Digitalis-Induced Signaling by Na$^+$/K$^+$-ATPase in Human Breast Cancer Cells

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ABSTRACT

Because beneficial effects of digitalis treatment in breast cancer patients have been suggested by epidemiological studies, we explored the mechanism of the growth inhibitory effects of these drugs on the estrogen receptor-negative human breast cancer cell line MDA-MB-435s. Ouabain concentrations (100 nM or lower) that caused less than 25% inhibition of the pumping function of Na$^+$/K$^+$-ATPase had no effect on cell viability but inhibited proliferation. At the same concentrations, ouabain 1) activated Src kinase and stimulated the interaction of Src and Na$^+$/K$^+$-ATPase with epidermal growth factor receptor (EGFR); 2) caused a transient and then a sustained activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2); 3) increased the expression of p21^{Cip1} but decreased that of p53; and 4) activated c-Jun NH$_2$-terminal kinase (JNK) but not p38 kinase. These data, in conjunction with our previous findings on the signaling role of Na$^+$/K$^+$-ATPase in other cells, suggest that ouabain-induced activation/transactivation of Src/EGFR by Na$^+$/K$^+$-ATPase leads to activation of ERK1/2, the resulting increase in the level of cell cycle inhibitor p21^{Cip1}, and growth arrest. Cooperation of JNK with ERK1/2 in this process is also suggested. Digoxin and digitoxin concentrations close to or at the therapeutic plasma levels had effects on proliferation and ERK1/2 similar to those of ouabain, supporting the proposed potential value of digitalis drugs for the treatment of breast cancer.

The extensively studied effects of digitalis drugs (cardiac glycosides) on the heart, and the uses of these drugs in the therapy of congestive heart failure and other cardiac disorders, are presented in standard textbooks. Less well known are the periodic reports that have suggested the potential value of these drugs in the treatment of various cancers (Repke et al., 1995; Haux, 1999; Stenkvist, 1999, and references therein). Although most of this research has been done on cancer cells in vitro, of particular interest are the studies that have suggested the beneficial effects of digitalis treatment in women with breast cancer (Stenkvist et al., 1979, 1980, 1982; Stenkvist, 1999). These studies were the prime stimulus for the work presented here.

Digitalis effect on cardiac contractility (the positive inotropic effect) is known to be caused by the drug's highly specific interaction with Na$^+$/K$^+$-ATPase; the enzyme that maintains the normal gradients of Na$^+$ and K$^+$ across the plasma membrane of most eukaryotic cells (Skou and Esmann, 1992). Decades ago, it was established that partial inhibition of the ion pumping function of cardiac Na$^+$/K$^+$-ATPase by digitalis leads to a modest increase in intracellular Na$^+$, which in turn affects the cardiac sarcolemmal Na$^+$/Ca^{2+} exchanger, causing a significant increase in intracellular Ca^{2+} and in the force contraction (Schwartz et al., 1975; Akera and Brody, 1978; Smith, 1988). This positive inotropic action of digitalis is generally assumed to be a basis of its utility in the treatment of congestive heart failure (Smith, 1988; Kjeldsen et al., 2002). More recently, it has been shown that the fraction of the cardiac myocyte Na$^+$/K$^+$-ATPase whose ion pumping function has been inhibited by digitalis participates in protein-protein interactions that lead to activation of multiple growth-related signal transduction pathways and that some of these activated pathways are also essential to digitalis-induced increase in contractility (Tian et al., 2001; Xie and Askari, 2002; Mohammadi et al., 2003). Thus, both the ion pumping function and the recently recognized signal-transducing function of Na$^+$/K$^+$-ATPase are involved in the classical digitalis effects on the heart. It has also been shown recently that digitalis-induced signaling by Na$^+$/K$^+$-ATPase is not limited to cardiac myocytes and that there are, as expected, different downstream consequences of such signaling in various cell types (Xie and Askari, 2002; Abramowitz et al., 2003).

ABBREVIATIONS: ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun NH$_2$-terminal kinase; EGFR, epidermal growth factor receptor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; LDH, lactic dehydrogenase; PAGE, polyacrylamide gel electrophoresis.

Because of the suggested value of digitalis in the treatment of human breast cancer, and because of the paucity of mechanistic studies on digitalis interaction with breast cancer cells, it seemed worthwhile to begin a systematic study of digitalis effects on the ion pumping and the signal transducing functions of Na^+/K^+-ATPase in human breast cancer cells. The results of our initial studies on the MDA-MB-435s cell line are presented here. Our findings point to novel and promising lines of research on the potential use of digitalis drugs in the treatment of breast cancer.

Materials and Methods

Materials. MDA-MB-435s cells were obtained from American Type Culture Collection (Manassas, VA), and Leibowitz's L-15 medium was from Invitrogen (Carlsbad, CA). Antibodies against ERK1/2, JNK, p38 kinase, and the phosphorylated (active) forms of these kinases, $p21^{Cip1}$ and p53, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against Src and EGFR, the Src kinase assay kit, and the TUNEL apoptosis detection kit were obtained from Upstate Biotechnology (Lake Placid, NY). Antibody against Na^+/K^+-ATPase α-subunit, α6F, was provided by Developmental Studies Hybridoma Bank (The University of Iowa, Iowa City, IA). CyQuant proliferation assay kit was from Molecular Probes (Eugene, OR). LDH assay kit, ouabain, digoxin, and digitoxin were purchased from Sigma-Aldrich (St. Louis, MO). [γ-^{32}P]ATP, ^{86}Rb$^+$, and ^{22}Na$^+$ were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA).

Cell Culture and Proliferation Assay. Cells were grown in Leibowitz's L-15 medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and incubated at 37°C in humidified air without added CO_2. For proliferation assays, cells were seeded in appropriate plates for 6 h before treatment. At indicated times after drug addition, cell counts were obtained either in a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA) or by the fluorimetric assay of cellular nucleic acids using the CyQuant kit according to manufacturer's instructions. For other experiments, cells were used when 70 to 80% confluence was reached. In some short-term experiments (Figs. 5, 6, 9, and 10), cells were serum-starved for 24 h before exposure to drugs.

Viability and Apoptosis Assays. Extent of release of LDH from the cells, i.e., ratio of medium LDH to total LDH, was used as a measure of loss of viability. LDH was assayed spectrophometrically by measuring the initial rate of conversion of NAD to NADH in the presence of lactate. For the detection of apoptotic cells, a TUNEL assay kit was used according to manufacturer's directions to label fragmented DNA and visualize apoptotic cells by fluorescence microscopy.

Active Uptake of Rb$^+$. The initial rate of ouabain-sensitive ^{86}Rb$^+$ uptake through Na^+/K^+-ATPase of the intact cells was assayed by the procedures we described previously (Kometiani et al., 2001). Uptake that was inhibited by 10 μM or higher concentrations of ouabain was taken as the maximal rate of active uptake.

Intracellular Na$^+$ Content. Estimation of relative change in intracellular Na^+ was done according to Kim et al. (1984). In brief, cells were incubated at 37°C in the culture medium containing trace amounts of ^{22}Na$^+$ for 45 min to fully equilibrate exchangeable intracellular Na^+ with ^{22}Na$^+$. The indicated concentrations of ouabain were then added, and incubations were continued for 30 min. Control and ouabain-treated cells were then washed in ice-cold solution of 100 mM $MgCl_2$, dissolved in SDS, and counted.

Assays of Src Kinase, ERK1/2, JNK, and p38 Kinase. Control cells, and those treated with drugs as indicated, were washed with ice-cold phosphate-buffered-saline and placed in the lysis buffer containing 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin,

and 50 mM Tris-HCl, pH 7.4. After homogenization and centrifugation at 16,000g for 10 min, the supernatant was assayed for Src kinase activity as we described previously (Mohammadi et al., 2003). The same supernatant was also used for the assay of total ERK1/2 and phospho-ERK1/2 by immunoblot analysis as we reported previously (Kometiani et al., 1998; Mohammadi et al., 2003). Likewise, to detect activation of JNK and p38 kinase, ratios of phosphorylated/activated kinases to total kinases were determined by Western analysis of the supernatants of the cell lysates.

Immunoblot Analysis and Coimmunoprecipitation. Supernatants of cell lysates, prepared as described above, were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with appropriate antibodies by standard procedures. The immunoreactive bands were detected using chemiluminescence. For quantitative comparisons, images were scanned with a densitometer. Different dilutions of samples were subjected to SDS-PAGE, and multiple exposures of films were used to ensure that quantitations were made within the linear range of the assays. The results were expressed in arbitrary units per unit of lysate protein.

Coimmunoprecipitation experiments were done on the supernatant of the cell lysates, using an anti-EGFR antibody and protein G-agarose beads, by standard procedures (Haas et al., 2000). Precipitated proteins were removed from the beads by SDS sample buffer and subjected to Western analysis using appropriate antibodies against the indicated proteins. Protein was determined by the Bio-Rad detergent-compatible protein colorimetric assay (Bio-Rad, Hercules, CA).

Data Analysis. Data points are mean $\pm$ S.E. of the results of the indicated number of experiments. Student's t test was used, and significance was accepted at $p < 0.05$.

Results

The human breast cancer cell line MDA-MB-435s was used in all of the studies presented below. This estrogen receptor-negative cell line was chosen to avoid the possibility of digitalis interaction with estrogen receptor. Ouabain was used in most of the experiments because the majority of the previous in vitro studies on cardiac glycosides have been done with this prototype drug. Selected experiments were also repeated with digoxin and digitoxin, the two digitalis drugs that are most widely used for therapeutic purposes.

Ouabain Sensitivity of the Ion Pumping Function of Na^+/K^+-ATPase in Breast Cancer Cells. Because our primary objective was to explore the consequences of digitalis-induced signaling at low drug concentrations that do not cause large changes in intracellular Na^+ and K^+ concentrations, it was necessary to assess the ouabain sensitivity of the pumping function of Na^+/K^+-ATPase in these breast cancer cells. The human isoforms of Na^+/K^+-ATPase, when isolated from intact cells or tissues and assayed for ouabain binding under optimal conditions, exhibit nearly identical K_d values in the range of 13 to 32 nM ouabain (Müller-Ehmsen et al., 2001; Wang et al., 2001). In an intact cell of human origin (HeLa cell) where the inhibitory potency of ouabain on the transport function of Na^+/K^+-ATPase has been measured before, the K_i value has been estimated to be 140 to 240 nM ouabain (Tepperman et al., 1997). That this value is higher than the above K_d values obtained with the isolated human enzyme is in keeping with the known antagonistic effect of extracellular K^+ on the affinity of the enzyme for ouabain (Tepperman et al., 1997; Müller-Ehmsen et al., 2001). In experiments, the results of which are summarized in Fig. 1, the effects of varying ouabain concentrations on the transport function of Na^+/K^+-ATPase of MDA-MB-435s cells, rep-

resented by the initial rate of active Rb^+ uptake, were determined. Half-maximal inhibition was obtained at about 430 nM ouabain (Fig. 1).

It is well established that because of the "reserve capacity" of Na^+/K^+-ATPase in an intact cell with normal gradients of Na^+ and K^+ across its membrane, the partial inhibition of the enzyme does not necessarily lead to significant changes in intracellular Na^+ and K^+ concentrations (Akera and Brody, 1978; Kim et al., 1984; Akera and Ng, 1991; Liu et al., 2000). Experiments were done to determine how the different degrees of ouabain inhibition of the transport function of Na^+/K^+-ATPase affect the intracellular Na^+ concentrations of the breast cancer cells (Fig. 2). The results showed that 100 nM ouabain, which produces about 20% inhibition of the transport function (Fig. 1), caused no significant change in intracellular Na^+ and that 500 nM ouabain, which causes about half-maximal inhibition of the transport function (Fig. 1), leads to an increase in intracellular Na^+ that is about 15% of that caused by a maximal inhibitory concentration of ouabain (Fig. 2). These findings which are in agreement with similar data obtained in other cell types (Kim et al., 1984; Liu et al., 2000) show that Na^+/K^+-ATPase of the intact cells used here also exhibits a reserve capacity as expected. It is noteworthy that the data also indicate that in the remaining experiments presented below, the ouabain effects obtained at ouabain concentrations of 100 nM or less cannot be caused by significant changes in intracellular ratio of Na^+/K^+.

Ouabain Effects on Cell Viability and Growth. Na^+/K^+-ATPase is a house-keeping enzyme; hence, its inhibition to the extent that alters the intracellular concentrations of Na^+ and K^+ significantly, is expected to be incompatible with cell survival. When the effects of varying concentrations of ouabain on loss of viability, represented by the release of LDH, were measured, dose-dependent toxic effects were noted at 250 nM or higher ouabain concentrations, but there were no effects at concentrations of 100 nM or lower (Fig. 3).
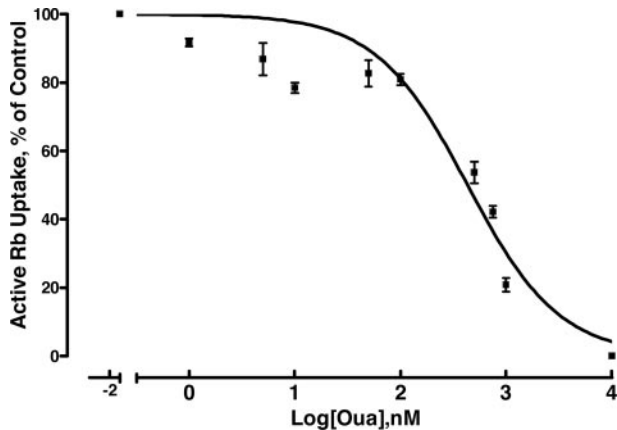
Because various concentrations of digitalis drugs have been reported to cause apoptosis in some, but not all, cell types other than breast cancer cells (Haux et al., 1999a; Chueh et al., 2001), we also tested the effects of ouabain on DNA fragmentation associated with apoptosis. Using the TUNEL assay (see *Materials and Methods*), we noted no clear evidence of ouabain-stimulated apoptosis at concentrations of 100 nM or less (not shown). This and the findings of Fig. 3 are consistent with those of Fig. 2, indicating that significant changes in intracellular Na^+ and K^+ concentrations must occur before ouabain induces cell death.

The effects of varying nonlethal concentrations of ouabain on cell proliferation were examined (Fig. 4). The results showed significant dose- and time-dependent antiproliferative effects of ouabain at concentrations in the range of 1 to 100 nM (Fig. 4). At 100 nM ouabain, almost complete growth arrest was noted (Fig. 4A).

Ouabain-Induced Signaling through Src and EGFR. The growth-related effects of ouabain in cardiac myocytes and several other cell types have been shown to be initiated by activation of Src kinase and transactivation of EGFR (Xie and Askari, 2002). In view of the well established involve-
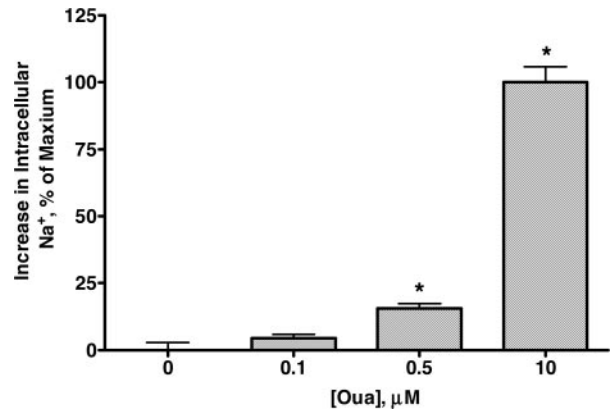

Fig. 2. Effects of varying concentrations of ouabain on intracellular Na^+ content. After equilibration of intracellular Na^+ with $^{22}Na^+$ (see *Materials and Methods*), the indicated concentrations of ouabain were added to the culture medium, and changes in intracellular $^{22}Na^+$ relative to that of untreated control were determined. $n = 6$; *, $p < 0.05$ compared with control.

Fig. 1. Inhibitory effect of varying concentrations of ouabain on the active uptake of Rb^+ by Na^+/K^+-ATPase of the intact breast cancer cells. Assay of the initial rate of ouabain-sensitive Rb^+ uptake was performed as described under *Materials and Methods* by incubating the cells in the culture medium containing traces of $^{86}Rb^+$ for 10 min and measuring the intracellular content of $^{86}Rb^+$. At each ouabain concentration, active uptake was expressed relative to that of untreated control. Each data point (mean ± S.E.; $n = 3$–12) is significantly different from control ($p < 0.05$). The indicated line is the nonlinear least-squares fit of the data to the Hill equation. K_i (nanomolar) = 431.4 ± 1.07, S.E. (95% confidence interval 373–498 nM); Hill coefficient = 0.99 ± 0.07, S.E. (95% confidence interval 1.15–0.84); R^2 (goodness of fit) = 0.93. Curve fitting was done with the help of Prism software (GraphPad Software Inc., San Diego, CA).
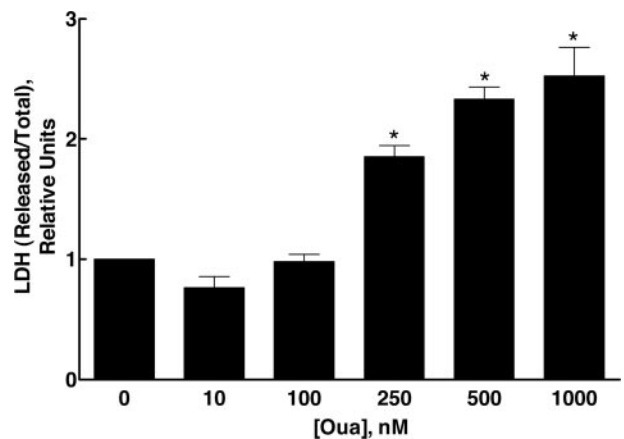

Fig. 3. Effects of varying concentrations of ouabain on the release of cellular LDH to the medium. Cells were cultured with the indicated ouabain concentrations for 48 h, and the extent of release of LDH was assayed as described under *Materials and Methods* and expressed relative to that of untreated control. $n = 4$; *, $p < 0.05$ compared with control.

ment of Src and EGFR in breast cancer development (Biscardi et al., 2000; Prenzel et al., 2001), it was of interest to determine whether the growth inhibitory effects of ouabain on breast cancer cells are accompanied by ouabain-induced interactions between Na^+/K^+-ATPase and these tyrosine kinases. Exposure of the cells to 100 nM ouabain
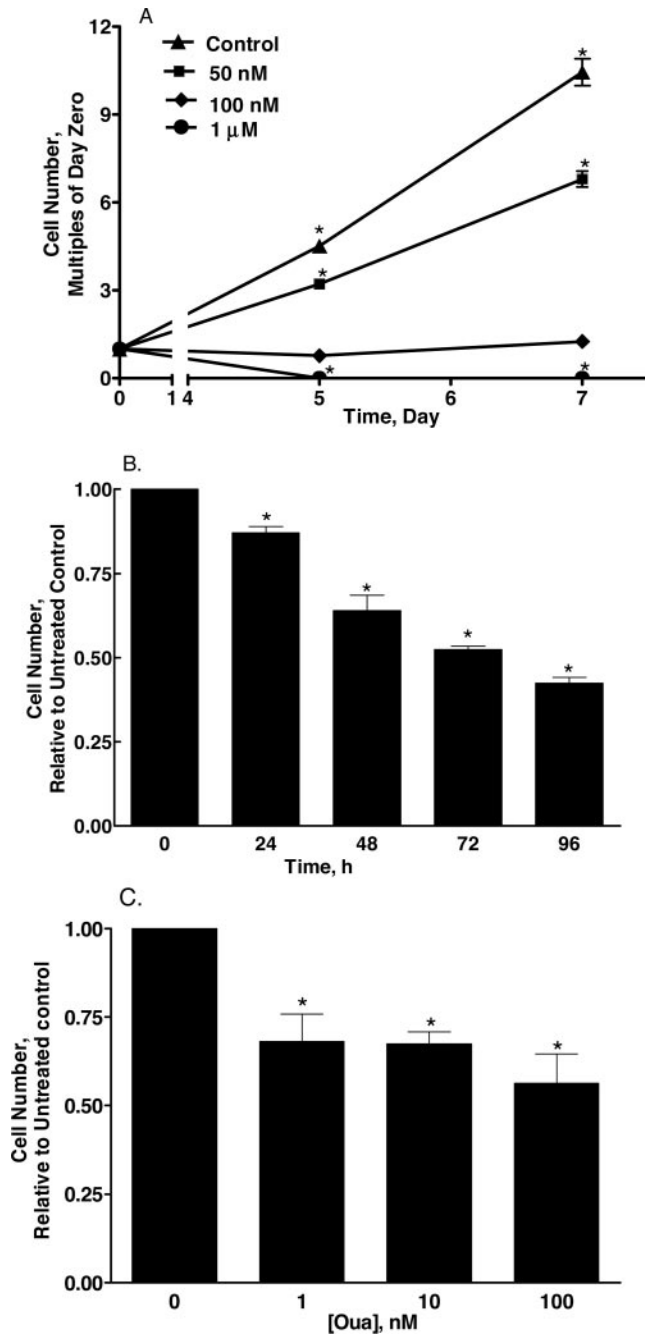
caused rapid and transient activation of Src kinase (Fig. 5A) and increased the coimmunoprecipitations of Src and Na^+/K^+-ATPase with EGFR (Fig. 5B), suggesting ouabain-stimulated interactions of the three proteins. In the range of 10 nM to 1 μM ouabain, rapid and transient activation of ERK1/2 were also noted (Fig. 6, A and B). Together, these findings indicate that the ouabain-activated pathways that have been fully identified to link Src/EGFR to Ras/Raf/MEK/ERK1/2 cascade in other cell types (Kometiani et al., 1998; Haas et al., 2000, 2002) are also activated in breast cancer cells at ouabain concentrations that arrest the growth of these cells. Because the duration and the level of ERK1/2 activation is often related to the nature of the growth-related response elicited by a signal (Marshall, 1995; Woods et al., 1997), effect of 100 nM ouabain on ERK1/2 was also examined up to 12 h after exposure. A sustained phase of activation that followed the initial rapid and transient phase was clearly evident (Fig. 6C).

Fig. 4. Inhibition of cell proliferation by ouabain. Cells were cultured in the presence of indicated ouabain concentrations, and cell numbers were determined at indicated times as described under *Materials and Methods*. A, time-dependent effects of varying ouabain concentrations on cells cultured for 7 days. Cell numbers were determined by the assay of total nucleic acids. $n = 8$; ∗, $p < 0.05$ relative to day 0. At 100 nM ouabain, the 5- and 7-day values were 0.76 ± 0.12 ($p > 0.05$) and 1.2 ± 0.06 ($p > 0.05$), respectively. B, time-dependent effects of 100 nM ouabain. Cell numbers were determined by Coulter counter. $n = 3$; ∗, $p < 0.05$ compared with control at the same time. C, effects of the indicated ouabain concentrations on cell numbers (counted) after 48 h. $n = 30$; ∗, $p < 0.05$ relative to 48 h control.
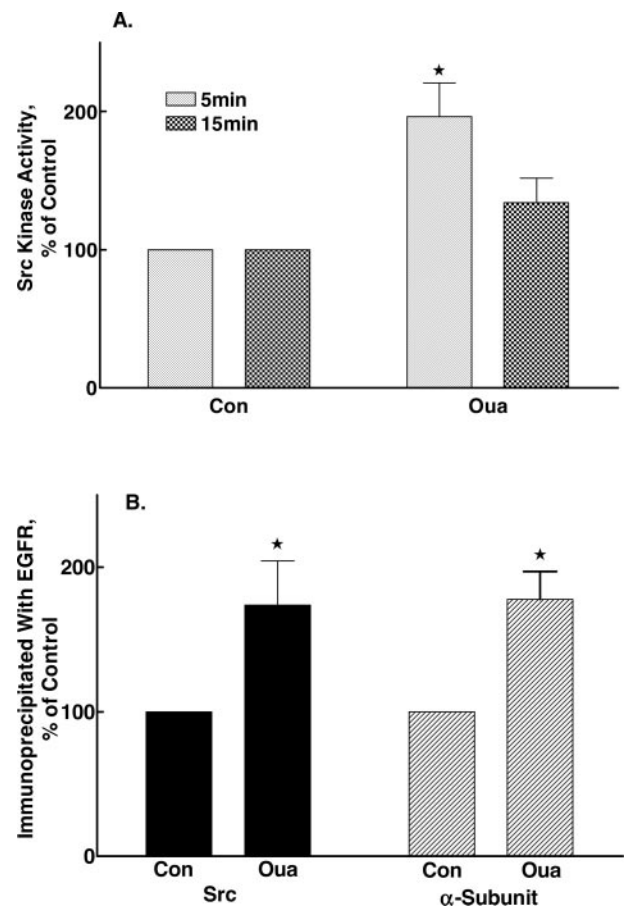

Fig. 5. A, ouabain-induced activation of Src kinase. After the exposure of the intact cells to 100 nM ouabain, lysates were prepared as described under *Materials and Methods* and subjected to immunoprecipitation by an anti-Src antibody. A portion of the precipitate was immunoassayed for Src, and another portion was assayed for Src kinase activity as indicated under *Materials and Methods*. Kinase activity per arbitrary unit of Src protein was determined and expressed relative to that of control. $n = 9$; ∗, $p < 0.05$. B, coimmunoprecipitation of Src and Na^+/K^+-ATPase α-subunit with EGFR. Lysates were prepared from control cells and those treated with 100 nM ouabain for 5 min and subjected to immunoprecipitation by an anti-EGFR antibody as indicated under *Materials and Methods*. The precipitates were then immunoassayed for EGFR, Src, and α-subunit. Results were calculated as arbitrary unit of Src or α-subunit per unit of EGFR and expressed relative to the values for the control cells. $n = 9$ (Src); $n = 3$ (α-subunit); ∗, $p < 0.05$.

Ouabain Effects on p21^{Cip1}, p53, JNK, and p38 Kinase. To begin the assessment of the mechanism of the ouabain-induced growth arrest in MDA-MB-435s cells, we examined the drug's effects on a number of established regulators of cell cycle and proliferation. Ouabain had a dose- and time-dependent stimulatory effect on the expression of p21^{Cip1} (Fig. 7) and an inhibitory effect on the expression of p53 (Fig. 8). Ouabain (100 nM) exhibited a time-dependent

activating effect on JNK (Fig. 9), but it had no effect on p38 kinase activity (not shown).

Digoxin and Digitoxin Effects. To compare the effects of ouabain with those of the two therapeutically used digitalis drugs, the following limited experiments were done. At concentrations lower than 100 nM, both digitoxin and digoxin caused activation of ERK1/2 (Fig. 10) and inhibition of proliferation (Fig. 11), although digitoxin seemed to be more effective than digoxin. This apparent superiority of digitoxin over digoxin has also been noted in experiments of others on the growth inhibitory effects of the two drugs on breast cancer cell lines other than the one used here (Kimijima et al., 1992; Haux et al., 1999a).
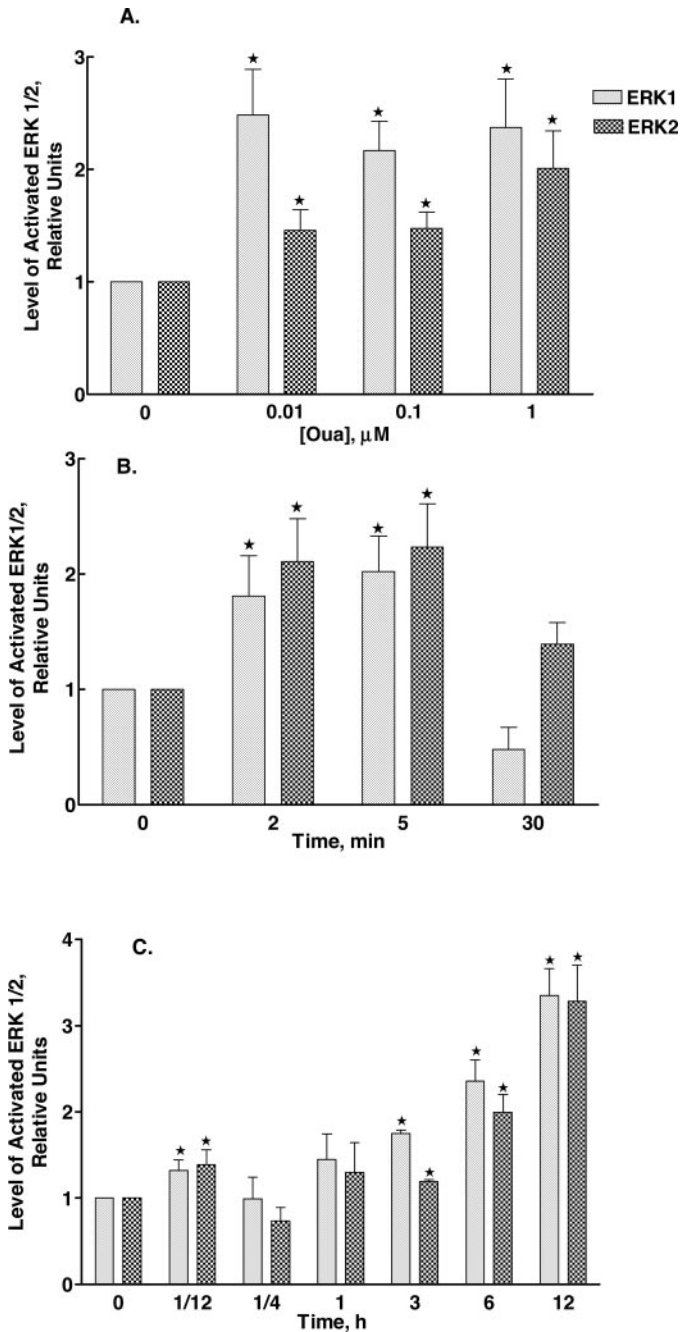

Fig. 6. Ouabain-induced activation of ERK1/2. Lysates from control and ouabain-treated cells were subjected to SDS-PAGE and immunoblot analysis (see *Materials and Methods*) using anti-ERK1/2 and anti-phosphorylated ERK1/2 antibodies. For each ERK band, the ratio of phospho-ERK to total ERK was calculated in arbitrary units. This ratio was expressed as unity for the untreated control, and other ratios were expressed relative to control. A, effects of different ouabain concentrations after 5 min of exposure. B and C, effects of 100 nM ouabain after the indicated exposure times. $n = 4–5$; *, $p < 0.05$.
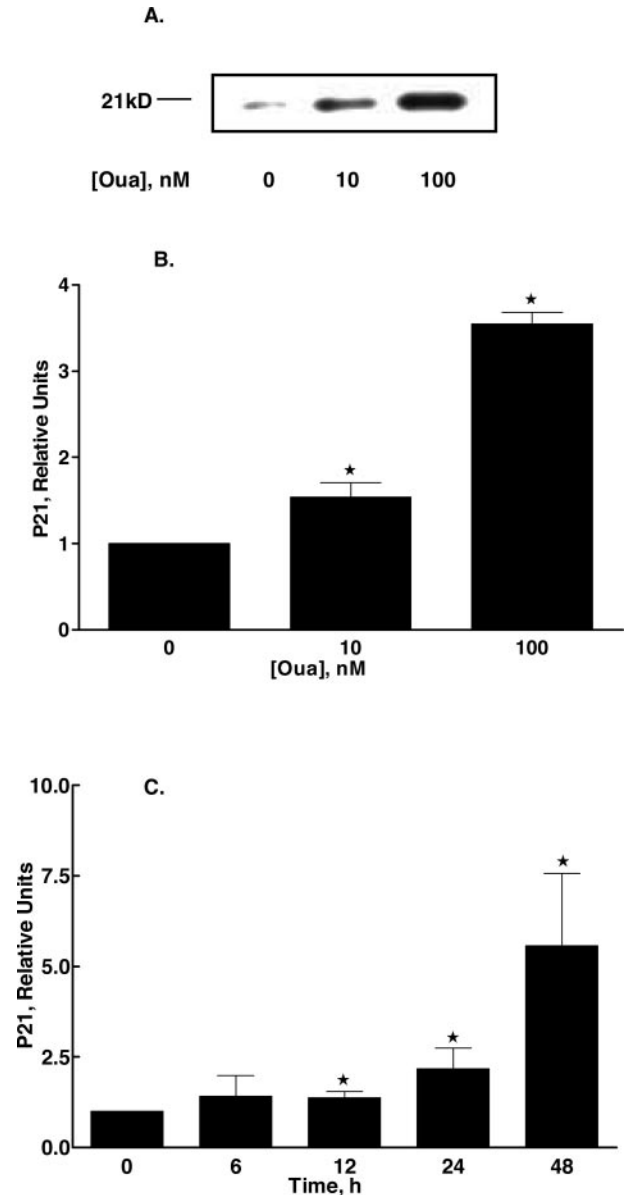

Fig. 7. Stimulation of p21^{Cip1} expression by ouabain. Lysates from control and ouabain-treated cells were subjected to immunoblot analysis as indicated under *Materials and Methods*. The results, per unit of lysate protein, are shown relative to untreated control. A, typical blot of the effects of two ouabain concentrations after 24 h of treatment. B, quantitation of blots such as those of A from five different experiments. *, $p < 0.05$. C, time-dependent effects of 100 nM ouabain. $n = 3$; *, $p < 0.05$.

Discussion

The epidemiological studies of Stenkvist et al. (1979, 1980, 1982) suggested that tumors of the breast cancer patients treated with digitalis drugs exhibited more benign characteristics and had reduced recurrence rates, than those of the patients not treated with digitalis. Prompted by these observations, others have reported growth inhibitory effects of these drugs, at concentrations at or near therapeutic blood levels, on human breast cancer cells in culture (Falconer et al., 1983; Kimijima et al., 1992; Haux et al., 1999a,b). We have now confirmed and extended these observations to explore molecular mechanisms for the growth inhibitory effects of digitalis drugs on breast cancer cells.
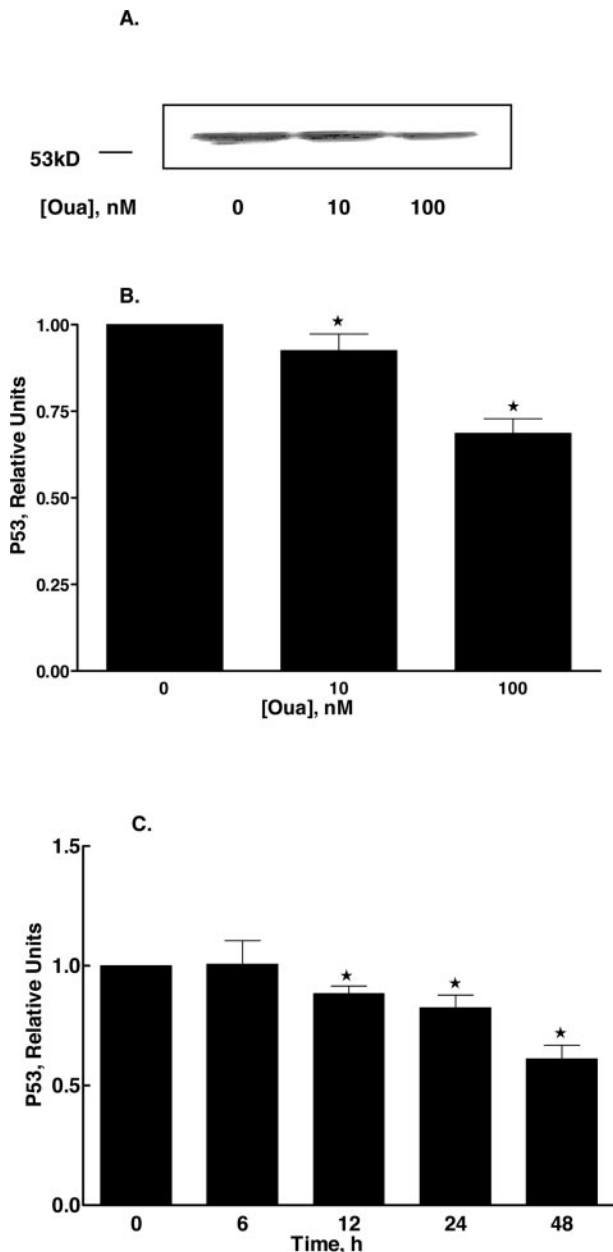

Concerning the growth inhibitory effects of digitalis drugs, it is important to note that when used at sufficiently high concentrations that alter the normal Na^+ and K^+ gradients, these drugs are lethal to nearly all mammalian cells. Although the nature of the cell death and the identity of the death signals generated by the reversal of intracellular Na^+/K^+ ratio are of interest (Mann et al., 2001), these are not the issues addressed in the present work. We have shown that at nonlethal concentrations of 100 nM or lower, ouabain causes growth arrest in breast cancer cells (Figs. 2–4), and we have focused attention on the mechanisms leading to this effect.

Our findings show that the same nonlethal concentrations of ouabain that inhibit proliferation of the breast cancer cells also induce signaling through Src and EGFR, leading to the activation of ERK1/2 (Figs. 5 and 6). Although these proximal ouabain-activated signaling events seem to be the same as those occurring in other cells (Xie and Askari, 2002), the growth-related consequences of the activations of these pathways vary in different cell types. In contrast to the antiproliferative effects noted here, nonlethal concentrations of ouabain cause hypertrophy in cardiac myocytes, and stimulation of proliferation in smooth muscle, epithelial, and endothelial cells (Xie and Askari, 2002; Abramowitz et al., 2003; Saunders and Scheiner-Bobis, 2004). It is clear that the initial ouabain-activated pathways must diverge significantly in different cell types, and the divergent pathways and consequences need to be identified in each cell type.

Ouabain-induced up-regulation of p21^{Cip1} (Fig. 7), an established cell cycle inhibitor (McMahon and Woods, 2001), is the likely explanation of the antiproliferative effect of the drug. The significance of the concomitant down-regulation of p53 (Fig. 8) is not clear, because there are both p53-dependent and -independent pathways of p21^{Cip1} induction (McMahon and Woods, 2001). In view of the known role of ERK1/2 in the regulation of p21^{Cip1} induction (Woods et al., 1997; McMahon and Woods, 2001; Todd et al., 2004), it is reasonable to assume that ouabain-induced activation of ERK1/2 is the link between the proximal ouabain-induced interactions between Na^+/K^+-ATPase, Src, and EGFR, and the cell cycle arrest caused by increased levels of p21^{Cip1}.

Fig. 8. Down-regulation of p53 expression by ouabain. Cell lysates were assayed as indicated in legend to Fig. 7 using the appropriate antibody. A, typical blot of the effects of different ouabain concentrations after 24 h of exposure. B, quantitation of multiple blots similar to A from four different experiments. $*$, $p < 0.05$. C, time-dependent effects of 100 nM ouabain. $n = 3$; $*$, $p < 0.05$.
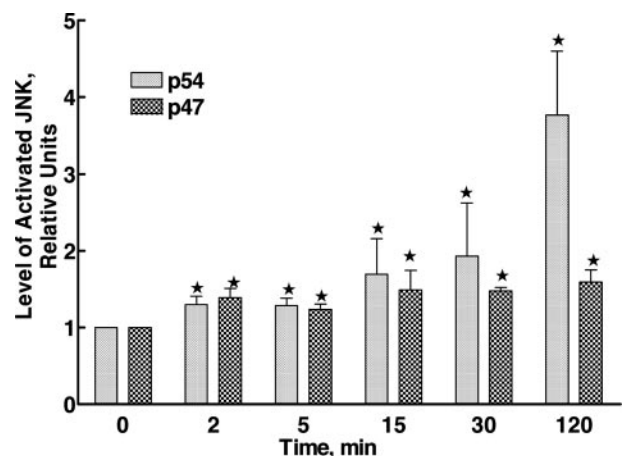

Fig. 9. Ouabain-induced activation of JNK. Cells were exposed to 100 nM ouabain for the indicated durations, lysates were subjected to immunoblot analysis of the phosphorylated and total JNK proteins, and the results were quantitated as indicated in legend to Fig. 6. $n = 3$; $*$, $p < 0.05$.

Whether the link is the rapid or the sustained phase of activation, or both, remains to be determined. Cooperation between ERK1/2, JNK, and p38 kinase pathways in the regulation of p21^{Cip1} induction has been noted (Todd et al., 2004). We find that ouabain activates JNK but not p38 kinase (Fig. 9; see *Results*). The pathways of ouabain-induced activation of JNK, and possible interactions of ERK1/2 and JNK pathways in the regulation of the growth of these cells, also remain to be explored.

Pharmacological and Clinical Implications. Like ouabain, the clinically used digoxin and digitoxin are highly specific inhibitors of Na$^+$/K$^+$-ATPase (Akera and Brody, 1978), and the potencies of the three drugs are about the same when tested against the enzymes isolated from human organs (Repke, 1997). This, and the fact that lower than 100 nM concentrations of digoxin and digitoxin have effects similar to those of ouabain on cell growth and ERK1/2 (Figs. 10 and 11), justifies the assumption that the three drugs cause growth arrest in breast cancer cells by similar mechanisms.

An important question is whether digoxin and digitoxin effects on breast cancer in vivo may be expected if patients are treated with the established dosage regimens that have been used for patients in congestive heart failure. Using such regimens, the therapeutic plasma levels of digoxin and digitoxin are considered to be in the ranges of 0.6 to 1.9 nM and 13 to 33 nM, respectively (Katzung and Parmley, 2001). Based on our data (Fig. 11) and those of others who have noted growth inhibitory effects of digoxin and digitoxin on several breast cancer cell lines other than MDA-MB-435s cells (Kimijima et al., 1992; Haux et al., 1999a), we conclude that in vitro the upper therapeutic levels of digoxin are at the threshold of being inhibitory but that therapeutic digitoxin concentrations cause significant inhibitory effects. Taking the liberty of extrapolating from in vitro to in vivo conditions, and considering the long-standing observations on digitalized breast cancer patients, we suggest that the previous pleas for further studies on the potential use of digitalis in the therapy of cancer (Haux, 1999; Stenkvist, 1999) were well justified, at least in the case of breast cancer. It is regrettable
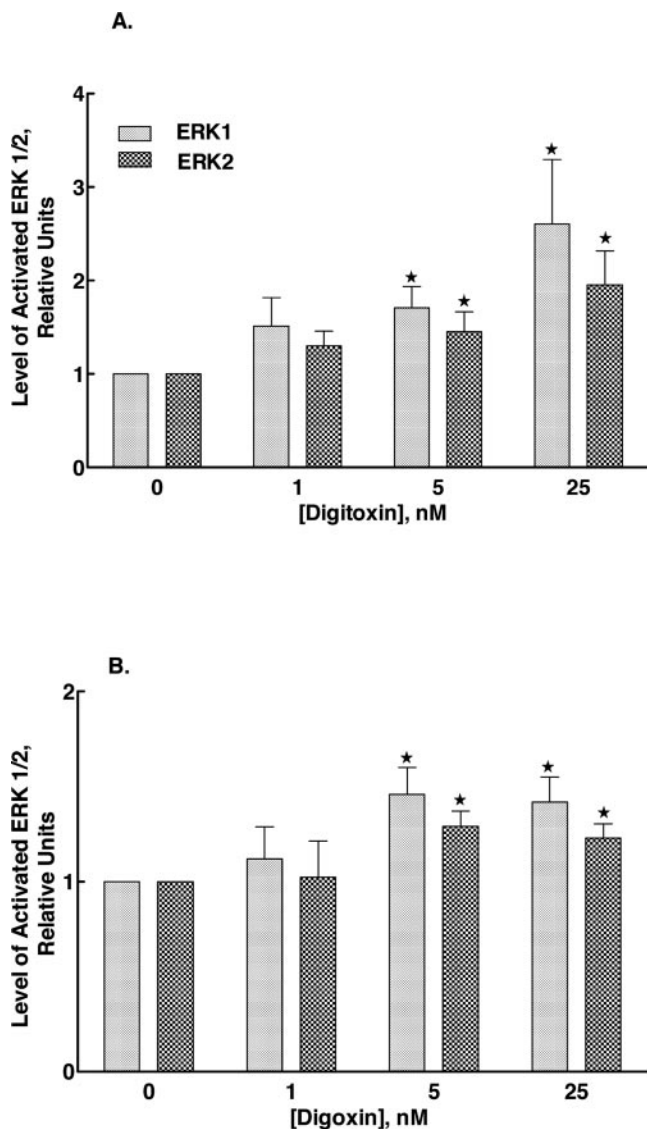

Fig. 10. Activation of ERK1/2 by digoxin and digitoxin. Cells were exposed to the indicated drug concentrations for 5 min, and the assays were done as described in legend to Fig. 6. $n = 6$; ∗, $p < 0.05$.
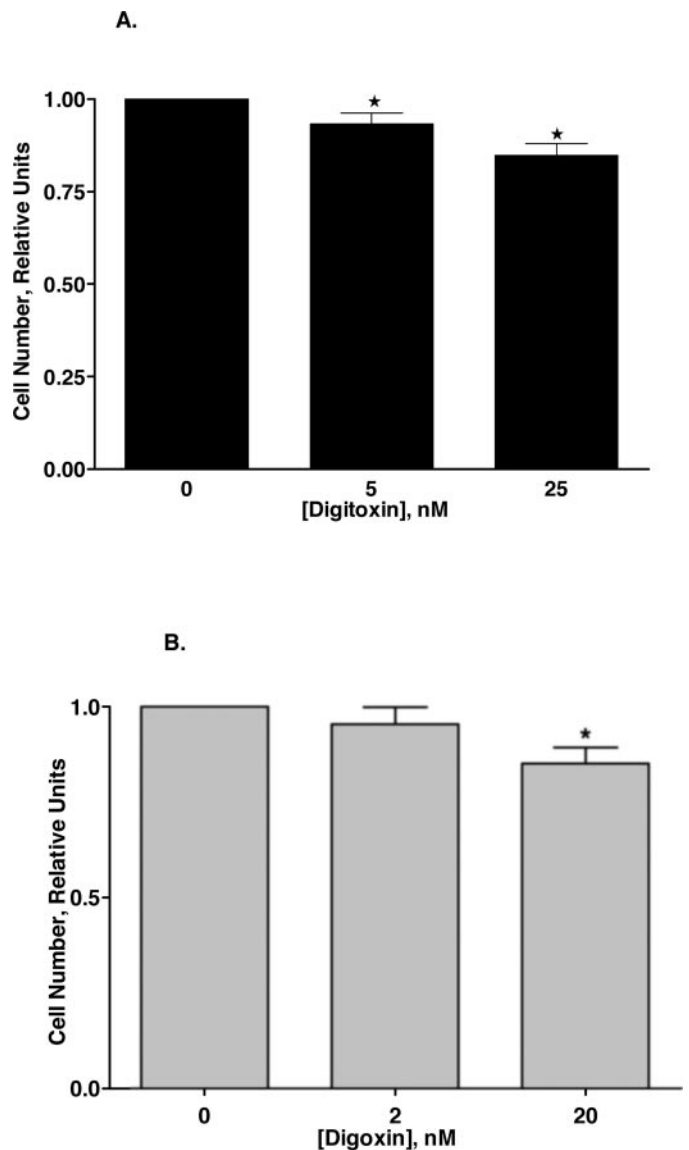

Fig. 11. Antiproliferative effects of digoxin and digitoxin. Experiments were done as indicated in legend to Fig. 4, and cell counts were determined after 48 h. $n = 30$ (digitoxin); $n = 6$ (digoxin); ∗, $p < 0.05$.

that the neglect of this potential of digitalis has been a side effect of the constant and legitimate search for new drugs.

Acknowledgments

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