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Tricyclodecan-9-yl-xanthogenate (D609) mechanism of actions: A Mini-review of literature

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Abstract

Tricyclodecan-9-yl-xanthogenate (D609) is known for its antiviral and antitumor properties. D609 actions are widely attributed to inhibiting phosphatidylcholine (PC)-specific phospholipase C (PC-PLC). D609 also inhibits sphingomyelin synthase (SMS). PC-PLC and/or SMS inhibition will affect lipid second messengers 1,2-diacylglycerol (DAG) and/or ceramide. Evidence indicates either PC-PLC and/or SMS inhibition affected the cell cycle and arrested proliferation, and stimulated differentiation in various *in vitro* and *in vivo* studies. Xanthogenate compounds are also potent antioxidants and D609 reduced A β -induced toxicity, attributed to its antioxidant properties. Zn²⁺ is necessary for PC-PLC enzymatic activity; inhibition by D609 might be attributed to its Zn²⁺ chelation. D609 has also been proposed to inhibit acidic sphingomyelinase or down-regulate hypoxia inducible factor-1 α ; however these are downstream events related to PC-PLC inhibition. Characterization of the mammalian PC-PLC is limited to inhibition of enzymatic activity (frequently measured using Amplex red assay with bacterial PC-PLC as a standard). The mammalian PC-PLC has not been cloned; sequenced and structural information is unavailable. D609 showed promise in cancer studies, reduced atherosclerotic plaques (inhibition of PC-PLC) and cerebral infarction after stroke (PC-PLC or SMS). D609 actions as an antagonist to pro-inflammatory cytokines have been attributed to PC-PLC. The purpose of this review is to comprehensively evaluate the literature and summarize the findings and relevance to cell cycle and CNS pathologies.

Keywords

Antioxidant; Cell cycle; Ceramide; Cytokines; Glutathione; Inflammation; Microglia/macrophage; Phosphatidylcholine; Phospholipase C; PC-PLC; Proliferation; Reactive oxygen species; Sphingomyelin synthase; Stroke

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Introduction

Tricyclodecan-9-yl-xanthogenate (D609) (Fig. 1) is known for its antiviral, antitumor properties and its effects are widely attributed to inhibiting phosphatidylcholine (PC)-specific phospholipase C (PC-PLC). However the effects of D609 on sphingomyelin synthase activity; antioxidant properties; chelation of Zn^{2+} , effects on various phospholipase A_2 s and anti-inflammatory effects are not ruled out. The purpose of this mini-review is to evaluate the literature and summarize the various findings and their relevance to cell cycle and central nervous system (CNS) pathologies.

Tricyclodecan-9-yl-xanthogenate (D609)

Xanthates including D609 were initially described as broad spectrum antiviral compounds [1]. Subsequent results provided evidence that D609 inhibited 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced phospholipase C activity in A431 human epidermal carcinoma cells [2]. D609 was shown to be a competitive inhibitor of bacterial phosphatidylcholine (PC)- specific phospholipase C (PC-PLC) and did not inhibit bacterial phosphatidylinositol (PI)-PLC, bovine pancreatic PLA_2 or phospholipase D from cabbage [3]. D609 exists as enantiomeric pairs of 4 diastereomers and commercial preparations may have undefined, variable mixtures of these 8 isomers (Amtmann, personnel communication). The isomers have different biological efficiency in anti-viral and PC-PLC inhibition assays. Lumavita (Basel, Switzerland) is currently developing LMV-601, an enantiomeric pure isomer of D609, for anti-viral therapy. LMV-601, an PC-PLC inhibitor, reduced expression of high-risk human Papillomavirus (HPV) types 16, 18, 31, and over time improved pre-cancerous cervical cell abnormalities [4].

Phosphatidylcholine (PC) specific – phospholipase C (PC-PLC)

PC-PLC (66 kDa) hydrolyzes PC to generate phosphocholine and 1,2-diacylglycerol (DAG). While bacterial PC-PLC has been purified, mammalian PC-PLC has not been cloned and its sequence has not been determined, imposing a limitation on identifying D609 actions on the mammalian enzyme. Rabbit polyclonal antibodies raised against the *Bacillus cereus* PC-PLC showed cross reactivity against mammalian PC-PLC [5]. Suppressing PC-PLC by D609 in presence or absence of basic fibroblast growth factor (bFGF) stopped proliferation and induced differentiation in various cell systems [6–9]. In vascular endothelia cells PC-PLC involvement in apoptosis and senescence has been demonstrated [10–12]. PC-PLC expression changed in a cell-cycle dependent manner inversely with cell division cycle 20 homolog (CDC20) and Cdc20 overexpression caused PC-PLC degradation mediated by ubiquitin-proteasome pathway [13]. PC-PLC inhibition by D609 down-regulated human epidermal growth factor receptor 2 (HER2) over-expression on plasma membrane of breast cancer cells [14]. Human epithelial ovarian cancer cells showed activation of PC-PLC and inhibition by D609 reduced the phosphocholine metabolite [15]. PC-PLC expression also regulated CD16 (highly glycosylated transmembrane protein) in NK cells and treatment with D609 caused dramatic decrease in CD16 receptor and PC-PLC expression on the plasma membrane [16]. Actions of D609 attributed to PC-PLC inhibition include suppressing expression of hypoxia-inducible factor 1 α (HIF-1 α) after stroke [17], reduced cytokine expression in lipopolysaccharide (LPS)-stimulated macrophages [18–20], prevention of tumor necrosis factor- α (TNF- α) or LPS-induced lethal shock in mice [21], and protection of immature neurons (do not express glutamate receptors) from oxidative glutamate toxicity by uncoupling the cystine/glutamate anti-porter [22]. Studies indicated that inhibition of PC-PLC by D609 enhanced phospholipase D (PLD) activity in UMR-106 osteoblastic cells either due to a compensatory effect or that D609 directly increased PLD activity [23]. A growing body of evidence implicates PC-PLC in metabolism, proliferation, differentiation,

senescence and apoptosis in mammalian cells as well as its role in atherosclerosis [24] and somewhat indirectly in stroke models [17, 25, 26].

Does Zn²⁺ chelation by D609 inhibit PC-PLC activity?

Bacterial PC-PLC is a monomeric enzyme which accommodates three Zn²⁺ ions in the active site likely to be involved in the binding to the substrate and essential for the enzymatic activity and protein conformational stability [27]. Xanthate derivatives that have the lipophilic chain would fit in the active center of PC-PLC, and the dithiocarbonate group could serve as a surrogate of the phosphate group and bind to active-site Zn²⁺ ions [28].

PC-PLC activity is often measured using the Amplex Red assay

The principle of the assay is: PC-PLC hydrolyzes PC to DAG and phosphocholine. Phosphocholine is hydrolyzed to choline by alkaline-phosphatase. Choline is oxidized by choline oxidase to betaine and H₂O₂. Amplex red reagent is oxidized stoichiometrically by H₂O₂ in the presence of horseradish peroxidase to generate the fluorescent product resorufin (Ex 530 nm and Em 590 nm). Activity is calculated against bacterial PC-PLC. Since the assay is indirect, proper controls need to be included to eliminate other sources of H₂O₂ to obtain the true PC-PLC activity. The contribution of phospholipase D, which hydrolyzes PC to phosphatidic acid and choline (Fig. 2), can be accounted for by elimination of alkaline phosphatase from the assay.

Sphingolipid metabolism

The first and rate-limiting step in *de novo* biosynthesis of ceramide is catalyzed by serine palmitoyltransferase (SPT). SMS transfers the phosphocholine group from PC to ceramide to form SM and DAG. There are two forms of SMS: SMS1 is located in the Golgi apparatus and SMS2 in the plasma membrane [29]. D609 inhibits both forms of SMS [30–32]. Many of the studies attributing the effects of D609 to PC-PLC inhibition may also involve SMS inhibition (Fig. 3). D609 also increased *de novo* ceramide synthesis, an effect that could be attributed to stimulation of SPT [33]. In our rat stroke model the neuroprotection offered by D609 was attributed to SMS inhibition; that caused ceramide accumulation and affected cell cycle events [34]. Scavenger receptor CD36/fatty acid translocase is associated with lipid microdomains/rafts and transport of fatty acids into cell. Ceramide can affect the CD36 translocation and function [35]. Ceramide levels may be critical to its signaling [36, 37] and can induce cell cycle arrest by dephosphorylation of retinoblastoma (Rb). D609 inhibited bFGF-stimulated astrocyte proliferation, which was attributed to SMS inhibition and increase in ceramide [38]. Through SMS inhibition and increased ceramide [31, 39], D609 can induce cell cycle arrest by up-regulating cyclin dependent kinase (Cdk) inhibitors p21 and p27 [39]. c-Myc suppresses expression of p21 [40] and p27 [41], and ceramide can induce expression of p21 and p27 through activation of protein phosphatase 2A (PP2A) that down-regulates c-myc [42, 43]. Since D609 did not block c-myc expression [44], these effects are likely down-stream of D609 induced increase in ceramide.

Antioxidant actions of D609

D609 has antioxidant/glutathione mimetic properties [45–47] due to the presence of the thiol function. Reduced glutathione (GSH) mimetic/antioxidant actions are based on A β toxicity studies [48]. The xanthate group of D609 can readily oxidize to form a disulfide [49], which is a substrate for glutathione reductase, regenerating D609 [48]. The crucial role of reactive oxygen species (ROS), lipid oxidation/peroxidation in tissue pathophysiology is demonstrated in many neurological disorders and CNS injuries [50]. D609 could attenuate formation of ROS and oxidized-PC (OxPC) through its antioxidant/glutathione mimetic properties [46, 47], providing benefit in these CNS pathologies.

Other miscellaneous effects of D609

D609 inhibited PC synthesis and may directly affect membrane translocation of cytidine triphosphate (CTP):phosphocholine cytidyltransferase (CCT), the rate-limiting enzyme in PC synthesis [51]. D609 prevented 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced increase in DAG, activation of nuclear CCT activity, and increase in nuclear PC synthesis [52]. Studies indicated that inhibition of PC-PLC by D609 enhanced PLD activity either due to a compensatory effect or that D609 directly increased PLD activity [23]. High concentrations of D609 (750 μ M) inhibited cPLA₂ in MDCK cells [53] while 350 μ M had no effect [54]. We have shown previously that D609 inhibited proliferation of cultured neuronal progenitor cells and increased metabolic activity in 264.7 macrophages (MTT assay) [55, 56]. The number of BrdU-positive cells was significantly reduced in comparison to controls, indicating that D609 inhibited proliferation of the neurospheres. This could suggest that D609 beneficial effects could arise from inhibiting proliferating microglia/macrophages that are the primary source for TNF- α and IL-1 and other pro-inflammatory cytokines [57]. A composite scheme representing the various actions of D609 has been presented in Fig. 4.

Relevance of D609 to inflammation, proliferation in neuroscience paradigm

D609 was initially developed as antiviral and anti-tumor agent that will affect cell proliferation [3]. In neurodegenerative diseases/disorders and CNS injuries, expression of cell cycle regulating proteins (cyclins, cyclin dependent kinases (Cdk) and their endogenous inhibitors) are altered [58–64]. In dividing non-neuronal cells such as astrocytes, microglia/macrophages, activation of the cell cycle machinery induces proliferation. However, in post-mitotic neurons, cell cycle entry results in their death [58–64]. Inhibiting the cell cycle will provide benefit by attenuating neuronal death. Hypothetically D609 may block the cell cycle, attenuating neuronal death and non-neuronal cell proliferation by increasing ceramide levels after CNS injury [34]. The role of microglia/macrophages in CNS pathologies remains controversial [65–70] as they are the source of pro-inflammatory TNF- α and IL-1 α/β [57] as well as trophic factors such as BDNF [68] and IGF-1 that offer protection. Studies indicate that ablation of proliferating microglia exacerbates ischemic injury in mice after transient cerebral ischemia [67]. However ablation of proliferating microglia resulted in an increase in cells expressing TNF-1 α , IL-1 β and IL-6, but the identity of these cells were unknown. This could suggest that D609 may prevent mature neurons from entering the cell cycle at the early reperfusion, however may not interfere with proliferation of microglia/macrophages that are the source of trophic factors and active during repair phase of stroke [34]. Resolving whether microglia/macrophages are the primary source of pro-inflammatory cytokines [57] or other cell(s) in the absence of activated microglia/macrophages will still provide the pro-inflammatory cytokines is challenge.

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Abbreviations

ASMase	Acidic sphingomyelinase
BDNF	Brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
CCT	Cytidine triphosphate (CTP):phosphocholine cytidyltransferase
Cdk	Cyclin dependent kinase

D609	Tricyclodecan-9-yl-xanthogenate
DAG	1,2-diacylglycerol
HER2	human epidermal growth factor receptor 2
IGF-1	Insulin-like growth factor
LPS	Lipopolysaccharide
OxPC	Oxidized phosphatidylcholine
PA	Phosphatidic acid
PC	Phosphatidylcholine
PC-PLC	PC-phospholipase C
PLC	Phospholipase C
PLD	Phospholipase D
ROS	Reactive oxygen species
SM	Sphingomyelin
SMS	Sphingomyelin synthase
SPT	Serine palmitoyl transferase
TPA	12- <i>O</i> -tetradecanoyl-phorbol-13-acetate

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Figure 1.
D609 chemical structure

Figure 2.

PC hydrolysis by PC-PLC and PLD. D609 inhibits PC-PLC, attenuating DAG release and subsequent PA formation [17]. PA can be directly formed *via* PC hydrolysis by PLD. Modified from ref [34].

Figure 3.

PC-PLC hydrolyzes PC to form DAG and phosphocholine. DAG stimulates ASMase and release of ceramide from SM. SM synthase transfers the phosphocholine head group from PC to ceramide to form SM and DAG. D609 inhibits PC-PLC [3] and/or SMS [31]. Modified from ref [25].

Figure 4.

Multiple pathways may be affected by D609 through targeting SMS/PC-PLC [3, 31]. Modified from ref [34]. D609 may increase *de novo* ceramide synthesis by stimulating SPT [33]. D609 antioxidant properties are based on A β induced oxidative stress [48], effects on cystine/glutamate antiporter are based on glutamate toxicity in immature hippocampal neurons [22], increase in metabolic activity in 264.7 macrophages is based on MTT assay. D609 could decrease formation of ROS and oxidized PC (OxPC) by inhibiting proliferation of microglia/macrophages. Alternatively, D609 could attenuate ROS/OxPC generation through its antioxidant/glutathione mimetic properties. D609 inhibited PC synthesis and may directly affect membrane translocation of CCT, the rate-limiting enzyme in PC synthesis [51]. Studies indicated that inhibition of PC-PLC by D609 enhanced PLD activity [23]. Bacterial PC-PLC is a monomeric enzyme which accommodates three Zn²⁺ ions in the active site likely to be involved in the binding to the substrate and essential for the enzymatic activity [27]. Does D609 inhibition of PC-PLC activity by Zn²⁺ chelation need to be addressed?

Table 1Mechanism of actions attributed to D609 protection in various *in vivo* and *in vitro* experimental settings

Mechanism	System	Action [Reference]
		IC50 = 94μM [71] D609 t _{1/2} in solution 19 min at 24C. Pro-drug modification may enhance chemical stability [49]
PC-PLC inhibition	A431 human epidermal carcinoma cells Bacterial PC-PLC Effect on HPV-16 and HPV-18 infected human cervical carcinoma cells Mesenchymal stem cells (MSC) Vascular endothelial cells Breast cancer cells NIH3T3 Natural killer (NK) cells Human epithelial ovarian cancer cells Rat hepatoma cells Bacterial PC-PLC Rat neural progenitor cells Human IL-3 dependent cells RAW 264.7 macrophages Mouse fibrosarcoma cells L929, Wehi164 Histiocytic lymphoma U937 cells Human acute leukemic T cells Jurkat Lipopolysaccharide (LPS)-stimulated macrophages immature cortical neurons, hippocampal cell line, HT22	Inhibited PLC activity [2] Competitive PC-PLC inhibition [3] Specific enantiomer of D609; LMV 601 (Lumivita AG, Switzerland) over time improved cervical cancer cell abnormalities [4] Affected ROS, NADPH oxidase differentiation and survival [7, 8, 72–74] Inhibited LPS induced IL-8 and MCP-1 [75] Inhibited PC-PLC, reduced phosphocholine content and arrested cell proliferation [14–16, 76–79] Effectuated Cdc20; ubiquitin proteasome pathway and cell cycle [13] PC-PLC inhibition through Zn ²⁺ chelation [27, 28] Inhibited proliferation by reducing cyclin D1 and pRb [56] Inhibited IL-3 induced expression of bcl-2 gene; c- myc unaffected [44] Peptidoglycan induced COX-expression was attenuated [80] Anti-inflammatory TNF antagonist; through PC- PLC, DAG and ASMase, Ceramide, NFκB pathway [21, 54, 71, 81–83] LPS induced PIP ₃ kinase activation reduced cytokine expression; PC-PLC⇒ DAG⇒ASMase⇒ceramide ⇒raf⇒ MEK⇒ERK [18–20] Inhibits glutamate induced cell death through cystine/glutamate anti-porter [22]
SMS inhibition	WI38 human lung fibroblasts Rat primary astrocytes U937 human monocytic leukemia cells	Inhibited SMS and suggested PC-PLC a putative SMS [31, 32] Inhibited bFGF induced proliferation of astrocytes [38] SMS inhibition by D609 induced apoptosis [84]

Mechanism	System	Action [Reference]
	Huh, HEK 293, CHO cells Kidney tubule cells	Decreased intracellular SM levels [85] Increased ceramide by inhibiting SMS. Augmenting Cd ²⁺ induced apoptosis [86, 87]
Stroke PC-PLC	Rat 2 hr middle cerebral artery occlusion; D609 50 mg/kg i.p.	Reduced cerebral infarction and improved behavioral outcome by decreasing HIF-1 α expression; the mechanism indirectly attributed to PC- PLC $\rightarrow$ DAG $\rightarrow$ PA $\rightarrow$ HIF-1 α [17]
PC-PLC	Mouse middle cerebral artery occlusion; D609 50 μ g/5 μ L i.c.v	Reduced cerebral infarction by decreasing ASMase $\rightarrow$ ceramide, and cytokines [26]
SMS	Rat 1 hr middle cerebral artery occlusion; D609 50 mg/kg i.p. <i>In vitro</i> stroke model (PC12 cells subjected to oxygen-glucose deprivation)	Reduced cerebral infarction by inhibiting sphingomyelin synthase (SMS); increasing ceramide levels and causing cell cycle arrest [34] Affected phospholipid metabolism during OGD [25]
Atherosclerotic plaques/PC-PLC	apoE ^{-/-} mice fed atherogenic diet Human umbilical vein endothelial cells	Inhibited progression of atherosclerotic lesions [24, 88] Reduced oxidized LDL-induced expression of vascular cell adhesion molecule-1, intercellular adhesion molecule-1 and monocytes chemotactic protein-1 [24, 88]
Antioxidant/lipid peroxidation	mice subjected to Ionizing radiation induced oxidative damage Rat primary neuronal culture Gerbil (in vivo)/Isolated mitochondria Gerbil (in vivo)/isolated synaptosomes Gerbil (ex vivo)/isolated synaptosomes	Inhibited formation of nitron (a free radical spin adduct) [89] Protection against A β induced oxidative stress [45, 46] Protection against oxidative stress induced by A β [90] reverse transcriptase inhibitor (NRTI)-2', 3'-dideoxycytidine [91] Protection against oxidative stress induced by A β [48] Fe ²⁺ /H ₂ O ₂ [92] A β induced loss of phospholipid asymmetry [93] Protection against free radical induced lipid peroxidation [94]
	Alveolar macrophages	Reduced Inflammatory response and H ₂ O ₂ [95]
Other mechanisms	CHO-K1, HEK-293, NIH-3T3 cells Purified enzymes, cPLA ₂ , sPLA ₂ , iPLA ₂ ; porcine pancreatic PLA ₂ Bovine pancreatic sPLA ₂ Purified bacterial PI-PLC and cabbage PLD NIH3T3 fibroblasts	Stimulated <i>de novo</i> ceramide synthesis [33] May also inhibit cPLA ₂ and stimulate sPLA ₂ [53] No inhibition [3] No inhibition [3] Inhibited PLD activity [96]

Mechanism	System	Action [Reference]
	Chinese hamster embryo fibroblasts (IIC9 cells, a subclone)	May inhibit PC synthesis by affecting translocation of CCT to membrane [51]
	Rat neurospheres	Increased the metabolic activity (MTT assay) [34, 56]
	UMR-106 osteoblastic cells	Increased PLD activity [23] The acid sphingomyelinase inhibitors block interferon- [alpha]-induced serotonin uptake via a COX-2/Akt/ERK/STAT-dependent pathway in T cells
	Jurkat T cells	Inhibited IFN- α induced ASMase activity, COX-2/Akt/ERK/STAT pathways [97]. PC-PLC inhibition cannot be ruled out