

Methadone, Commonly Used as Maintenance Medication for Outpatient Treatment of Opioid Dependence, Kills Leukemia Cells and Overcomes Chemoresistance

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Abstract

The therapeutic opioid drug methadone (D,L-methadone hydrochloride) is the most commonly used maintenance medication for outpatient treatment of opioid dependence. In our study, we found that methadone is also a potent inducer of cell death in leukemia cells and we clarified the unknown mechanism of methadone-induced cell killing in leukemia cells. Methadone inhibited proliferation in leukemia cells and induced cell death through apoptosis induction and activated apoptosis pathways through the activation of caspase-9 and caspase-3, down-regulation of Bcl-x_L and X chromosome-linked inhibitor of apoptosis, and cleavage of poly(ADP-ribose) polymerase. In addition, methadone induced cell death not only in anticancer drug-sensitive and apoptosis-sensitive leukemia cells but also in doxorubicin-resistant, multidrug-resistant, and apoptosis-resistant leukemia cells, which anticancer drugs commonly used in conventional therapies of leukemias failed to kill. Depending on caspase activation, methadone overcomes doxorubicin resistance, multidrug resistance, and apoptosis resistance in leukemia cells through activation of mitochondria. In contrast to leukemia cells, nonleukemic peripheral blood lymphocytes survived after methadone treatment. These findings show that methadone kills leukemia cells and breaks chemoresistance and apoptosis resistance. Our results suggest that methadone is a promising therapeutic approach not only for patients with opioid dependence but also for patients with leukemias and provide the foundation for new strategies using methadone as an additional anticancer drug in leukemia therapy, especially when conventional therapies are less effective. [Cancer Res 2008;68(15):6059–64]

Introduction

Anticancer drugs such as doxorubicin have been used in the treatment of leukemias (1). In leukemia cells, anticancer drugs have been shown to induce apoptosis and to activate apoptosis pathways (1–3). The therapeutic opioid drug methadone (D,L-methadone) is a highly effective and safe medication for the opioid dependence of outpatients (4–7). It suppresses opioid withdrawal and blocks the effects of illicit opioids (4). Methadone, which was introduced in the 1960s, is a long-acting μ opioid receptor agonist (4). In addition to the treatment of opioid withdrawal, methadone is of significant use as a long-acting analgesic, particularly for neuropathic pain syndromes (4–6). Furthermore,

in human lung carcinoma cell lines, methadone can induce cell death (8). However, it was unknown whether methadone could be used as a therapeutic anticancer drug in leukemia therapy.

Different pathways such as ligand/receptor-driven amplifier systems or direct mitochondria activation without ligand/receptor interaction, resulting in cleavage and activation of effectors, are involved in anticancer drug-induced apoptosis (9). Caspases play a critical role in apoptosis induction (1, 10). The mitochondrial pathway can be activated through the ligand/receptor system, or directly by apoptotic stimuli, and is regulated by Bcl-2 family members (10, 11). Deficiencies in the apoptotic pathways may lead to chemoresistance and apoptosis resistance, which is one of the primary causes for therapeutic failure in the chemotherapy of leukemia and solid tumors (12, 13).

In this study, we found that methadone kills leukemia cells and overcomes chemoresistance and apoptosis resistance by inducing apoptosis and activation of apoptosis pathways.

Materials and Methods

Drugs and reagents. D,L-Methadone hydrochloride (methadone; Sigma) was freshly dissolved in sterile distilled water prior to each experiment to ensure the constant quality of the preparations.

Cell culture. The human myeloid leukemia cell line HL-60 and the human lymphoblastic leukemia T-cell line CEM were grown in RPMI 1640 (Life Technologies, Invitrogen) containing 10% FCS (Biochrom), 10 mmol/L of HEPES (pH 7.3; Biochrom), 100 units/mL of penicillin (Life Technologies), 100 μ g/mL of streptomycin (Life Technologies), and 2 mmol/L of L-glutamine (Biochrom) at 37°C and 5% CO₂. CEM^{CD95^R} are resistant to 1 μ g/mL of anti-CD95 (2) and CEM^{Doxo^R} are resistant to 0.1 μ g/mL of doxorubicin (14). CEM^{CD95^R} is apoptosis-resistant and multidrug-resistant, and is cross-resistant to several anticancer drugs such as methotrexate, cytarabine, cisplatin, etoposide, vincristine, as well as being resistant to γ -irradiation and β -irradiation (2, 3, 15). All cell lines used in this study were *Mycoplasma*-free.

Induction of apoptosis. Leukemia cells (1×10^5 cells/mL) were treated with 30, 20, 15, and 10 μ mol/L of methadone in 150 mL flasks or 96-well plates. After 24 and 48 h, quantification of apoptosis was measured by flow cytometry as previously described (16, 17). In brief, to determine apoptosis, cells were lysed with Nicoletti buffer containing 0.1% sodium citrate plus 0.1% Triton X-100 and 50 μ g/mL of propidium iodide as described by Nicoletti and colleagues (16). The percentage of apoptotic cells was measured by hypodiploid DNA (sub-G₁) or forward scatter/side scatter (FSC/SSC) analysis (16, 17). Propidium iodide-stained nuclei or FSC/SSC profiles of cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

Isolation of peripheral blood lymphocytes. Peripheral blood lymphocytes (PBLs) were isolated from fresh blood of healthy persons. PBLs (1×10^6 cells in 1 mL) were treated with 30, 20, 15, and 10 μ mol/L of methadone in 96-well plates. After 24 and 48 h, quantification of apoptosis was measured by flow cytometry as previously described (17).

Inhibition of methadone-induced caspase activation by zVAD.fmk. Inhibition of caspase activation was performed as previously described (15). In brief, the broad spectrum tripeptide inhibitor of caspases zVAD.fmk

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(benzoylcarbonyl-Val-Ala-Asp-fluoromethyl ketone; Enzyme Systems Products) was used at a concentration of 50 $\mu\text{mol/L}$. HL-60 and CEM cells were preincubated with zVAD.fmk 1 h before methadone treatment. After 24 and 48 h, the percentage of apoptotic cells was measured by hypodiploid DNA (sub-G₁) or FSC/SSC analysis. Propidium iodide-stained nuclei (16) or the FSC/SSC profile of cells (17) were analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

Western blot analysis. Western blot analyses were done as previously described (14, 18). Immunodetection of PARP, caspase-3, caspase-9, caspase-8, X chromosome-linked inhibitor of apoptosis (XIAP), CD95, CD95-L, Bax, Bcl-x_L, and β -actin was done using rabbit anti-PARP polyclonal antibody (1:5,000; Roche), mouse anti-caspase-3 monoclonal antibody (1:1,000; Cell Signaling), mouse anti-caspase-8 monoclonal antibody (1:1,000; Cell

Signaling), rabbit anti-active caspase-9 polyclonal antibody (1:1,000; Cell Signaling), mouse anti-XIAP monoclonal antibody (1:1,000; Transduction Laboratories), mouse anti-Fas (anti-CD95) monoclonal antibody (1:1,000; Transduction Laboratories), mouse anti-Fas ligand (anti-CD95 ligand) monoclonal antibody (1:250; BD, PharMingen), rabbit anti-Bax polyclonal antibody (1:250; Oncogene), rabbit anti-Bcl-X_{s/L} polyclonal antibody (1:1,000; Santa Cruz Biotechnology), rabbit anti-p21 polyclonal antibody (1:1,000; Santa Cruz Biotechnology), and mouse anti- β -actin monoclonal antibody (Sigma). Peroxidase-conjugated goat anti-mouse IgG or peroxidase-conjugated goat anti-rabbit IgG (1:5,000; Santa Cruz Biotechnology) as secondary antibody were used for the enhanced chemoluminescence system (Amersham-Pharmacia). Equal protein loading was controlled by β -actin detection.

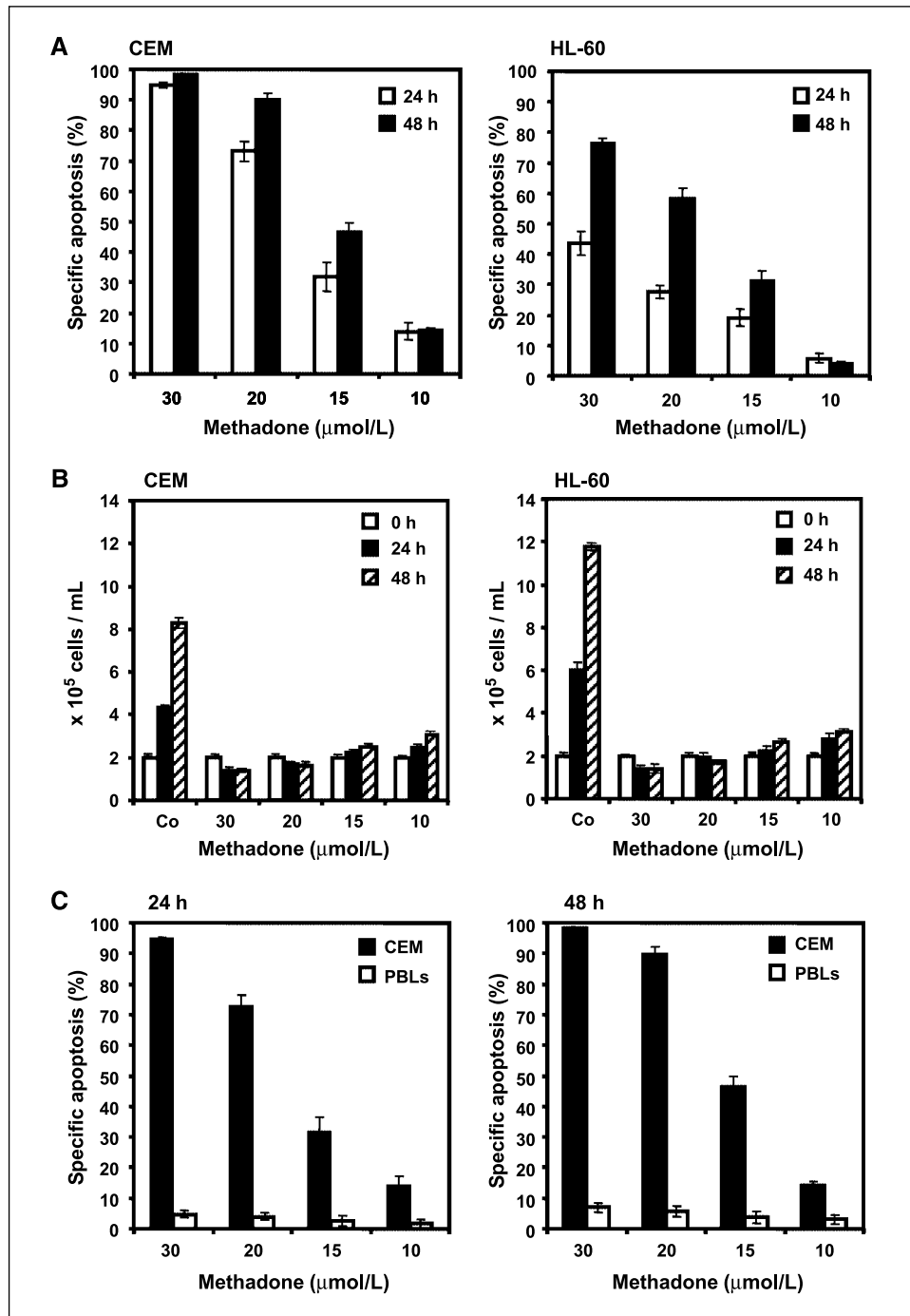
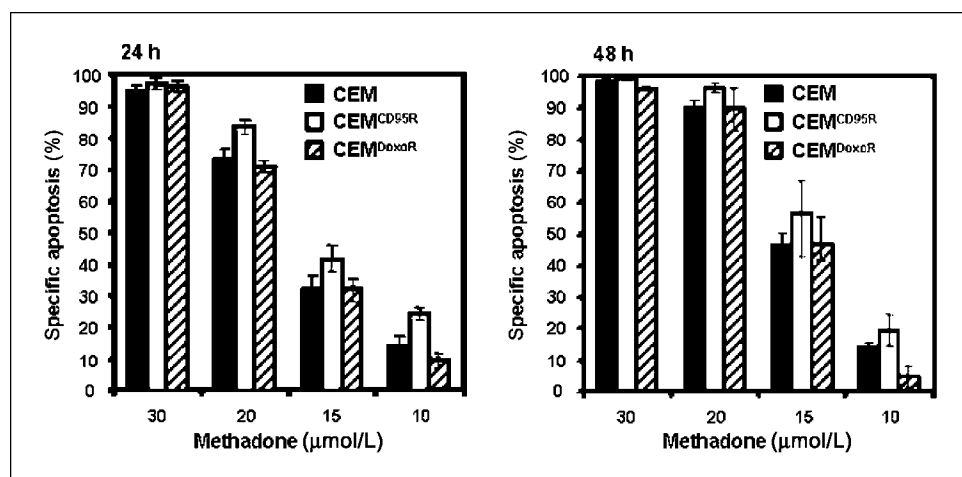


Figure 1. Methadone induces apoptosis in leukemia cells but not in nonleukemic PBLs. **A**, CEM and HL-60 cells were treated with different concentrations of methadone as indicated. After 24 h (white columns) and 48 h (black columns), the percentages of apoptotic cells were measured by hypodiploid DNA analysis. The percentage of specific cell death was calculated as follows: $100 \times [\text{experimental dead cells} (\%) - \text{spontaneous dead cells in medium} (\%)] / [100 - \text{spontaneous dead cells in medium} (\%)]$. Columns, mean of triplicates; bars, SD <10%. Similar results were obtained in three independent experiments. **B**, CEM and HL-60 cells (2×10^5 cells/mL) were treated with different concentrations of methadone as indicated or left untreated (Co, control). After 0 h (white columns), 24 h (black columns), and 48 h (hatched columns), the number of cells in 1 mL of blood was counted. Columns, mean of triplicates; bars, SD <10%. Similar results were obtained in three independent experiments. **C**, CEM cells (black columns) and PBLs (white columns) were treated with different concentrations of methadone as indicated. After 24 and 48 h, the percentages of apoptotic cells were measured by hypodiploid DNA analysis. The percentage of specific cell death was calculated as described in **A**. Columns, mean of triplicates; bars, SD <10%. Similar results were obtained in three independent experiments.

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Figure 2. Methadone induces apoptosis in CD95-resistant (CEM^{CD95R}) and in doxorubicin-resistant (CEM^{DoxoR}) cells with comparable apoptosis rates to parental sensitive CEM leukemia cells. CEM (black columns), CEM^{CD95R} (white columns), and CEM^{DoxoR} (hatched columns) leukemia cells were treated with different concentrations of methadone as indicated. After 24 and 48 h, the percentages of apoptotic cells were measured by the hypodiploid DNA analysis. The percentage of specific cell death was calculated as described in Fig. 1A. Columns, mean of triplicates; bars, SD <10%. Similar results were obtained in three independent experiments.



Results

Methadone induces cell killing in CEM and HL-60 leukemia cells through apoptosis with nontoxic effects in nonleukemic PBLs. In leukemias and solid tumors, anticancer drugs were shown to induce apoptosis and inhibit proliferation (1). Therefore, we analyzed if the therapeutic opioid drug methadone could also inhibit proliferation and trigger apoptosis in the human lymphoblastic leukemia T-cell line CEM and the human myeloid leukemia cell line HL-60, comparable to established and well-known anticancer drugs (Fig. 1A and B). Twenty-four and 48 hours after treatment with different concentrations of methadone (30, 20, 15, and 10 μmol/L), a strong induction of apoptosis (Fig. 1A) and a strong inhibition of growth (Fig. 1B) were detected in CEM and HL-60 cells. We next analyzed if methadone also induces apoptosis in nonleukemic PBLs (Fig. 1C). We incubated isolated PBLs with different concentrations of methadone (30, 20, 15, and 10 μmol/L). Twenty-four and 48 hours after methadone treatment, we found that methadone could not kill PBLs at concentrations comparable to that used for the treatment of leukemia cells such as CEM (Fig. 1C). This shows that methadone induces apoptosis in leukemia cells with nontoxic effects on nonleukemic PBLs.

Methadone breaks doxorubicin resistance, CD95 resistance, and multidrug resistance in leukemia cells. Resistance to anticancer drugs is a limiting factor in the treatment of patients with leukemias and/or tumors (13, 18). We have found that methadone exhibits a potent antileukemic activity and efficiently kills leukemia cells. Therefore, we analyzed if methadone could also induce cell death in doxorubicin-resistant leukemia cells, which were apoptosis-resistant. We treated doxorubicin-resistant CEM leukemia cells CEM^{DoxoR} with different concentrations of methadone (30, 20, 15, and 10 μmol/L; Fig. 2). Twenty-four and 48 hours after methadone treatment, cell death was measured by flow cytometry. After treatment with 30, 20, or 15 μmol/L of methadone, we measured a strong induction of apoptosis in doxorubicin-resistant cells CEM^{DoxoR}, which was similar to that in sensitive leukemia cells CEM (Fig. 2), indicating that methadone overcomes doxorubicin resistance and apoptosis resistance.

We next examined if methadone can also kill leukemia cells which were multidrug-resistant. Therefore, we treated CD95-resistant leukemia cells CEM^{CD95R}, which were resistant to several anticancer drugs such as etoposide, cisplatin, methotrexate, cytarabine, doxorubicin, and vincristine with different concentrations of methadone (30, 20, 15, and 10 μmol/L; Fig. 2). After treatment with

30, 20, and 15 μmol/L of methadone, we measured a strong induction of apoptosis in CD95-resistant leukemia cells CEM^{CD95R} after 24 and 48 hours, which was similar to that in sensitive leukemia cells CEM (Fig. 2). This suggests that methadone induces not only a strong induction of apoptosis in sensitive leukemia cells, but it also kills doxorubicin-resistant and CD95-resistant leukemia cells, which commonly used anticancer drugs failed to kill (2, 3).

Methadone induces caspase-dependent cell death and activates mitochondria in sensitive, doxorubicin-resistant, CD95-resistant, and multidrug-resistant leukemia cells. Methadone induces apoptosis in sensitive and in resistant leukemia cells with unknown molecular mechanisms. Therefore, we wanted to identify the mechanism and effector molecules which may be altered by methadone-triggered cell death in leukemia cells.

Caspases play a critical role in apoptosis induction by anticancer drugs (1, 10). Therefore, using Western blot analyses, we examined if methadone activates caspases in HL-60 and CEM leukemia cells as well as in doxorubicin-resistant leukemia cells CEM^{DoxoR}, which were apoptosis-resistant, and in CD95-resistant leukemia cells CEM^{CD95R}, which were multidrug-resistant and apoptosis-resistant. After treatment with different concentrations of methadone (20 and 15 μmol/L), caspase-3 and PARP were cleaved in HL-60 and CEM leukemia cells (Fig. 3A), as well as in doxorubicin-resistant leukemia cells CEM^{DoxoR} and in CD95-resistant leukemia cells CEM^{CD95R} (Fig. 3B). Activation of caspase-8, which has been shown to be induced by anticancer drugs in leukemia cells, was not found after methadone treatment. To investigate the critical role of methadone in the activation of caspases, we preincubated CEM and HL-60 cells with the broad spectrum inhibitor of caspases, zVAD.fmk. Incubation with zVAD.fmk almost completely inhibited methadone-induced apoptosis (Fig. 3C), suggesting that caspases are central for methadone-induced apoptosis in leukemia cells.

Anticancer drugs have been shown to activate the mitochondrial pathway as well as the ligand/receptor pathway in leukemia and tumor cells (1). We investigated if mitochondria could also play a role in methadone-induced apoptosis in leukemia cells. CEM, HL-60, doxorubicin-resistant leukemia cells CEM^{DoxoR}, and CD95-resistant leukemia cells CEM^{CD95R} were treated with different concentrations of methadone (20 and 15 μmol/L; Fig. 4). After 24 and 48 hours, we found a strong cleavage (37 kDa fragment) of caspase-9 and a strong down-regulation of the caspases inhibiting XIAP in HL-60 and CEM leukemia cells (Fig. 4A), as well as in doxorubicin-resistant leukemia cells CEM^{DoxoR} and in

CD95-resistant leukemia cells CEM^{CD95R} (Fig. 4B), which were multidrug-resistant and apoptosis-resistant.

Mitochondrial changes are regulated by proapoptotic and antiapoptotic Bcl-2 family members. After 24 and 48 hours, a strong down-regulation of Bcl-x_L was found in HL-60 and in CEM leukemia cells (Fig. 4A), as well as in doxorubicin-resistant leukemia cells CEM^{DoxoR} and in CD95-resistant leukemia cells CEM^{CD95R} (Fig. 4B) after treatment with different concentrations of methadone (20 and 15 μmol/L). Up-regulation of Bax was not found after methadone treatment in leukemia cells.

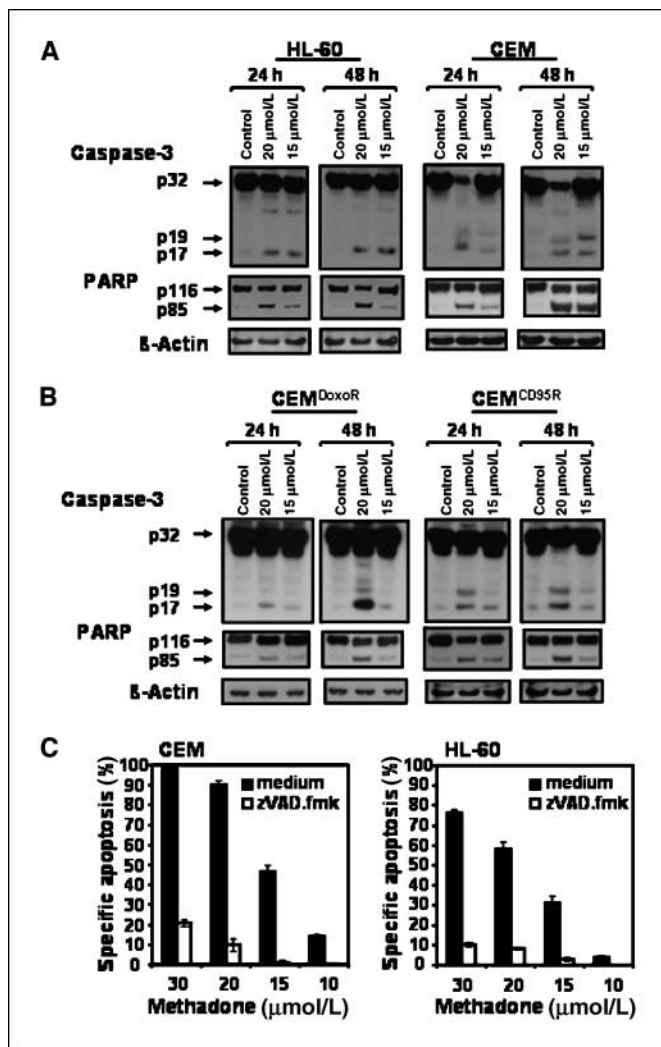


Figure 3. Methadone induces caspase-dependent death in sensitive (HL-60 and CEM), in doxorubicin-resistant (CEM^{DoxoR}), and in CD95-resistant (CEM^{CD95R}) leukemia cells, which were multidrug-resistant and apoptosis-resistant. A and B, methadone-induced activation of caspase-3 and PARP cleavage in HL-60, CEM, CEM^{DoxoR} and CEM^{CD95R} cells. HL-60 and CEM (A), and CEM^{DoxoR} and CEM^{CD95R} (B) cells were treated with different concentrations of methadone as indicated or left untreated (Control). After 24 and 48 h, Western blot analyses for caspase-3 and PARP were performed. The active fragment of caspase-3 was detected at ~19 and 17 kDa, and the cleaved product of PARP at ~85 kDa. Equal protein loading was controlled by anti-β-actin antibody. C, inhibition of caspase activation with zVAD.fmk blocks methadone-induced apoptosis in CEM and HL-60 cells. CEM and HL-60 cells were treated with different concentrations of methadone as indicated in the absence (black columns, medium) or presence (white columns, 50 μmol/L zVAD.fmk) of 50 μmol/L of zVAD.fmk. After 48 h, the percentages of apoptotic cells were measured by hypodiploid DNA analysis. The percentage of specific cell death was calculated as described in Fig. 1A. Columns, mean of triplicates; bars, SD <10%. Similar results were obtained in three independent experiments.

Furthermore, up-regulation of death-inducing ligands and death-inducing receptors such as CD95, which have been shown to be up-regulated by anticancer drugs, were not found after methadone treatment in leukemia cells (data not shown). This indicates that methadone induces apoptosis by direct activation of the intrinsic mitochondrial pathway in sensitive as well as in resistant leukemia cells.

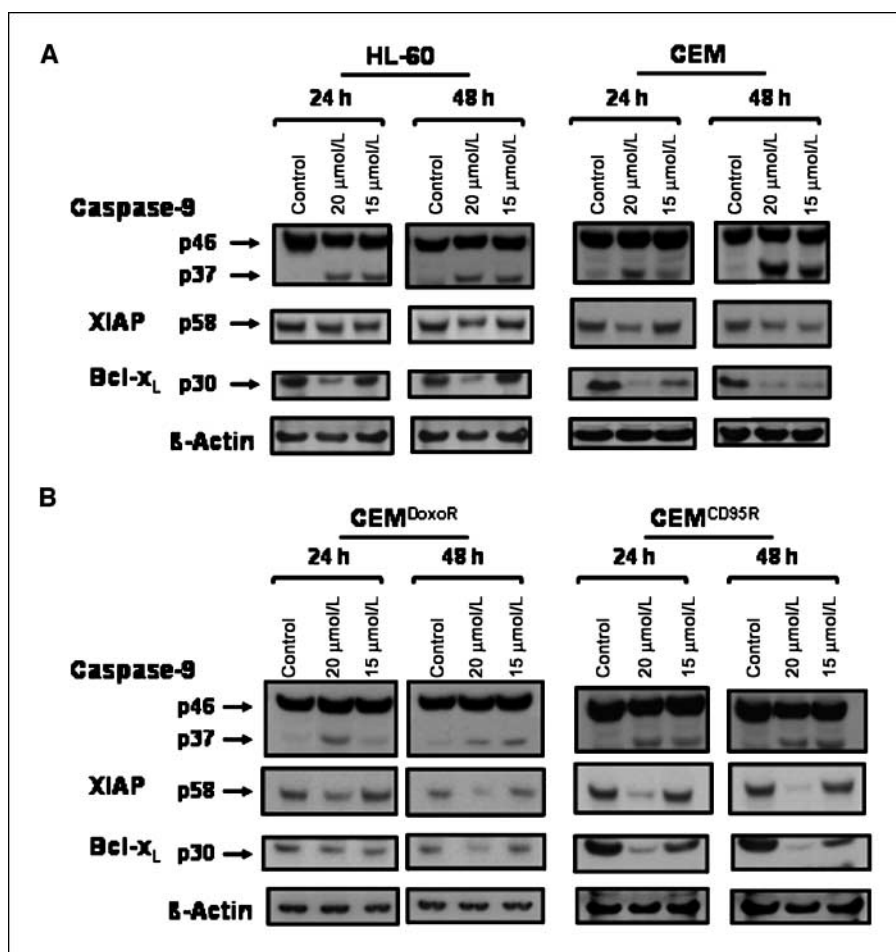
Discussion

Different anticancer drugs have been used in the treatment of leukemias and solid tumors in conventional therapies (1). The therapeutic opioid methadone has been used in therapies to treat opioid addiction (4). In addition to treatment of opioid withdrawal, methadone is of significant use as a long-acting analgesic, particularly for neuropathic pain syndromes (4–6). Leukemia cells such as HL-60 cells express the μ opioid peptide receptor, which was regulated by morphine (19). Anticancer drugs induce apoptosis in leukemias and solid tumors (1). We have found that methadone inhibited the growth of leukemias and killed potent leukemia cells through apoptosis induction, indicating a potent antileukemic activity of methadone in leukemias. In contrast to leukemia cells, nonleukemic PBLs survived after methadone treatment at comparable concentrations and time points. Resistance to anticancer drugs is a major problem and a limiting factor in treatment of patients with leukemias and tumors (1, 13). Cross-resistance has been noted between different commonly used anticancer drugs, involving defects in apoptosis signaling (1–3). Therefore, new options are needed to improve therapeutic success in the treatment of leukemias and solid tumors. We provide evidence that methadone induces not only a strong apoptosis in sensitive leukemia cells but that it also kills potent doxorubicin-resistant as well as CD95-resistant and apoptosis-resistant leukemia cells, which cannot be killed by several anticancer drugs commonly used in leukemia therapies. This suggests that methadone is a promising agent for the treatment of leukemia, especially when conventional therapies are less effective because methadone kills leukemia cells and breaks chemoresistance and apoptosis resistance in leukemia cells with nontoxic effects on nonleukemic lymphocytes. In addition, methadone is a commonly used maintenance medication for outpatient treatment of opioid dependence (4). The side effects of methadone therapy in patients are well known (7).

We have found that methadone induces apoptosis in leukemia cells similar to commonly used anticancer drugs. However, the molecular mechanism of methadone-induced cell killing is unknown. Anticancer drugs activate apoptosis pathways in leukemias and solid tumors involving receptor-driven pathways and/or mitochondrial pathways (1, 9). We have shown that methadone activates apoptosis pathways in leukemia cells by activating the intrinsic mitochondrial pathway. Anticancer drugs activate caspases in leukemia cells (1). Methadone-induced cell death was completely dependent on the activation of caspases in leukemia cells, involving the activation of caspase-3 and caspase-9. This indicates that methadone induces cell death via caspase activation similar to anticancer drugs used in leukemia therapy. However, activation of caspase-8, which was induced by anticancer drugs in leukemia cells, could not be found after methadone treatment.

Deficient activation of caspases was found in chemoresistant and radioresistant leukemia cells following anticancer drug treatment (1, 3, 14). Methadone activated caspases in doxorubicin-resistant and in CD95-resistant leukemia cells, which are apoptosis-resistant and multidrug-resistant, suggesting that methadone reverses

Figure 4. Methadone activates the mitochondrial pathway in sensitive (*HL-60* and *CEM*), in doxorubicin-resistant (*CEM^{DoxoR}*), and in CD95-resistant (*CEM^{CD95R}*) leukemia cells, which were multidrug-resistant and apoptosis-resistant. *A* and *B*, methadone-induced activation of caspase-9, down-regulation of XIAP, and down-regulation of Bcl-x_L in *HL-60*, *CEM*, *CEM^{DoxoR}*, and *CEM^{CD95R}* cells. *HL-60* and *CEM* (*A*) and *CEM^{DoxoR}* and *CEM^{CD95R}* (*B*) cells were treated with different concentrations of methadone as indicated or left untreated (*Control*). After 24 and 48 h, Western blot analyses for caspase-9, XIAP, and Bcl-x_L were performed. The active fragment of caspase-9 was detected at ~37 kDa, XIAP was detected at ~58 kDa, and Bcl-x_L was detected at ~30 kDa. Equal protein loading was controlled by anti-β-actin antibody.



deficient activation of caspases in resistant leukemia cells. In addition, anticancer drugs trigger apoptosis through mitochondria activation or receptor/ligand interaction (2, 9, 20). In *CEM* and *HL-60* leukemia cells, anticancer drugs such as doxorubicin have been shown to induce apoptosis through CD95 receptor/ligand interaction (2, 18). In doxorubicin-resistant and apoptosis-resistant *CEM* and *HL-60* cells, which have a defect in the apoptosis pathway, CD95 receptor/ligand interaction was blocked after treatment with several anticancer drugs used in conventional leukemia therapies. We recently published that α -irradiation, in contrast with β -irradiation and γ -irradiation, induces apoptosis and overcomes resistance by direct activation of mitochondria in sensitive, chemoresistant, and radioresistant leukemia cells (15). Consistent with these data, we found that methadone activates the mitochondrial pathway in sensitive as well as in doxorubicin-resistant and CD95-resistant leukemia cells, which were apoptosis-resistant and multidrug-resistant. Mitochondria-directed apoptotic stimuli induce a variety of mitochondrial changes, among them, the production of oxygen radicals and the opening of membrane pores, thereby releasing apoptogenic factors such as cytochrome *c*, apoptosis-inducing factor, and caspase-2 or caspase-9 from the mitochondria into the cytosol triggering caspase-3 activation through the formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex (1). Methadone activated caspase-9 and inhibited XIAP. Mitochondria are regulated by Bcl-2 family members. The antiapoptotic protein Bcl-x_L was down-regulated in leukemia cells after methadone treatment. Up-regulation of death-inducing ligands and death-

inducing receptors such as CD95 were not found. This indicates that methadone induces apoptosis and overcomes doxorubicin resistance, chemoresistance, and apoptosis resistance by direct activation of the intrinsic mitochondrial pathway.

Taken together, these results suggest that methadone is a promising agent in leukemia therapy, especially when conventional therapies are less effective. Notably, methadone kills sensitive leukemia cells and breaks chemoresistance and apoptosis resistance in leukemias with nontoxic effects on nonleukemic PBLs. By providing the molecular basis of methadone-induced cell death in sensitive as well as in chemoresistant and apoptosis-resistant leukemia cells and by finding comparable activation of apoptosis pathways of commonly used anticancer drugs but with more potent effects in cell killing, especially in chemoresistant and apoptosis-resistant leukemia cells, this study provides the foundation for new strategies establishing methadone as an additional therapeutic anticancer drug in leukemia therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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