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The Aminosteroid Phospholipase C Antagonist U-73122 (1-[6-[[17-β-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione) Potently Inhibits Human 5-Lipoxygenase in Vivo and in Vitro

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ABSTRACT

U-73122 (1-[6-[[17-β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino] hexyl]-1*H*-pyrrole-2,5-dione) is a widely used antagonist of phosphoinositide-specific phospholipase C (PLC) and is frequently used to define a role of PLC in receptor-mediated elevation of intracellular calcium concentration ($[Ca^{2+}]_i$). In human polymorphonuclear leukocytes (PMNLs), U-73122 inhibited increases in $[Ca^{2+}]_i$ induced by G protein-coupled receptor (GPCR) agonists (*N*-formyl-methionyl-leucyl-phenylalanine or platelet-activating factor; IC_{50} of ≈2 to 4 μM), but it failed to suppress responses induced by ionomycin or thapsigargin. 5-Lipoxygenase (5-LO) is a Ca^{2+}-regulated enzyme that can be activated in leukocytes by stimuli that elevate $[Ca^{2+}]_i$. Attempts to investigate the involvement of PLC in cellular 5-LO activation revealed that U-73122 suppresses 5-LO product synthesis regardless of the stimulus and independently of Ca^{2+}. Thus, U-73122 blocked 5-LO product synthesis induced by cell stress, involving 5-LO phosphorylation pathways in the absence of Ca^{2+} with an IC_{50} of ≈2 μM. Direct inhibition of 5-LO by U-73122 was evident in PMNL homogenates (IC_{50} of ≈2.4 μM), and isolated human recombinant 5-LO enzyme was potently inhibited by U-73122 (IC_{50} of ≈30 nM). Thiols (glutathione) strongly blunted the effect of U-73122 on isolated 5-LO. On the other hand, depletion of cellular thiols by *N*-ethylmaleimide strongly increased the efficacy of U-73122 to inhibit 5-LO in intact cells or corresponding homogenates, suggesting that U-73122 may interfere with sulfhydryl groups on 5-LO. Since 5-LO products induce increases in $[Ca^{2+}]_i$ via GPCRs, caution should be used when interpreting data where U-73122 is used as tool to determine a direct role of PLC in receptor-mediated Ca^{2+} mobilization.

The aminosteroid U-73122 is widely used as a tool to investigate the involvement of the phosphoinositide-specific phospholipase C (PLC) in signal transduction, particularly in studies attempting to characterize pathways leading to intracellular Ca^{2+} mobilization upon agonist challenge (Bleasdale et al., 1989, 1990; Smith et al., 1990). PLC isoenzymes are activated in response to many extracellular stimuli and hydrolyze phosphatidylinositol bisphosphate (PIP_2) to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3); the latter releases Ca^{2+} from internal stores (Rhee, 2001). Detailed analysis of U-73122 actions confirmed that the compound directly blocks PLC isoenzymes in vivo and in vitro (Bleasdale et al., 1990; Smith et al., 1990; Hou et al., 2004). U-73343, a closely related inactive analog, where the pyrrolidindione was substituted for pyrroledione, is commonly used as a negative control that fails to block agonist-induced Ca^{2+} mobilization (Bleasdale et al., 1990).

Several reports have described actions of U-73122 that could not be clearly connected to PLC inhibition, and it was suggested that molecular targets for U-73122 other than PLC may exist (Alter et al., 1994; Berven and Barritt, 1995; Grierson and Meldolesi, 1995; Wang, 1996; Pulcinelli et al., 1998; Walker et al., 1998; Hughes et al., 2000). For example,

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ABBREVIATIONS: U-73122, 1-[6-[[17-β-3-methoxyestra-1,3,5(10)-trien-17-yl] amino]hexyl]-1*H*-pyrrole-2,5-dione; PLC, phospholipase C; PIP_2, phosphatidylinositol bisphosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; LO, lipoxygenase; LT, leukotriene; MAPK, mitogen-activated protein kinase; GPCR, G protein-coupled receptor; $[Ca^{2+}]_i$, intracellular calcium concentration; U-73343, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidine-dione; AA, arachidonic acid; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GSH, glutathione; NEM, *N*-ethylmaleimide; PAF, platelet-activating factor; HPLC, high-performance liquid chromatography; PMNL, polymorphonuclear leukocyte; PBS, phosphate-buffered saline; RT, room temperature; DMSO, dimethyl sulfoxide; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; AM, acetoxymethyl ester.

much higher doses are required to suppress PLC activity compared with those needed to inhibit Ca^{2+} mobilization under the same assay conditions (Smith et al., 1990; Muto et al., 1997; Pulcinelli et al., 1998), and evidence was provided for separate effects of U-73122 on PLC and Ca^{2+} channels (Berven and Barritt, 1995; Grierson and Meldolesi, 1995; Wang, 1996; Pulcinelli et al., 1998). Nevertheless, despite the numerous observations of PLC-independent effects, the molecules that are targeted by U-73122 are less defined. Thus far, only the histamine H_1 receptor was unraveled as an additional molecular target of U-73122 (Hughes et al., 2000).

5-Lipoxygenase (5-LO) is the key enzyme in the biosynthesis of leukotrienes (LTs), which are important mediators of inflammatory and allergic reactions (Funk, 2001). Activation of 5-LO in the cell involves the initial conversion of the active site iron from the ferrous to the ferric state and is accompanied by a translocation process of the enzyme to the nuclear membrane, where it cofunctions with the 5-LO-activating protein (for review, see Werz, 2002). Such an initial cellular activation of 5-LO can be mediated by elevated Ca^{2+} levels and/or 5-LO phosphorylation at serine residues by members of the mitogen-activated protein kinase (MAPK) family (Werz, 2002), as well as by DAGs, such as 1-oleoyl-2-acetylglycerol (Bauldry et al., 1991; Albert et al., 2003). In fact, several stimuli capable to induce 5-LO product synthesis, including ligands of G protein-coupled receptors (GPCRs) but also nonreceptor-coupled stimuli such as thapsigargin or ionophores, elevate $[Ca^{2+}]_i$, cause formation of DAGs, and stimulate MAPKs.

Since elevation of $[Ca^{2+}]_i$, generation of DAGs, and MAPK activation are known to occur via PLC-dependent pathways, it was conceivable to investigate the role of PLC in agonist-induced 5-LO product synthesis using U-73122 as a pharmacological tool. We found that 5-LO product synthesis is potently suppressed by U-73122 in intact cells at similar concentrations required to inhibit the mobilization of Ca^{2+} by ligands transducing signals via G protein/PLC pathways. However, cellular 5-LO also was suppressed by U-73122 under conditions involving PLC-independent activation pathways, and U-73122 also blocked 5-LO activity in cell-free systems. Detailed analysis of 5-LO inhibition demonstrates that in vitro, U-73122 is a direct and potent inhibitor of 5-LO at concentrations that are far below those needed to inhibit PLC.

Materials and Methods

Materials. The plasmid pT3–5LO was kindly provided by Dr. Olof Rådmark (Karolinska Institutet, Stockholm, Sweden). U-73122 was from Calbiochem (San Diego, CA); U-73343 and Fura-2 were from Alexis (Grünberg, Germany); AA, ionomycin, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), glutathione (GSH), *N*-ethylmaleimide (NEM), platelet-activating factor (PAF), and thapsigargin were from Sigma Chemie (Deisenhofen, Germany); [^{3}H]PIP_2 was from GE Healthcare Bio-Sciences (Freiburg, Germany); and HPLC solvents were from Merck (Darmstadt, Germany).

Cells. Human platelets and polymorphonuclear leukocytes (PMNLs) were freshly isolated from leukocyte concentrates obtained at St. Markus Hospital (Frankfurt, Germany) as described previously (Albert et al., 2002). In brief, venous blood was taken from healthy adult donors, and leukocyte concentrates were prepared by centrifugation at 4000*g*, 20 min, 20°C. PMNLs were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories GmbH, Linz, Austria), and hypotonic lysis of erythrocytes. PMNLs (10^7 cells/ml; purity >96–97%) were finally resuspended in PBS containing 1 mg/ml glucose (PG buffer), and 1 mM $CaCl_2$ (PGC buffer) or 1 mM EDTA was added, as indicated.

Platelets were isolated from supernatants (800*g*, 10 min, RT) after centrifugation of leukocyte concentrates on Nycoprep cushions (see above). The supernatant was mixed with PBS, pH 5.9 [3:2 (v/v)], centrifuged (2000*g*, 15 min, RT), and the pelleted platelets were resuspended in PBS, pH 5.9/0.9% NaCl [1:1 (v/v)], washed by centrifugation (2000*g*, 10 min, RT) and finally resuspended in PBS, pH 7.4.

Measurement of Intracellular Ca^{2+} Levels. PMNLs (10^7/ml PGC buffer) were incubated with 2 μM Fura-2/AM for 30 min at 37°C, washed, resuspended in 1 ml of PGC buffer, and transferred into a thermally controlled (37°C) fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman series 2) with continuous stirring. The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively. Intracellular Ca^{2+} levels were calculated according to the method of Grynkiewicz et al. (1985). F_{max} (maximal fluorescence) was obtained by lysing the cells with 1% Triton X-100 and F_{min} by chelating Ca^{2+} with 10 mM EDTA.

Expression and Purification of 5-LO from *Escherichia coli*. Expression of 5-LO, performed in *E. coli* JM 109 cells transfected with pT3–5LO, and purification of 5-LO by ATP affinity chromatography (A2767; Sigma Chemie) was performed as described previously (Fischer et al., 2003). Partially purified 5-LO was immediately used for in vitro activity assays.

Determination of 5-Lipoxygenase Products in Intact PMNLs. To assay 5-LO product formation in intact cells, 7.5×10^6 freshly isolated PMNLs were finally resuspended in 1 ml of PG or PGC buffer. After preincubation with U-73122 or vehicle (DMSO) at 37°C, 5-LO product formation was started by addition of the indicated stimuli with or without exogenous AA as indicated. The concentration of DMSO was kept to <0.3% (v/v), and the final volume was 1 ml. After 10 min at 37°C, the reaction was stopped, and formed 5-LO metabolites were extracted and analyzed by HPLC as described previously (Werz et al., 2002b). 5-LO product formation is expressed as nanograms of 5-LO products per 10^6 cells, which includes LTB_4 and its all-*trans* isomers 5(*S*),12(*S*)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid and 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid. Cysteinyl LTs (LTC_4, D_4, and E_4) were not detected, and oxidation products of LTB_4 were not determined.

Determination of Product Formation of 5- and 15-Lipoxygenase in Cell-Free Systems. For determination of 5- and 15-LO activity in homogenates, freshly isolated PMNLs (7.5×10^6) were resuspended in 1 ml of PBS containing 1 mM EDTA, sonicated (3×10 s) at 4°C, and 1 mM ATP was added. For determination of the activity of recombinant isolated 5-LO, partially purified 5-LO (0.5 μg in 5 μl) was added to 1 ml of a 5-LO reaction mix (PBS, pH 7.4, 1 mM EDTA, 1 mM ATP, and 25 μg/ml γ-globulin), and test compounds or vehicle (DMSO) was added as indicated. The concentration of DMSO was kept to <0.3% (v/v). After 5 to 10 min at 4°C, samples were prewarmed for 30 s at 37°C, and 2 mM $CaCl_2$ and AA at the indicated concentrations were added to start 5- and 15-LO product formation. The reaction was stopped after 10 min at 37°C, and the formed metabolites were analyzed by HPLC as described for intact cells. 15(*S*)-Hydro(pero)xy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid was analyzed as products of 15-LO.

Determination of 12-Lipoxygenase Product Formation in Homogenates of Platelets. Platelets (1×10^8) were resuspended in 1 ml of PBS (containing 1 mM EDTA) and cooled on ice for 5 min. After sonication (3×5 s), U-73122 was added (5–10 min at 4°C), the samples were preincubated for 30 s at 37°C, and the incubation was started by the addition of Ca^{2+} and AA (2 mM and 40 μM final concentrations, respectively). After 10 min at 37°C, incubations were stopped, and 12(*S*)-hydro(pero)xy-5,8-*cis*-10-*trans*-14-*cis*-eicosatetraenoic acid was extracted using C18 solid phase extraction columns and analyzed by HPLC as described previously (Albert et al., 2002).

Determination of PLC Activity in Vitro. PLC activity in 100,000*g* supernatants of PMNLs was assayed by measuring the hydrolysis of PIP_2 into inositol phosphates as described previously (Wang and Kuo, 1997).

Statistics. The program GraphPad Prism 3.0 was used for statistical comparisons (GraphPad Software Inc., San Diego, CA). Statistical evaluation of the data was performed using Student's *t* test for unpaired observations. A *P* value of <0.05 was considered significant.

Results

U-73122 Selectively Inhibits Receptor-Coupled Ca^{2+} Mobilization in PMNLs and Blocks PLC Activity in Vitro. The efficacy and selectivity of U-73122 to suppress PLC-mediated Ca^{2+} mobilization in PMNLs was determined. For control, U-73343, the inactive analog of U-73122, was used. In agreement with others (Bleasdale et al., 1990; Smith et al., 1990; Hou et al., 2004) and with our recent report (Feisst and Werz, 2004), U-73122 dose dependently inhibited the mobilization of Ca^{2+} induced by the GPCR ligands fMLP and PAF in PMNLs, with IC_{50} of ≈2 and 4 μM, respectively (Fig. 1A). In contrast, U-73122 was hardly effective in cells stimulated by ionomycin or thapsigargin, which circumvent PLC signaling for Ca^{2+} mobilization (Fig. 1A). U-73343 up to 10 μM failed to significantly inhibit Ca^{2+} mobilization, regardless of the stimulus (data not shown).

Next, the effect of U-73122 on soluble PLC activity in 100,000*g* supernatants from PMNLs was analyzed. The compound concentration dependently inhibited total PLC activity, with an IC_{50} of ≈10 μM (Fig. 1B), which is in agreement with the values determined in previous studies (Smith et al., 1990; Pulcinelli et al., 1998).

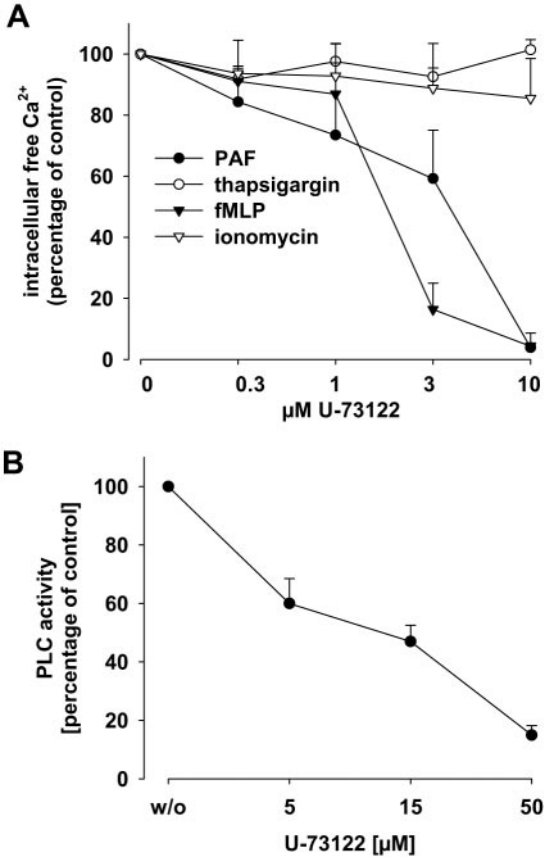

Fig. 1. U-73122 inhibits receptor-mediated Ca^{2+} mobilization in PMNLs and suppresses PLC activity in vitro. A, Ca^{2+} mobilization. Freshly isolated PMNLs (10^7/ml PGC buffer) were loaded with 2 μM Fura-2/AM. Cells were preincubated with U-73122 at the indicated concentrations for 20 min at RT and stimulated with 100 nM fMLP, 100 nM PAF, 1 μM ionomycin, or 1 μM thapsigargin at 37°C. The fluorescence was measured and $[Ca^{2+}]_i$ was calculated as described in text. Results are expressed as the percentage of the Ca^{2+} level (mean + S.E.; $n = 3–4$) versus control cells without inhibitor (100%). Stimulation with fMLP, PAF, ionomycin, and thapsigargin elevated the $[Ca^{2+}]_i$ levels from 53.7 ± 3.1 nM to 174 ± 13.4, 249 ± 35, 412.7 ± 61.4, and 167 ± 34 nM, respectively. B, PLC activity in vitro. Supernatants of PMNLs (100,000*g*) were prepared, and the hydrolysis of PIP_2 into inositol phosphates was assayed as described in text. Results are given as mean + S.E.; $n = 4$.

U-73122 Generally Inhibits 5-LO Product Formation in Intact PMNLs. Elevation of $[Ca^{2+}]_i$ is a determinant for 5-LO activation, and diverse agonists capable to increase $[Ca^{2+}]_i$ induce the formation of 5-LO products in various cell types (Werz, 2002). PMNLs were stimulated with agonists

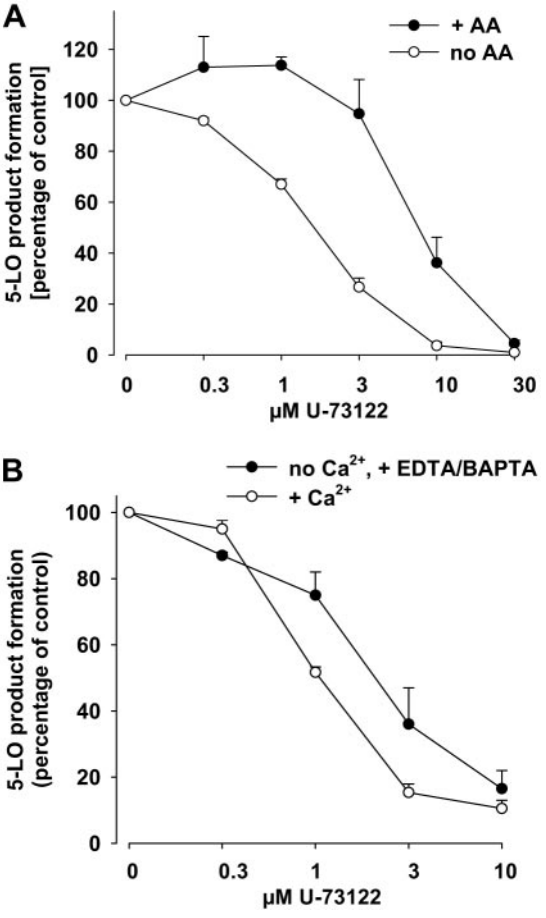

Fig. 2. U-73122 inhibits 5-LO product formation in intact PMNLs. Freshly isolated PMNLs (7.5×10^6/ml PG buffer) were preincubated with U-73122 at the indicated concentrations for 20 min at 37°C. A, cells were supplemented with 1 mM $CaCl_2$ and stimulated with ionomycin (1 μM) in the presence or absence of 40 μM AA. 5-LO products formed in the absence of U-73122 (control) were 29.3 ± 2.5 and 107.3 ± 25.7 ng/10^6 cells for stimulation with ionomycin and ionomycin plus AA, respectively. B, five minutes after addition of U-73122, cells were preincubated with 1 mM EDTA plus 30 μM BAPTA/AM or with 1 mM $CaCl_2$ for 15 min at 37°C as indicated. Cells were then stimulated with 300 mM NaCl. After 3 min, 40 μM AA was added and after another 10 min, 5-LO products were extracted and determined by HPLC as described under *Materials and Methods*. 5-LO products formed in the absence of U-73122 (control) were 55 ± 4.6 and 48.3 ± 6.4 ng/10^6 cells for stimulation with NaCl plus 1 mM EDTA and 30 μM BAPTA/AM and NaCl plus $CaCl_2$, respectively. Results are given as mean + S.E.; $n = 3$ to 4.

that do (fMLP and PAF) and do not (ionomycin) use PLC-dependent pathways to induce Ca^{2+} mobilization. Also, to circumvent effects of U-73122 on AA supply by $cPLA_2$, 5-LO product synthesis was examined in the presence of exogenously added AA (40 μM).

U-73122 suppressed 5-LO product synthesis in PMNLs under all stimulation conditions. In agreement with previous findings (for review, see Werz, 2002), for PMNLs stimulated with fMLP or PAF, 5-LO product formation was low (<1 ng/10^6 cells). Thus, attempts to obtain reliable concentration-response curves for U-73122 and to determine IC_{50} values with high precision failed. For PMNLs stimulated with ionomycin (that causes substantial 5-LO product synthesis; 29.3 ± 2.5 ng/10^6 cells), the concentration-response curves are presented in Fig. 2A. As can be seen, U-73122 concentration dependently inhibited 5-LO product synthesis with an IC_{50} value of ≈1.8 μM. Also, in the presence of exogenous AA, U-73122 suppressed 5-LO product synthesis (IC_{50} of ≈6 μM). U-73343 was less efficient and the IC_{50} value was >10 μM (data not shown). Moreover, when 5-LO was activated by phosphorylation using a stimulus [hyperosmotic (300 mM) NaCl] that does not elevate $[Ca^{2+}]_i$ in PMNLs (Werz et al., 2002a,b), U-73122 still suppressed 5-LO product formation

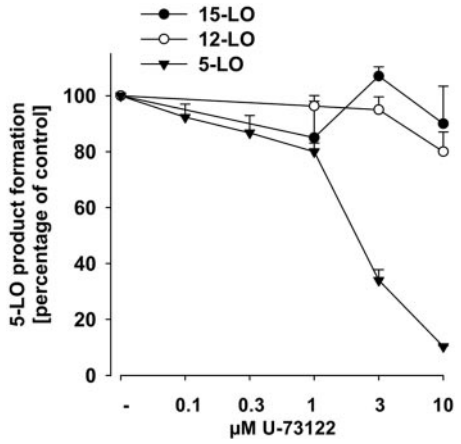

Fig. 3. U-73122 inhibits 5-LO product formation in broken cells: effects on 12- and 15-LO. Whole homogenates of human PMNLs or platelets, corresponding to 7.5 × 10^6 PMNL/ml or 1 × 10^8 platelets/ml PG buffer containing 1 mM EDTA were prepared as described in text. To homogenates of PMNLs, 1 mM ATP was added. Homogenates were preincubated with the indicated concentrations of U-73122 for 5 to 10 min on ice. Samples were prewarmed at 37°C for 30 s, and AA (40 μM) together with 2 mM $CaCl_2$ was added. After another 10 min at 37°C, 5-, 12-, and 15-LO activities were determined as described in text. Product formation of 5-, 12-, and 15-LO without U-73122 (controls) was 156 ± 16.5, 3.1 ± 0.6, and 25.3 ± 4.4 ng/10^6 cells. Results are given as mean + S.E.; n = 3 to 4.

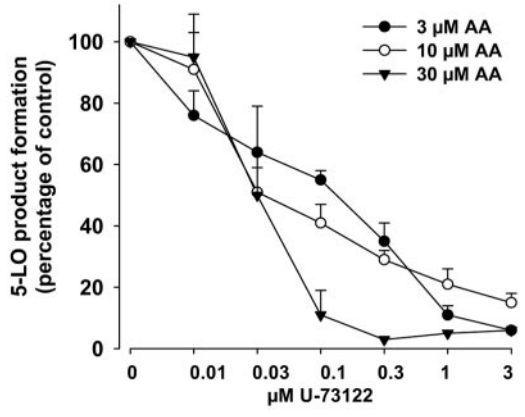

Fig. 4. U-73122 inhibits the activity of isolated 5-LO in vitro. Human recombinant 5-LO was expressed in *E. coli* and partially purified as described in text. 5-LO (0.5 μg) was added to a 5-LO reaction mix containing the indicated concentrations of U-73122. After 5 to 10 min on ice, the samples were prewarmed for 30 s at 37°C. The 5-LO reaction was started by addition of AA (3, 10, or 30 μM as indicated) in the presence of 2 mM $CaCl_2$. After 10 min at 37°C, formed 5-LO products were extracted and determined by HPLC. 5-LO products formed in control incubations (no U-73122) at 3, 10, or 30 μM AA were 68.9 ± 4.7, 219.9 ± 25.7, and 244.4 ± 78.7 ng/ml, respectively. Results are given as mean + S.E.; n = 3.

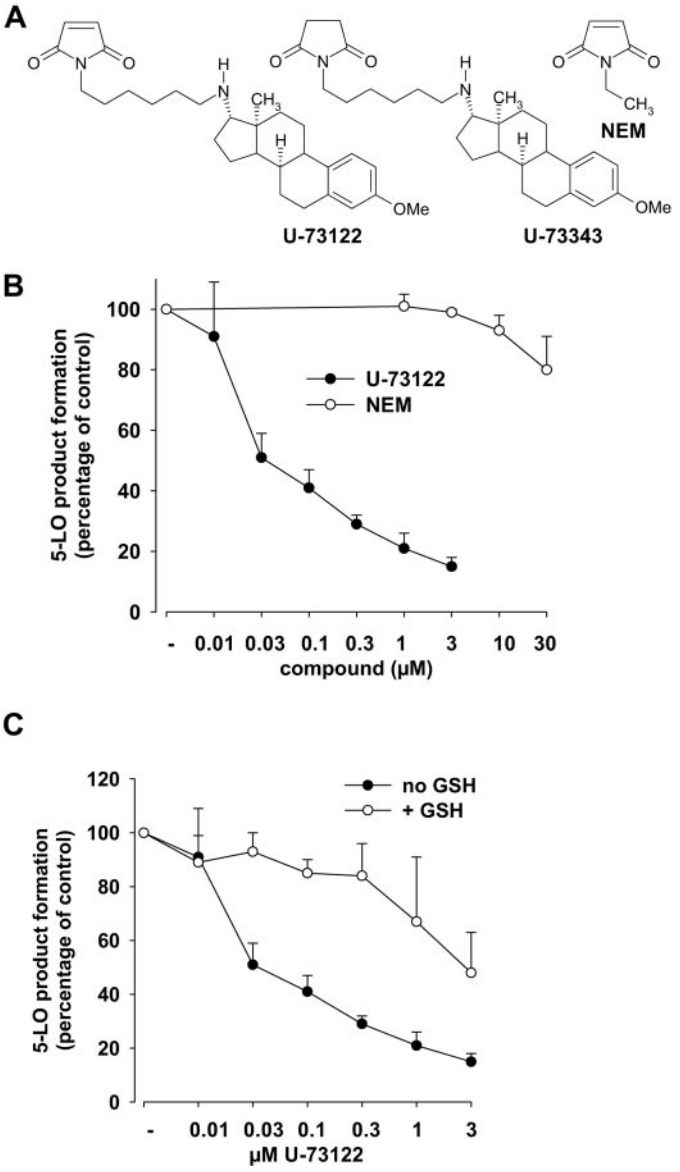

Fig. 5. Comparison of NEM with U-73122 in 5-LO inhibition: effects of glutathione. A, chemical structures of U-73122, U-73343, and NEM. B, inhibition of 5-LO by NEM and U-73122. Partially purified recombinant 5-LO (0.5 μg) was added to a 5-LO reaction mix containing the indicated amounts of U-73122 or NEM, respectively. C, effects of GSH. Partially purified recombinant 5-LO (0.5 μg) was added to a 5-LO reaction mix with or without 1 mM GSH containing the indicated amounts of U-73122. After 5 to 10 min on ice, the samples were prewarmed for 30 s at 37°C, the 5-LO reaction was started by addition of 10 μM AA in the presence of 2 mM $CaCl_2$ and after another 10 min at 37°C, formed 5-LO products were extracted and determined by HPLC. 5-LO product formation of controls (no U-73122) was 219.9 ± 25.7, 252.7 ± 72.2, and 226.7 ± 52.1 ng/ml in the absence of additive, in presence of NEM, and in presence of GSH, respectively Results are given as mean + S.E.; n = 3.

(Fig. 2B). Finally, when PMNLs were depleted from extra- and intracellular Ca^{2+} using EDTA and BAPTA/AM, respectively, U-73122 reduced 5-LO product synthesis induced by 300 mM NaCl with the same potency (Fig. 2B).

U-73122 Inhibits 5-LO Activity under Cell-Free Assay Conditions: Effects on 12- and 15-LOs. Direct inhibition of 5-LO under cell-free conditions by U-73122 was investigated using whole homogenates of human PMNLs. Under optimized assay conditions (presence of Ca^{2+}, ATP, and 40 μM AA as substrate), U-73122 dose-dependently inhibited 5-LO activity in homogenates with an IC_{50} of ≈2.4 μM (Fig. 3), whereas U-73343 (10 μM) was less efficient (39 ± 8% inhibition). Also, in the absence of the stimulatory cofactors ATP and Ca^{2+}, U-73122 inhibited 5-LO with same efficacy (data not shown). Thus, U-73122 seems to directly inhibit enzymatic activity of 5-LO.

Besides 5-LO, the effect of U-73122 was tested for its ability to inhibit the closely related human platelet-type 12-LO as well as 15-LO from (eosinophilic) PMNLs. U-73122 up to 10 μM failed to significantly suppress the activity of 12-LO in platelet homogenates (Fig. 3). Similarly, crude 15-LO in homogenates of (eosinophilic) PMNLs was not significantly inhibited by U-73122 up to 10 μM.

U-73122 Inhibits the Activity of Isolated 5-LO. Next, U-73122 was tested for inhibition of isolated human recombinant 5-LO expressed in *E. coli*. U-73122 concentration dependently and potently inhibited product formation of isolated 5-LO at a substrate concentration of 10 μM AA, with an IC_{50} of ≈30 nM (Fig. 4). U-73343 up to 10 μM caused no suppression of 5-LO activity (data not shown). To check whether the potency of U-73122 is affected by the levels of substrate, 5-LO inhibition was determined at various AA concentrations (3, 10, and 30 μM). As shown in Fig. 4, high AA concentrations (30 μM) rather improved the potency of U-73122, indicating that U-73122 does not act in a substrate-competitive manner.

U-73122 represents a pyrrole-2,5-dione, capable to react with thiol groups. In contrast, the inactive analog U-73343 possesses a pyrrolidin-2,5-dione moiety, which does not react with thiols (Fig. 5A). NEM represents a truncated pyrrole-2,5-dione analog of U-73122, which is highly thiol-reactive. However, NEM up to 30 μM failed to inhibit isolated 5-LO (Fig. 5B), indicating that the thiol-reactive pyrrole-2,5-dione moiety is not solely responsible for 5-LO inhibition and that the aminosteroid backbone of U-73122 also is of importance.

Inhibition of 5-LO by U-73122 was examined in presence of 1 mM GSH to provide excess of thiols to saturate the reactive pyrrole-2,5-dione moiety. In fact, GSH strongly decreased the potency (≈100-fold) of U-73122 to inhibit isolated 5-LO, shifting the IC_{50} value from 30 nM to about 3 μM (Fig. 5C).

Depletion of Cellular Thiols Restores Potent 5-LO Inhibition by U-73122 in Intact Cells and Corresponding Homogenates. Since the potency of U-73122 for inhibition of 5-LO in intact cells and corresponding homogenates is much reduced compared with isolated 5-LO, it seemed possible that cellular thiols could impair the efficacy of U-73122. Depletion of intact PMNLs from endogenous thiols by pretreatment with 50 μM NEM strongly improved the potency of U-73122. Thus, the IC_{50} for 5-LO in PNMLs stimulated with ionomycin plus 10 μM AA shifted from 7 to 0.6 μM, when cells had been depleted from thiols by NEM (Fig. 6A). In analogy to intact cells, removal of thiols by NEM (30 μM) from PMNL homogenates also shifted the IC_{50} of U-73122 to significantly lower values (2.2 versus 0.45 μM; Fig. 6B). In contrast, NEM (50 μM) did not at all affect the potency of U-73122 for inhibition of isolated 5-LO in the absence of thiols (data not shown).

Discussion

U-73122 was originally described as a research tool that selectively inhibits receptor-coupled PLC-dependent processes (Bleasdale et al., 1989, 1990; Smith et al., 1990), and a correlation between the effects of U-73122 and a role for PLC in agonist-induced Ca^{2+} mobilization has been drawn in more than a thousand publications to date. In fact, U-73122

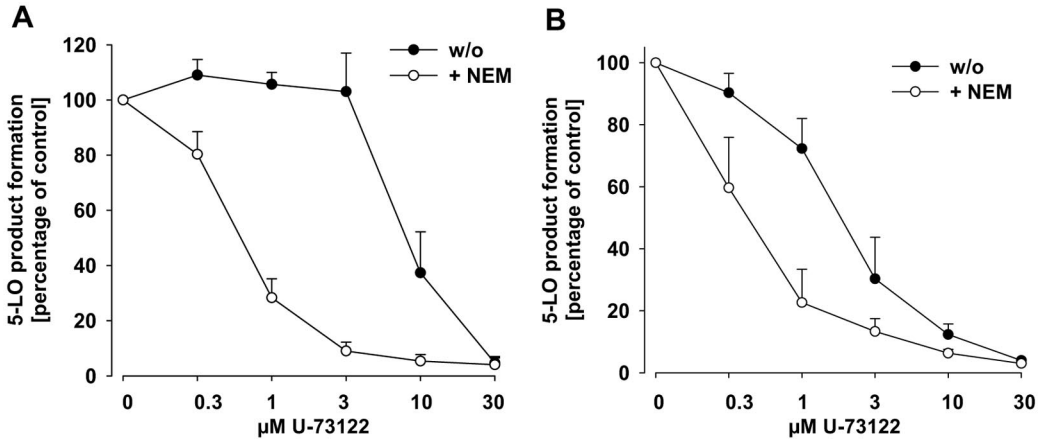

Fig. 6. Depletion of cellular thiols by NEM increases the potency of U-73122 for 5-LO inhibition. A, effects of NEM in intact cells. Freshly isolated PMNLs (7.5×10^6/ml PG buffer) were preincubated with U-73122 at the indicated concentrations in the presence or absence of 50 μM NEM for 20 min at 37°C. Then, cells were supplemented with 1 mM $CaCl_2$ and stimulated with ionomycin (1 μM) plus 10 μM AA. After 10 min, 5-LO products were extracted and determined by HPLC as described in text. 5-LO product formation of controls was 134.3 ± 34.2 and 77.1 ± 16.5 ng/10^6 cells in the absence and presence of NEM, respectively. B, effects of NEM in homogenates. Whole homogenates of human PMNLs, corresponding to 7.5×10^6 cells/ml PG buffer containing 1 mM EDTA, were supplemented with 1 mM ATP, NEM (30 μM), and U-73122 as indicated. After 5 to 10 min on ice, samples were prewarmed at 37°C for 30 s, AA (20 μM) together with 2 mM $CaCl_2$ was added, and after another 10 min, 5-LO products were determined as described in text. 5-LO product formation of controls (no U-73122) was 76.8 ± 10.1 and 60.4 ± 16.9 ng/10^6 cells in the absence and presence of NEM, respectively. Results are given as mean + S.E.; n = 3 to 4.

blocks agonist-induced IP_3 formation by cellular PLC and suppresses the activity of PLC in cell-free assays (Bleasdale et al., 1990; Smith et al., 1990; Alter et al., 1994; Pulcinelli et al., 1998; Hou et al., 2004), confirming that U-73122 is a direct PLC inhibitor. However, much higher concentrations of U-73122 are necessary to inhibit PLC activity in vitro (IC_{50} = 9–70 μM) compared with those required to suppress IP_3-mediated release of Ca^{2+} in intact cells (IC_{50} = 0.5–5 μM) (Bleasdale et al., 1990; Smith et al., 1990; Muto et al., 1997; Pulcinelli et al., 1998; Feisst and Werz, 2004). In some studies, U-73122 even blocked receptor-coupled Ca^{2+} mobilization without reducing IP_3 production (Alter et al., 1994; Hellberg et al., 1996). Therefore, U-73122 may act at other targets than PLC related to Ca^{2+} signaling, for example, at Ca^{2+} channels (Berven and Barritt, 1995; Grierson and Meldolesi, 1995; Macrez-Lepretre et al., 1996; Pulcinelli et al., 1998).

In this study, we identified 5-LO as a molecular target of U-73122, with substantially superior sensitivity toward the drug compared with PLC isoenzymes. Ca^{2+} and/or phosphorylation activates 5-LO (Werz et al., 2002a,b,c), and indeed, typical stimuli known to induce 5-LO product synthesis (e.g., fMLP, PAF, cytokines, or immune complexes) are all capable to elevate $[Ca^{2+}]_i$ and to evoke MAPK activation (Werz, 2002). Since these agonists transduce signals via G proteins leading to the stimulation of PLC (Rhee, 2001), a role for PLC in Ca^{2+}-mediated 5-LO activation seems conceivable. However, aiming to demonstrate a role of PLC in agonist-induced 5-LO activation, we found that U-73122 inhibits 5-LO also in cell-free assays, even at concentrations that are more than 100-fold lower than those required to inhibit PLC in vitro (Muto et al., 1997; Pulcinelli et al., 1998; Hou et al., 2004).

In intact cells or homogenates, 5-LO inhibition by U-73122 was significantly reduced compared with isolated 5-LO, presumably related to the ability of U-73122 to react with endogenous cellular thiols. Thus, the pyrrole-2,5-dione moiety is highly thiol-reactive (Majima et al., 1995), and inclusion of GSH strongly impaired the potency of U-73122 for inhibition of isolated 5-LO. Moreover, the inactive pyrrolidin-2,5-dione analog, lacking thiol-reactivity (Bleasdale et al., 1990; Smith et al., 1990), was not active on isolated 5-LO. On the other hand, depletion of cellular thiols restored efficient 5-LO inhibition in intact cells and homogenates. Therefore, the pyrrole-2,5-dione moiety seems to be a determinant. Nevertheless, since NEM caused no 5-LO inhibition, also the aminosteroid backbone might be essential. Direct 5-LO inhibitors are categorized as redox inhibitors, iron-chelating compounds, and nonredox (competitive)-type inhibitors (Werz, 2002). At present, the molecular mode of action of how U-73122 inhibits 5-LO is not clear. Presumably, U-73122 reacts with an essential SH-group on the 5-LO protein, thereby rendering 5-LO catalytically inactive.

In conclusion, U-73122 is a potent inhibitor of 5-LO in vivo and in vitro and may represent a new lead for the development of novel 5-LO inhibitors. U-73122 was shown to suppress inflammatory responses in neutrophils (Smith et al., 1990, 1996), typically induced by 5-LO products (Samuelsson, 1983; Funk, 2001). In animal models, U-73122 blocked carrageenan-induced hind paw edema, leukocyte accumulation, and infiltration, and it was effective in models of peritonitis and ear edema (Hou et al., 2004). Since 5-LO products (i.e., LTs) bind to specific GPCRs, resulting in PLC activation and thus mobilization of Ca^{2+} (Yokomizo et al., 1997; Lynch et al., 1999; Falgueyret and Riendeau, 2000), it is conceivable that a certain agonist may first induce (PLC-independent) formation of LTs, that in turn induce Ca^{2+} mobilization via a GPCR/PLC pathway. Under these conditions, U-73122 may abolish the Ca^{2+} signal due to inhibition of 5-LO independently of PLC. Thus, caution should be taken when interpreting results using U-73122 as a tool to identify a role of PLC in signaling pathways leading to Ca^{2+} mobilization.

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