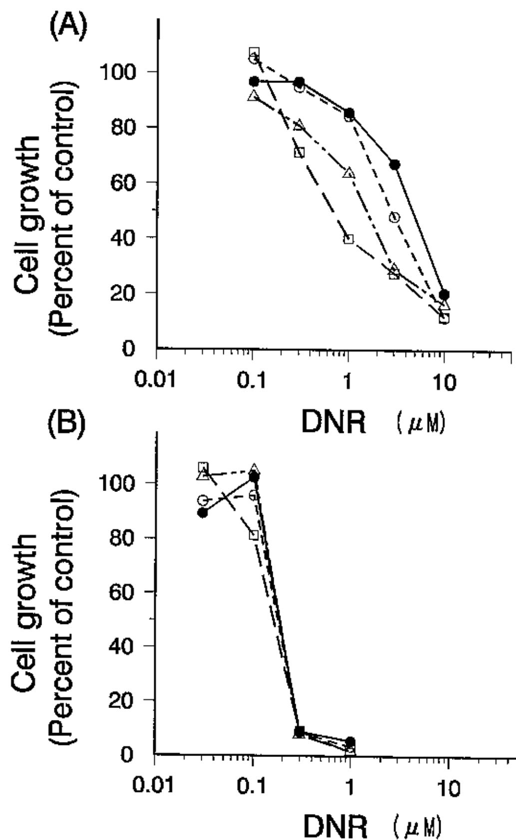


Fig. 2. Growth-inhibitory effect of DNR with or without toremifene on K562 and its drug-resistant cell line. Cells ($1\times10^5$/ml) were seeded and treated with DNR and toremifene (NK622). After being cultured for 72 h the number of viable cells was counted. (A) K562/D1-9 cells. (B) K562 cells. Without toremifene (●). With toremifene at concentrations of 1.0 (○), 3.0 (△) and 10.0 (□) $\mu M$. Points indicate the means of triplicate determinations.

K562/D1-9 also showed cross resistance to VCR, DOX, VP-16 and IDA. As to the mechanism of MDR, a PGP phenotype was detected in K562/D1-9, but not in the wild-type K562, by the APAAP technique, with both MRK16 and C219. By FCM methods, PGP was also detected with MRK16 only in K562/D1-9 (Fig. 1).[13)]

**Cytotoxicity of toremifene** The anti-proliferative effect of toremifene on K562 and K562/D1-9 cell lines was examined. A concentration of less than 5 $\mu M$ showed substantially no antiproliferative effect in either cell line (less than 5% inhibition). Toremifene had a small anti-proliferative effect at 10 $\mu M$, and was clearly cytotoxic at 30 $\mu M$. From these data, we selected a concentration of less than 5 $\mu M$ for the following experiments, including cell growth inhibition study. The concentration of 10 $\mu M$ was used for the short-term uptake study. Every experiment included a control with toremifene alone in the medium.

**Effect of toremifene on DNR sensitivity** The addition of toremifene to the culture medium decreased the resistance of K562/D1-9 to DNR in a dose-dependent manner, but the resistance of K562 was unaffected (Fig. 2). A similar effect could be obtained when tamoxifen was added to the medium with K562/D1-9. However, the effect of toremifene seemed to be greater than that of tamoxifen at a concentration of 3.0 $\mu M$ (Table I).

**Effect of toremifene on DNR accumulation** The amount of DNR accumulated in K562/D1-9 was less than that accumulated in K562 (Fig. 3). The addition of toremifene dose-dependently increased DNR accumulation in K562/D1-9, but not in K562 (Fig. 3). The addition of tamoxifen also increased DNR accumulation in K562/D1-9.

Verapamil could also reverse the decreased DNR accumulation in K562/D1-9. However, the simultaneous addition of both verapamil and toremifene at the concentration of 10 $\mu M$ did not increase DNR accumulation to a level higher than that produced by the addition of verapamil alone (Fig. 4). Concentrations of 3 $\mu M$ each of both verapamil and toremifene also showed no additive effect on intracellular DNR accumulation.

**Efflux of DNR** Accumulated DNR was effluxed rapidly from K562/D1-9 but not from K562 at 37°C (data not

Table I. Effect of Toremifene and Tamoxifen on DNR Sensitivity in K562/D1-9

| Drug added | $IC_{50}\pm SD$ ($\mu M$) | x-fold decrease[b)] |
|---|---|---|
| None | 7.3±1.4 | 1.0 |
| Toremifene | 1.6±1.5[a)] | 4.6 |
| Tamoxifen | 2.9±1.9[a)] | 2.5 |

Concentration of added drug was 3 $\mu M$. Each value is the mean of at least three experiments.

*a)* Significantly different from control group ($P<0.05$ by paired *t* test).

*b)* The *x*-fold decrease was obtained by dividing the $IC_{50}$ value in the absence of toremifene and tamoxifen by that in the presence of each of them.

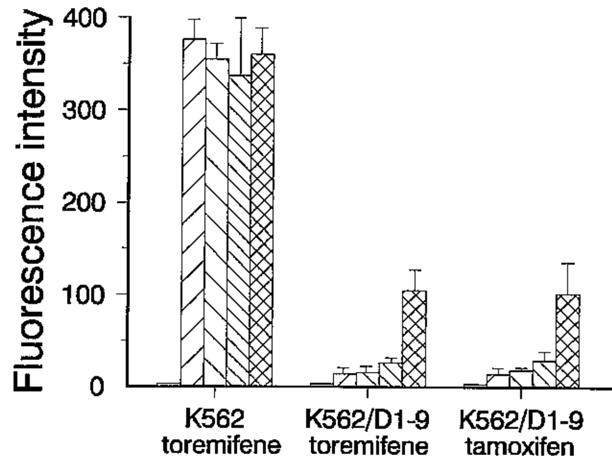


Fig. 3. DNR accumulation in K562 and its DNR-resistant cell line with or without toremifene and tamoxifen. Cells ($1\times10^5$/ml) were incubated with 1.0 $\mu M$ DNR without (▨) or with 1.0 (▧), 3.0 (▧), 10.0 (⊠) $\mu M$ toremifene or tamoxifen for 60 min and fluorescence intensity was measured by FCM. (□) shows cells in a drug-free medium. Values are means±SD.

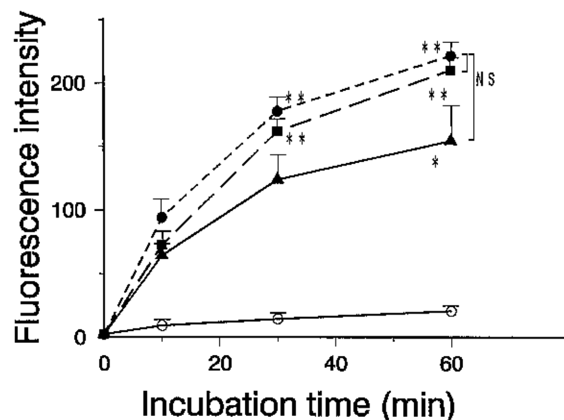


Fig. 4. Effect of toremifene and/or verapamil on DNR accumulation in the DNR-resistant cell line. Cells ($1\times10^5$/ml) were incubated with 1.0 $\mu M$ DNR as a control (○), or 1.0 $\mu M$ DNR in the presence of 10 $\mu M$ toremifene (▲), 10 $\mu M$ verapamil (●) or both (■). Then cells were harvested at a designated time (10, 30, 60 min) and fluorescence intensity was determined by FCM. Values are mean±SD. *: $P<0.05$, **: $P<0.01$, compared with the control group by using the paired *t* test.

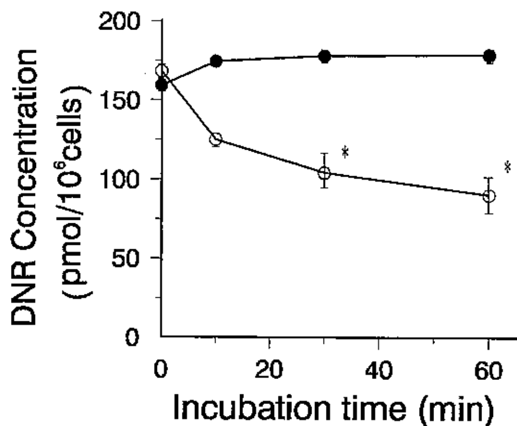


Fig. 5. The effect of toremifene on DNR efflux in DNR-resistant K562 cell line. Cells were cultured with 10 $\mu M$ DNR for 60 min. Then cells were resuspended in DNR-free medium without (○) or with (●) 10 $\mu M$ toremifene. Intracellular DNR were determined as described in "Materials and Methods." Values are mean±SD. *: $P<0.05$, compared with the toremifene group by using the paired $t$ test.

Table II. Toremifene Uptake by K562 and K562/D1-9

| Cell line | Drug added | Intracellular toremifene ± SD (pmol/2 × $10^6$ cells) |
|---|---|---|
| K562 | None | 380±45 |
| K562/D1-9 | None | 449±89 |
| | Daunorubicin | 516±79 |
| | Verapamil | 445±70 |

Concentrations of daunorubicin, verapamil and tamoxifen were 10 $\mu M$. Each value is the mean of at least triplicate determinations.

shown). This outward transport to the DNR-free resuspended medium was suppressed by the addition of 10 $\mu M$ toremifene (Fig. 5).

**Toremifene accumulation** When K562/D1-9 or K562 cells were incubated with 1.0 $\mu M$ $^3$H-toremifene for 60 min, accumulation of $^3$H-toremifene was the same in K562/D1-9 and K562, within the limits of experimental error. The accumulation in K562/D1-9 did not increase upon addition of 10 $\mu M$ verapamil or DNR (Table II).

**Partition coefficients** The octanol/water partition coefficient for toremifene was $(9.1\pm3.2)\times10^2$ (mean and SD of triplicate measurements). This result indicates that toremifene is highly lipophilic.

## DISCUSSION

We developed a DNR-resistant K562 cell line, K562/D1-9, for investigation of drug resistance in acute leukemia chemotherapy, because DNR has been used in clinical practice more frequently than DOX. K562/D1-9 possesses typical characteristics of a multidrug-resistant cell line,[1] that is, K562/D1-9 expressed PGP and exhibited cross-resistance to other anti-cancer drugs such as DOX or VCR. The addition of toremifene to culture medium of K562/D1-9 in the presence of DNR reversed the resistance to DNR in a dose-dependent manner. Toremifene had no effect on DNR retention or sensitivity in the wild-type K562.

Both the rate and extent of intracellular DNR accumulation were reduced in resistant cells, and both reductions were reversed by the addition of toremifene, which decreased the efflux of DNR from resistant cells. Thus, toremifene appears to suppress the multidrug-resistance mechanism of PGP, which acts as an efflux pump of drugs.

Verapamil is a representative MDR-reversing drug. Yusa and Tsuruo[16] reported that verapamil was strongly bound to PGP, and competitively inhibited the efflux of antitumor agents through PGP, resulting in increased intracellular accumulations of antitumor agents. Consequently, the uptake of varapamil itself was also less in PGP-associated resistant cells than in parent cells, and retention of verapamil in the resistant cells was increased by addition of VCR or ADR.[16] In contrast, uptake of toremifene in resistant cells was almost equal to that of the parent cells, and intracellular retention of toremifene was not affected by the addition of DNR or verapamil in resistant cells. Toremifene showed no additive effect with verapamil on DNR accumulation. Nevertheless, this drug inhibited the efflux pump of PGP and could reverse both the DNR efflux and drug resistance. These results suggested that toremifene itself was not effluxed through PGP competitively at the same binding site as DNR or verapamil. Toremifene could thus have a different mechanism of MDR reversal from verapamil. Tamoxifen, the parent compound of toremifene, also has the ability to reverse MDR. It is possible that toremifene does not bind to PGP in the same manner as tamoxifen.[17] Their MDR-reversing mechanism might not depend on PGP. Ramu *et al.*[18] suggested that the increased membrane rigidity reported in MDR cell membranes was decreased by tamoxifen, which could account for accelerated diffusion of doxorubicin into the cells and enhancement of its cytotoxicity. This effect of tamoxifen may have some relation to its lipophilicity and not to its antiestrogenic effect. Toremifene has a high octanol/water partition coefficient and is highly lipophilic, so it might also have a high affinity for the membrane. This may be important for its MDR-reversing mechanism, just as in the action of $N^4$-behenoyl-1-$\beta$-D-arabinofuranosylcyosine against hematologic malignant cells.[19]

Tamoxifen inhibited calmodulin[20] and protein kinase C,[21] both of which have been suggested to be possible points at which drug resistance could be modified. We intend to examine whether toremifene has similar effects. Toremifene showed a reversing effect on MDR at concentrations of at least 3.0 $\mu M$. It is known that patients have plasma toremifene concentrations of 10–15 $\mu M$ following doses of 360 mg/day for 5 days.[22] Toremifene can be administered at higher doses than tamoxifen[11, 23] and higher serum levels can be achieved.[24, 25]

Thus, toremifene could be more effective for reversing MDR than tamoxifen at clinically available levels. In addition, toremifene has no severe side effects, is easily self-administered and is efficacious orally.[26] Toremifene could be a promising agent for circumventing DNR resistance in leukemia chemotherapy.

ACKNOWLEDGMENTS

We thank Dr. Takashi Tsuruo for providing the MRK16 antibody. This study was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare of Japan.

*(Received November 20, 1993/Accepted March 8, 1994)*

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