The Phospholipase C Inhibitor U73122 Inhibits Phorbol Ester-Induced Platelet Activation

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Accepted for publication December 21, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

Activation of phospholipase C (PLC) is a central component of the signal transduction process in numerous cells, including platelets. U73122 has been widely used as a selective PLC inhibitor. In the present study, the effects of U73122 on platelet function have been further examined. Platelets were stimulated with collagen (via PLC-γ), the stable thromboxane mimetic U46619 (via PLC-β), or phorbol myristate acetate (PMA) via protein kinase C (PKC). Consistent with inhibition of PLC, U73122 inhibited platelet aggregation and [3H]-serotonin release in response to collagen and U46619 in a concentration-dependent manner. Similarly, U73122 blocked collagen-induced release of thromboxane A2. U73122 also inhibited U46619-induced [32P]phosphatidic acid production and phosphorylation of the major PKC substrate, pleckstrin. U73122 had no effect on PMA-induced pleckstrin phosphorylation, [3H]-serotonin release, or intracellular vacuole formation. However, U73122 did inhibit PMA-induced platelet aggregation and fibrinogen binding. Overall, these results suggest that U73122, in addition to its inhibition of PLC, also affects PKC-independent events that interfere with platelet aggregation.

The activation of phospholipase C (PLC) is a key early component in the stimulation of a number of cell types, including platelets, induced by a wide variety of agonists (Siess, 1989; Brass et al., 1997). Of particular importance are two families of PLC enzymes, the PLC-β and PLC-γ isoenzymes. PLC-β activation is associated with stimulation of the serpentine seven transmembrane type of receptor via heterotrimeric GTP-binding proteins. In contrast, PLC-γ is primarily activated by the tyrosine kinase growth factor-type family of receptors (Cockcroft and Thomas, 1992; Rhee and Choi, 1992).

Human platelets contain members of both families, including PLC-β1, PLC-β2, PLC-β3, PLC-β4, PLC-γ1, and PLC-γ2 (Banno et al., 1996). Platelet activation by high concentrations of collagen stimulate the tyrosine phosphorylation and activation of PLC-γ2 (Daniel et al., 1994; Blake et al., 1994). However, at lower, physiologically relevant, collagen concentrations, PLC activity is apparently inhibited by cyclooxygenase inhibitors and, therefore, probably mediated by thromboxane A2 (TXA2) (Rittenhouse and Allen, 1982; Pollock et al., 1986; McNicol and Nickolaychuk, 1995). Platelet stimulation with the stable TXA2 analog, U46619, does not stimulate tyrosine phosphorylation of PLC-γ2, and conflicting reports exist concerning the effect of thrombin on the stimulation of tyrosine phosphorylation of PLC-γ2 (Tate and Rittenhouse, 1993; Blake et al., 1994).

In contrast to collagen, agonists such as TXA2, platelet-activating factor, vasopressin, and thrombin all stimulate PLC-β, leading to the hydrolysis of phosphatidylinositol 4,5 bisphosphate which, in turn, results in the formation of two second messengers, 1,2-diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP3) (Siess, 1989; Brass et al., 1997). DAG has been shown to stimulate protein kinase C (PKC) (Nishizuka, 1984) and IP3 mobilizes intracellular Ca2+ (Berridge, 1993). Together these messengers act synergistically to induce platelet activation including aggregation, the secretion of alpha- and dense-granule contents, the formation of TXA2, and the expression of adhesive receptors (Kaibuchi et al., 1983; Siess, 1989; Brass et al., 1997).

The relationships among the intracellular messengers are complex, and selective inhibitors have been widely used to examine these relationships. For example, several studies have used PKC inhibitors to demonstrate the specific role that this enzyme plays in platelet activation, including the membrane fusion associated with intracellular granule release and feedback inhibition of arachidonic acid release (Gerrard et al., 1989; Murphy and Westwick, 1992; Hargreaves et al., 1996).

The amino steroid, 1-{[6-{[17β-3-Methoxyestra-1,3,5(10)-tri-en-17-yl]-amino[hexyl]-1H-pyrrole-2,5-dione (U73122), was initially reported to selectively inhibit PLC-dependent processes in...
Fig. 1. The effects of U73122 on agonist-induced aggregation. Washed human platelets were preincubated for 2 min with U73122 at the concentrations indicated or with 0.25% DMSO vehicle control before the addition (†) of 1 µg/ml collagen (Fig. 1A), 1 µM U46619 (Fig. 1B), or 30 nM PMA (Fig. 1C). In addition, samples were preincubated for 2 min with 25 µM U73122, 25 µM U73343, or 0.25% DMSO before the addition of 30 nM PMA (Fig. 1C, insert). Aggregation was monitored continuously as an increase in light transmission. Each series of tracings is representative of three experiments.
human platelets and neutrophils (Smith et al., 1990; Bleasdale et al., 1990) and, therefore, has subsequently proven useful in the evaluation of the role that PLC plays in cell activation. In platelets, U73122 was shown to inhibit aggregation, increases in intracellular Ca\(^{2+}\), and production of \[^{3}H\]-IP\(_3\) in response to thrombin and U46619 (Bleasdale et al., 1990). Similarly, in polymorphonuclear neutrophils, U73122 inhibited increases in intracellular Ca\(^{2+}\), IP\(_3\) production, and DAG production induced by N-formyl-methionyl-leucyl-phenylalanine (Smith et al., 1990, Bleasdale et al., 1990). These data are consistent with U73122 selectively inhibiting PLC stimulation. Based on studies of acetylcholine receptor sequestration in SK-N-SH neuroblastoma cells, the proposed mechanism of action of U73122 lies at the level of regulation of the heterotrimeric G-protein, G\(_q\) (Thompson et al., 1991), implying that U73122 preferentially inhibits PLC-\(\beta\) over PLC-\(\gamma\).

Several other studies have, however, reported additional effects of U73122 on nonPLC-dependent processes. Vickers demonstrated that U73122 affected the equilibria between membrane phosphoinositides in resting and in thrombin-stimulated platelets, likely through inhibiting inositol phosphate and phosphatidylinositol 4-phosphate kinase activity (Vickers, 1993). U73122 has also been reported to alter Ca\(^{2+}\) homeostasis in rat neutrophils (Wang, 1996) and mouse fibroblasts (Grierson and Meldolesi, 1995), possibly through inhibition of the Ca\(^{2+}\) ATPase.

In the present study, we have further evaluated the effects of U73122 on platelet function. As the platelet-activating effects of low concentrations of collagen are mediated by the production of TxA\(_2\), we have used U73122 to compare the potential roles that PLC plays in response to low concentrations of collagen and the stable Tx mimetic, U44619. In addition, we have determined the effects of U73122 on platelet activation in response to the PKC activator, phorbol myristate acetate (PMA).

**Materials and Methods**

U73122, U73343, and Ro 31–8220 were all obtained from Calbiochem (La Jolla, CA). U46619 was obtained from Cayman Chemicals.
Platelet Isolation. Blood was collected into acid citrate dextrose (ACD) anticoagulant (3.8 mM citric acid, 7.5 mM trisodium citrate, and 125 mM dextrose; 1.9 ml:8.1 ml of blood) by venipuncture of healthy volunteers who had not taken medication known to interfere with platelet function for at least 10 days before the experiment. Platelet rich plasma (PRP) was obtained by centrifugation at 800g for 5 min. Plasma-free platelet suspensions were obtained by centrifugation of the PRP at 2000g for 10 min and the subsequent pellet was resuspended in the appropriate buffer (McNicol, 1996).

Platelet Aggregation. Plasma-free platelet suspensions were prepared in HEPES-Tyrode’s buffer (134 mM NaCl, 12 mM NaHCO3, 2.9 mM KCl, 0.34 mM Na2HPO4, 1 mM MgCl2, 10 mM HEPES, 5 mM dextrose, 0.3% bovine serum albumin; pH 7.4). Aliquots (0.4 ml) containing CaCl2 (1 mM final) were dispensed into siliconized aggregometer cuvettes. Aggregation, in the presence of inhibitor or vehicle control, was monitored photometrically for 3 min after the addition of U46619, 4 min after addition of collagen, and 7 min after addition of PMA from Sigma (St. Louis, MO). [3H]-serotonin and [32P]-ortho-phosphate were obtained from Amersham (Oakville, ON). All other materials were of the highest grade available.

Platelet Aggregation.

Fig. 3. The effects of U73122 on agonist-induced [32P]-phosphatidic acid production. Prelabeled platelets were preincubated for 1 min with U73122 at the concentrations indicated or with 0.25% DMSO vehicle control before the addition of 1 μM U46619. The reaction was terminated 2 min after the addition of U46619. The [32P]-phosphatidic acid was extracted, separated by TLC, and quantified by radiochromatographic scanning. Data are mean values (± S.E.M.) of five experiments each with triplicate samples, expressed as a percentage of unstimulated values (solid bars; 135.5 ± 15.5 cpm). *p < .05 with respect to unstimulated control platelets (solid bar); †p < .05 with respect to agonist-stimulated platelets (open bar).

[View of figure 3]

Electronic Microscopy. Aliquots (0.4 ml) of plasma-free platelet suspensions in HEPES-Tyrode’s buffer (containing 1 mM CaCl2) were incubated with agonist in the presence of inhibitor or vehicle control. At the time indicated, the reaction was terminated by transferring the entire sample to 2 ml of chloroform/methanol/10 M HCl (25:50:4, v/v/v). The phospholipids were extracted, dried under nitrogen, resuspended in 50 ml of chloroform/methanol (1:1, v/v), separated by thin-layer chromatography, and subjected to radiographic scanning as described previously (McNicol et al., 1993; McNicol and Nickolaychuk, 1995). The phospholipids were visualized by exposure to iodine and identified by comparison with standards. Under the experimental conditions used, [32P]-ortho-phosphate is preferentially incorporated in phosphoinositides and the formation of [32P]-phosphatidic acid reflects the action of phosphoinositide-specific PLC. [32P]Phosphatidic acid formation, expressed as a percentage of the unstimulated control, is therefore an index of phosphoinositide-specific PLC activity (McNicol et al., 1993; McNicol and Nickolaychuk, 1995).

Pleckstrin Phosphorylation. Plasma-free platelet suspensions were prepared in phosphate-free HEPES-Tyrode’s buffer as outlined above and incubated with 1 μCi of [32P]-ortho-phosphate for 60 min at 37°C. Platelets were isolated by centrifugation and resuspended in HEPES-Tyrode’s buffer as outlined above for aggregation. Aliquots (0.4 ml containing 1 mM CaCl2) were incubated at 37°C with the agonist in the presence of inhibitor or vehicle control. At the time indicated, the reaction was terminated by transferring an aliquot to a denaturing solution (6% SDS, 2% mercaptoethanol, 30% glycerol, 3 mM EDTA, 12 mM EGTA, 0.03% bromophenol blue, 450 mM Tris; pH 6.8). The samples were denatured by boiling for 3 min and separated on a 10% SDS gel; the gel was subsequently dried and subjected to autoradiography as described previously (McNicol et al., 1993). The protein band corresponding to pleckstrin was identified, excised, and counted for radioactivity. Pleckstrin is a 40- to 47-kDa protein that is the major PKC substrate in platelets (Castaagna et al., 1982; Nishizuka, 1984, 1986), and as such, its phosphorylation is regarded as an index of PKC activity.

Electron Microscopy. Aliquots (0.4 ml) of plasma-free platelet suspensions in HEPES-Tyrode’s buffer (containing 1 mM CaCl2) were incubated with agonist in the presence of inhibitor or vehicle control. The reaction was stopped by the addition of an equal volume of 0.1% glutaraldehyde in White’s saline. The samples were subsequently fixed in 3.0% glutaraldehyde for 1 h and 1.0% osmium tetroxide solution for 90 min. After washing, the samples were dehydrated overnight in 3.0% aqueous uranyl acetate then dehydrated by successive washes in 70, 90, and 100% ethanol. Finally, the samples were impregnated in 1.1 propylene oxide/resin for 1 h and pure resin for 4 h (Israels and Gerrard, 1996). Thin sections were mounted and examined on a Philips (Eindhoven, the Netherlands) EM400 electron microscope. Sixteen to 25 platelets from four to six micrographs of each treatment were digitized by computer-assisted image analysis. For each platelet the total platelet area and area encompassed by vacuoles were measured.

Fibrinogen Binding. Fibrinogen binding was analyzed by flow cytometry using fibrinogen labeled with the fluorescent probe FITC (Shattil et al., 1987). Aliquots (0.4 ml) of plasma-free platelet
suspensions in HEPES-Tyrode’s buffer (containing 1 mM CaCl₂) were incubated at 37°C with purified FITC-labeled fibrinogen in the presence of inhibitor or vehicle control for 1 min before the addition of agonist for 10 min. The reaction was stopped by the addition an aliquot of treated platelets (0.04 ml) to 0.1% paraformaldehyde (0.36 ml). Fibrinogen binding was detected by flow cytometry in an EPICS model 753 flow cytometer (Becton Dickinson, Mississauga, ON) equipped with an argon laser (500 mW, 488 nm). Forward and 90° light scatter measurements were used to establish gates for intact viable platelets. Single parameter, 255-channel, log integral green fluorescent histograms were obtained, and the negative and positive fluorescent populations were resolved.

**Statistical Analysis.** Data are reported as means ± S.E.M. Raw data were analyzed by analysis of variance. Where appropriate, the least significant difference test was used to test for significance differences at p < .05.

**Results**

**Aggregation.** The effects of pretreatment with U73122 on agonist-induced platelet aggregation were measured by light transmission. Preincubation for 2 min with U73122 (0.25–3 μM) inhibited, in a concentration-dependent manner, aggregation induced by either collagen (1 μg/ml; Fig. 1A) or U46619 (1 μM; Fig. 1B). It has been shown previously that U73343 has no effect on aggregation in response to either agonist (Bleasdale et al., 1990). These data are consistent with the known PLC-mediated activation of platelets by collagen and U46619. However, U73122 (0.25–10 μM) also inhibited aggregation in response to the PKC stimulant PMA (30 nM; Fig. 1C). In contrast U73343 at concentrations up to 25 μM had no effect on PMA-induced aggregation (30 nM; Fig. 1C, inset).

**Dense Granule Secretion.** Collagen (1 μg/ml; Fig. 2A) and U46619 (1 μM; Fig. 2B) caused the secretion of significant amounts of [³H]-serotonin from [³H]-serotonin-prelabeled platelets, consistent with dense granule release in response to these agonists. Collagen-induced [³H]-serotonin release was significantly inhibited by 0.5 μM U73122 and abolished by 1.0 to 3.0 μM U73122. In contrast U46619-induced [³H]-serotonin secretion was abolished by all concentrations of U73122 tested (0.5–10.0 μM). There was minimal [³H]-serotonin secretion (15.7 ± 5.9% versus 10 ± 3.6% in unstimulated control) in response to PMA (30 nM), and this secretion was unaffected by pretreatment with up to 10 μM U73122 (Fig. 2C).

**TxA₂ Release.** The effects of U73122 on collagen-induced synthesis of TxA₂ was measured as the release of the stable TxA₂ metabolite, TxB₂. The basal level of TxB₂ was 183 ± 74 pg/ml and increased to 6350 ± 2616 pg/ml after the addition of collagen (1 μg/ml). Pretreatment with U73122 (1.5 μM; 2 min) abolished collagen-induced TxB₂ production (185 ± 21 pg/ml). It has been shown previously that neither U46619 (Faili et al., 1994; Murthy et al., 1995) nor PMA (Mobley and Tai, 1985; Halenda et al., 1985) release significant levels of TxB₂.

**Phosphatidic Acid Production.** The effects of U73122 on agonist-induced [³²P]-phosphatidic acid formation were measured as an index of PLC activity. U46619 (1 μM; 2 min) stimulated [³²P]-phosphatidic acid levels to 240.5 ± 16.0% of unstimulated levels (Fig. 3), confirming that TxA₂ stimulated PLC, as previously established (Siess, 1989; Brass et al., 1997). Pretreatment with U73122 (0.5–10 μM) inhibited, in a concentration dependent manner, U46619-induced [³²P]-

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**Fig. 4.** The effects of U73122 on PMA- and U46619-induced [³²P]-pleckstrin phosphorylation. Prelabeled platelets were preincubated for 1 min with U73122 (at the concentrations indicated), Ro 31–8220, or 0.25% DMSO (vehicle control) before the addition of 15nM PMA (Fig. 4A) or 1 μM U46619 (Fig. 4B). The reaction was stopped 7 min after addition of PMA or 3 min after the addition of U46619. The samples were denatured and separated by 10% SDS gel electrophoresis. The gel was dried and subjected to autoradiography, and the band corresponding to pleckstrin was excised and counted for radioactivity. Results are mean values ± S.E.M. of three to five experiments, expressed as a percentage of unstimulated values (2225.1 ± 777.6 cpm for PMA, 3516.0 ± 192.4 for U46619).
Fig. 5. The effects of U73122 on PMA-induced vacuole formation. Washed human platelets were preincubated for 2 min with 0.25% DMSO vehicle control (Fig. 5, A and B), 1.0 μM (Fig. 5C), or 10.0 μM (Fig. 5D) U73122 before the addition of either 0.25% DMSO vehicle control (Fig. 5A) or 30nM PMA (Fig. 5, B-D). The reaction was stopped 10 min after the addition of PMA and the samples were fixed and stained. Thin sections were mounted on formvar-coated grids and examined by electron microscopy. Bar corresponds to 1 μm. Image analysis software was used to measure cross-sectional platelet and vacuole areas from electron micrographs (Fig. 5E). Results are mean values (± S.E.M.) of 16 to 25 platelets from four to six micrographs, expressed as a percentage of unstimulated values. *p < .05 with respect to unstimulated control platelets (solid bar).
phosphatidic acid formation (Fig. 3), consistent with the inhibition of PLC by U73122.

**Pleckstrin Phosphorylation.** The effects of U73122 on agonist-induced pleckstrin phosphorylation were measured as an index of PKC activity. PMA (15 nM) increased the phosphorylation of pleckstrin to 191.0 ± 27.7% of unstimulated levels. Pretreatment with U73122 (0.5–10 μM; 2 min) did not affect PMA-induced pleckstrin phosphorylation (Fig. 4A). In contrast, the PKC inhibitor Ro 31–8220 (3 μM) caused pleckstrin phosphorylation, which was significantly inhibited by 10.0 μM U73122 and by 3 μM Ro 31–8220 (Fig. 4B).

**Electron Microscopy.** PKC activation by PMA is known to cause the formation of intracellular vacuoles. The effect of U73122 on the area of platelet occupied by vacuoles was evaluated in PMA-stimulated platelets. Unstimulated platelets were found to have 9.3 ± 1.1% of their cross-sectional area occupied by vacuoles (Fig. 5A), whereas PMA (30 nM)-stimulated platelets had 43.3 ± 2.7% of their area occupied by vacuoles (Fig. 5B). Pretreatment with 1 μM U73122 (41.5 ± 2.7%; Fig. 5C), 3 μM U73122 (39.4 ± 2.5%; data not shown), or 10 μM U73122 (34.7 ± 2.6%; Fig. 5D) had no significant effect on the area occupied by vacuoles (Fig. 5E).

**Fibrinogen Binding.** The effects of U73122 on PMA stimulation of fibrinogen binding were evaluated by flow cytometry. PMA (200 nM) stimulated fibrinogen binding to platelets, which was significantly inhibited, but was not abolished, by pretreatment with U73122 (1–10 μM; 10 min; Fig. 6).

### Discussion

In platelets, stimulation of PLC-β by agonists such as thrombin and TxA2 results in the hydrolysis of phosphatidylinositol 4,5 bisphosphate to form DAG and IP3 (Siess, 1989). The amino steroid U73122 has been widely used as an inhibitor of PLC-β and consequently has been shown in platelets to inhibit DAG and IP3 formation and increases in intracellular Ca2+ in response to agonists known to stimulate PLC-β (Bleasdale et al., 1990). The present study has confirmed many of these effects of U73122 on PLC-β-mediated signal transduction in platelets. For example, U73122 inhibited aggregation, [32P]-phosphatidic acid production (an index of PLC activity), and pleckstrin phosphorylation (a consequence of PLC activity) induced by the stable TxA2 mimetic U46619, which is known to activate PLC-β via a heterotrimeric GTP binding protein, Gq.

Platelets contain multiple PLC isoforms, including PLC-β1, PLC-β2, PLC-β3, PLC-β4, PLC-γ1, and PLC-γ2 (Banno et al., 1996). In the present study, we examined the effects of U73122 on collagen-induced platelet activation to determine its potential action on the PLC-γ. U73122 inhibited collagen-induced aggregation and dense granule release. As platelet activation induced by low concentrations of collagen is TxA2-dependent, these inhibitory effects of U73122 may be due to an action on the post-TxA2 receptor PLC-β pathway. However, as collagen-induced TxA2 production was abolished by U73122, the inhibition must occur before the synthesis of TxA2 by collagen. The mechanism of collagen-induced TxA2 production under these conditions is unclear. However it has been suggested that the early effects of collagen are due to the activity of PLC-γ. The present study therefore provides evidence that U73122 inhibits PLC-γ in addition to PLC-β, as has been suggested previously (Heemskerk et al., 1997). However, the study does not address whether a common mechanism is involved to account for the inhibition of these two isoforms of PLC.

Of particular interest, the present study further demonstrated, unexpectedly, that U73122 also inhibited PMA-induced aggregation. This inhibition cannot result from U73122 inhibiting a TxA2-mediated component of PMA-induced aggregation, as PMA is known to release minimal amounts of TxA2 (Halenda et al., 1985; Mobley and Tai, 1985) and indeed cyclooxygenase inhibitors have no effect on PMA-induced aggregation (Jerushalmi et al., 1988). Therefore, the effects of U73122 on PMA-induced activation were examined in further detail.

In contrast to its effects on aggregation, U73122 did not affect PMA-induced pleckstrin phosphorylation. As pleckstrin is the primary endogenous substrate for PKC in platelets these data are consistent with U73122 not directly attenuating PKC activation. Similarly PMA-induced dense granule secretion and vacuole formation are both events which are largely dependent on PKC activity (Gerrard et al., 1989; Walker and Watson, 1993). Neither dense granule secretion nor vacuole formation were affected by U73122. Consistent with this observation, Bleasdale and colleagues reported that U73122 had no effect on PKC-stimulated diglyceride production in polymorphonuclear neutrophils (Bleasdale et al., 1990). Taken together, the data suggest that U73122 does not directly affect PKC.

Similarly we can conclude that the inhibition of PMA-induced platelet aggregation by U73122 occurred at a point distal to PKC activation. Consequently, as PMA does not stimulate PLC, an inhibitory mechanism of U73122 unrelated to either PLC or PKC must be involved.

An important component in the platelet activation process is the exposure of adhesive receptors, notably the integrin αIIBβ3, on the surface of the plasma membrane and the sub-

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**Fig. 6.** The effects of U73122 on PMA-induced fibrinogen binding. Washed human platelets were preincubated for 2 min with FITC-labeled fibrinogen and U73122, at the concentrations indicated, or with 0.25% DMSO vehicle control before stimulation with 200 nM PMA for 10 min. Fibrinogen binding was measured by flow cytometry. Results are mean values (± S.E.M.) of four experiments, expressed as a percentage of unstimulated values. *p < .05 with respect to unstimulated control platelets (solid bar); †p < .05 with respect to agonist-stimulated platelets (open bar).
sequent binding of fibrinogen to cross link platelets and thereby consolidate the aggregate (Plow and Ginsberg, 1989; Du and Ginsberg, 1997). PMA has been shown to stimulate the expression of αIIbβ3 on the surface of activated platelets; this expression is unaffected by preincubation with cyclooxygenase inhibitors and is therefore not TxA2-mediated (Shattil and Brass, 1987). In the present study, U73122 inhibited PMA-induced fibrinogen binding by approximately 50%. The observed inhibition of PMA-induced fibrinogen binding and aggregation, with little effect on release, is similar to the effects of the fibrinogen-derived peptide RGDS, which also inhibits PMA-induced aggregation without an effect on PMA-induced [14C]-serotonin secretion (Patel et al., 1994).

In conclusion, in the present study U73122 has been shown to have inhibitory effects on PLC-β activity in platelets. However, in addition, U73122 has effects in platelets that occur distal to both PLC and PKC. Specifically, U73122 inhibits fibrinogen binding in a fashion resembling the peptide inhibitor RGDS. This study does not detract from the use of U73122 as a PLC inhibitor but does infer that caution should be exercised when using this compound and interpreting its effects.

Acknowledgments

We thank Tracy Shibou and Catherine Robertson for technical assistance, and Dr. Norman Fleming and Dr. Gary Glavin for their critical reading of this manuscript.

References


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