A reflection of the lasting contributions from Dr Robert Bittman to sterol trafficking, sphingolipid and phospholipid research

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ABSTRACT-With the passing of Dr Robert Bittman from pancreatic cancer on the 1st October 2014, the lipid research field lost one of the most influential and significant personalities. Robert Bittman's genius was in chemical design and his contribution to the lipid research field was truly immense. The reagents and chemicals he designed and synthesised allowed interrogation of the role of lipids in constituting complex biophysical membranes, sterol transfer and in cellular communication networks. Here we provide a review of these works which serve as a lasting memory to his life.

Introduction

Robert Bittman (Fig. 1) published 340 peer-reviewed papers, contributed 64 book chapters and filed 19 US patents. He was funded continuously by the National Institute of Health from 1973 to the beginning of 2014 and he was awarded distinguished MERIT funding from the National Heart Lung and Blood Institute from 1986. International recognition included him receiving The Avanti Award of the American Society of Biochemistry and Molecular Biology in 2003 and a Fellowship from the American Association for the Advancement of Science in 2004. For the purpose of this review we have focused on Bittman's contribution to understanding lipid trafficking and the role of sphingolipids (e.g. ceramide, sphingosine and sphingosine 1-phosphate (S1P), ceramide 1-phosphate (C1P)) and phospholipids (e.g. lysophosphatidic acid

(LPA), ether glycerol lipids) in cellular communication networks governing cancer biology, inflammation and other diseases. We treat these works in a reflective manner to provide some measure of their impact on the key issues that were facing the field at the time. Indeed, Bittman's research has influenced all the major areas concerning these bioactive lipids and one would hope that his work might ultimately influence the development of therapeutics in the future that will tackle the terrible disease that took his life.

Lipid trafficking

Bittman published extensively on sterol biochemistry. Examples include the assessment of the distribution of cholesterol between the outer and inner halves of the lipid bilayer of mycoplasma cell membranes [1]. He also determined the rates of rapidly exchanging cholesterol and phospholipid pools in sphingomyelin- and phosphatidylcholine-containing membranes [2]. Bittman also investigated how sphingomyelin modulates trans-bilayer distribution of galactosylceramide in phospholipid containing membranes [3]. Using fluorescence quenching assays, he established a role for ceramide in lipid raft organisation and as a determinant of sterol content [4]. Bittman also synthesised BODIPY (boron-dipyrromethene)-cholesterol, which was then used to visualise sterol trafficking in cells [5]. The egress of BODIPY cholesterol (BC) from late endosomal (LE) organelles is dependent on acid lipase and Niemann-Pick C1 (NPC1) protein [6]. NPC1 was shown to recruit Rab8a to BC-containing LEs and to thereby enhance the motility and segregation of BC- and CD63-positive organelles from lysosomes. Low density lipoprotein (LDL) increased the number and dynamics of focal adhesions and stimulated cell migration in an acid lipase-, NPC1- and Rab8a-dependent manner. Further advances include the demonstration that N-myc downstream-regulated gene 1 (NDRG1) regulates LDL uptake by LDL receptor [7].

Bittman and colleagues also demonstrated that the Sonic Hedgehog receptor, Patched might contribute to cholesterol efflux from cells, thereby affecting intracellular cholesterol concentration [8]. This activity likely accounts for the inhibition of sonic hedgehog signalling receptor, Smoothened, enrichment at the plasma membrane [8] and which is an important step in activation of the sonic hedgehog signalling pathway. Sonic hedgehog signalling has a critical function in regulating growth and patterning during embryonic development, and also in stem cell maintenance and tissue regeneration in adults.

Bittman and Ikonen used sphingomyelin, ceramide and sphingosine labelled with [^{3}H] in carbon-3 of the sphingosine backbone and targeted them to the late endosomes and lysosomal compartment (LE/LY) in low-density lipoprotein (LDL) particles. These probes were routed through the LE/LY sphingolipid degradation and recycling pathway. They demonstrated that NPC1 does not play a significant role in LE/LY sphingosine export. Instead, NPC1-deficient cells displayed an increased affinity for sphingosine independently of protein-mediated lipid transport [9]. Bittman and Pagano and colleagues also demonstrated that the endocytosis of fluorescent glycosphingolipid (GSL) analogues occurs via a caveolar-dependent mechanism [10]. Collaboration with Wilschut and colleagues demonstrated that the alphavirus Semliki Forest virus (SFV) enters cells through receptor-mediated endocytosis that involves stereospecific interaction of the viral fusion protein with D-*erythro* sphingolipids in the target membrane [11]. These studies collectively advanced the lipid trafficking field made possible by ingenious synthesis of highly specific lipid tools.

Sphingolipid Signalling

In the mid 1990's the concept of the sphingolipid rheostat was proposed, where inter-conversion of ceramide, sphingosine and S1P by various enzymes was suggested to regulate cellular fate. In this model, shifting the balance toward ceramide induces apoptosis, while predominance of S1P formation promotes cell survival. However, the sphingolipid rheostat exhibits complexity and has recently been re-evaluated [12], as there is temporal and spatial regulation, where functionality is governed by compartmentalised signalling [13]. A measure of this complexity is evident as conversion of S1P to (*E*)-2 hexadecenal and phosphoethanolamine can result in formation of phospholipids that have additional defined signalling functions in cells [14] and ceramide can be converted to C1P which has opposing actions to ceramide. Therefore, the regulation of the sphingolipid rheostat in different cellular compartments is likely to provide finer tuning of cell biology than was previously considered. Bittman made a significant contribution to our understanding of sphingolipid signalling by developing reagents and chemical tools that allowed some of the functions of ceramide, C1P, sphingosine, S1P and hexadecenal to be identified.

Ceramide: stress sensor and responder--In the late 1980s, the hydrolysis of sphingomyelin by sphingomyelinase was demonstrated to constitute a novel signal transduction pathway in mammalian cells. The product, ceramide, can be acylated to sphingosine, which functions to inhibit protein kinase C (PKC) [15], or phosphorylated to C1P [16] by a calcium-dependent ceramide kinase, the function of which was unknown at the time. Moreover, 1,25-dihydroxyvitamin D3 was shown to activate sphingomyelinase to stimulate differentiation of HL60 cells [17, 18]. Sphingosine had also been demonstrated to promote the phosphorylation of the EGF receptor on Thr 669 in A431 human epidermoid carcinoma cells via a mechanism that was independent of PKC [19].

Diacylglycerol and ceramide were shown to have opposing cellular actions. Thus, activation of PKC by diacylglycerol promotes cell survival whereas apoptosis is induced by ceramide [20, 21]. Bittman synthesised a cell-permeable ceramide, N-octanoyl sphingosine (C8-Cer) that was used in collaboration with Kolesnick's group to induce a concentration- and time-dependent increase in diacylglycerol levels in cells and which was associated with a reduction in phosphatidic acid levels [22]. C8-Cer was not metabolized to sphingomyelin and promoted PKC translocation from the cytosol to membrane, due to the increase in intracellular diacylglycerol. The mechanism of action of ceramide was resolved by demonstration that it is a competitive (with diacylglycerol) inhibitor of diacylglycerol kinase, the enzyme that catalyses the conversion of diacylglycerol into phosphatidate [22]. Therefore, these studies identified that functional cross-talk between phospholipid- and ceramide-dependent signalling pathways exist in mammalian cells. The significance of these findings contextualised prior studies, which had demonstrated that ceramide promotes apoptotic cell death in the human myeloid leukaemia cell lines HL-60 and U937 [21] and this is suppressed by diacylglycerol [23].

The role of ceramide in the induction of apoptotic death had become evident from attempts to decipher the signalling mechanisms activated by 1,25-dihydroxyvitamin D3 [17, 18] and which was significantly helped by use of ceramide analogues, such as C2-ceramide [20]. Others demonstrated that ceramide was also able to activate protein phosphatase 2A (PP2A) [24, 25]. Activation of heterotrimeric PP2A was specific for ceramide because related sphingolipids had no effect. Indeed, dihydro-C2-ceramide, which lacks the trans-double bond in the sphingoid base inhibited PP2A activity. Later studies showed, for instance, that the ceramide-activated protein phosphatase is involved in deactivating the PKB/Akt pathway [26, 27], down-

regulating c-Myc [25] and promoting dephosphorylation of retinoblastoma protein to induce growth arrest [28] thereby providing molecular detail to ceramide-dependent signalling and biological responses.

Bittman made contributions in this area with studies investigating the effect of N-octanoyl-sphingosine (C8-Cer) stereoisomers, N-octanoyl-DL-*erythro*-dihydrosphingosine (DL-*e*-DHC8-Cer), and N-octyl-D-*erythro*-sphingosine (D-*e*-C8-Ceramine) on apoptosis in U937 cells [29]. D- and L-*threo* stereoisomers were demonstrated to be more potent than the *erythro* analogues in inducing apoptosis. D-*e*-C8-Cer, D-*t*-C8-Cer and D-*e*-C8-Ceramine induced the elevation of endogenous ceramide, but this did not account for their apoptotic effect. In addition, okadaic acid (PP2A inhibitor) failed to protect U937 cells from apoptosis induced by D-*e*-C8-Cer. There was also controversy at the time as to the significance of the 4,5-*trans*-carbon-carbon double bond in short chain ceramides concerning cytotoxicity. These studies resolved that the absence of the 4,5-*trans* double bond in DHC8-Cer reduced cytotoxicity.

Bittman also synthesised analogues of ceramide with a view to exploiting their potential anti-cancer activity. For instance, (2*S*,3*R*)-(4E,6E)-2-octanoylamidooctadecadiene-1,3-diol (4,6-diene-Cer) was synthesised [30]. This analogue contains an additional trans double bond at C(6)-C(7) of the sphingoid backbone and was demonstrated to be more potent in inducing apoptosis than ceramide in TNFα-resistant and TNFα-sensitive MCF-7 cells. 4,6-diene-Cer caused a prolonged elevation of intracellular ceramide levels in MCF-7 cells and induced apoptosis as evidenced by the release of cytochrome c, loss of membrane asymmetry and reduced mitochondrial membrane potential. Bittman also synthesised an analogue of short chain ceramides with a disulfide linkage, N-(4',5'-dithiaheptanoyl)-D-*erythro*-ceramide) and in collaboration with Arthur, demonstrated that this compound induced significantly more

cytotoxicity than short chain ceramides in BT549, A549, and DU145 cancer cells [31]. The activity was associated with a reduction in cellular glutathione (GSH) level, which had been proposed some time before to be a critical step in neutral sphingomyelinase activation and subsequent ceramide-induced apoptosis [32]. Bittman and colleagues also demonstrated that the C(4)-C(5) trans-double bond has little influence on the ability of apoptosis regulatory proteins, Bax and Bcl-xL, to bind to the ceramide channels [33]. However, the stereochemistry of the head group and access to the amide group of ceramide is essential for Bax, but not Bcl-xL binding. Bcl-xL was shown to also optimally bind long-chain apoptotic ceramides.

Bittman synthesised many FTY720 analogues some of which targeted various key enzymes involved in sphingolipid metabolism such as ceramide synthases and sphingosine kinase. FTY720 modulates the immune response by preventing egress of T-lymphocytes from lymph nodes [34]. FTY720 (Gilenya^{TM}) is licenced for oral treatment of relapsing multiple sclerosis and is a pro-drug, which is phosphorylated by sphingosine kinase 2 (SK2). FTY720 phosphate is a functional antagonist of the $S1P_1$ receptor, causing its proteasomal degradation and removal from T-lymphocytes [34]. Since T-lymphocytes use an S1P gradient to egress from lymph nodes, FTY720 is able to prevent this by creating $S1P_1$ null T-lymphocytes that do not respond to the S1P gradient, thereby ablating their action on the central nervous system in multiple sclerosis. However, it is clear that FTY720 can affect other targets in cells. Indeed, Bittman was involved in two studies that demonstrated that FTY720 modulates *de novo* synthesis of ceramide. In collaboration with Futerman and colleagues, FTY720 was shown to inhibit ceramide synthase activity in a non-competitive manner with acyl-CoA and an uncompetitive manner with sphinganine [35]. The inhibitory effect of FTY720 was dependent on acyl-CoA chain length. Ceramide synthesis was inhibited at high (0.5-5 μM) but not low (< 200 nM) sphinganine concentrations in cells. Ceramide, sphingomyelin, and hexosylceramides levels

were increased in response to FTY720, possibly a consequence of additional effects on, for instance, sphingosine kinase 1 (SK1). These findings were highly informative in defining the complexity of the mechanism of action of FTY720. FTY720 is known to induce apoptosis of, for instance, cancer cells, and therefore modulation of ceramide levels might underlie, in part, this mechanism of action. In collaboration with Natarajan's group, stable isotope pulse labelling of human pulmonary artery endothelial cells was used to establish that FTY720 modulates *de novo* ceramide synthesis [36].

Alpha-galactosylceramide (alphaGalCer) and its isosteric C-glycoside analogue activate natural killer T (NKT) cells [37]. Bittman synthesised a truncated non-isosteric C-alphaGalCer analogue that, in common with its isosteric C-glycoside analogue, is not enzymatically labile at the glycosidic linkage. Instead the analogues have the anomeric carbon directly bonded to the C1 of the phytoceramide backbone. Comparison of the three ligands using human dendritic cells as the antigen-presenting cells and human NKT cells as the responding cells demonstrated that these lipids preferentially promote Th1-immune responses and might therefore serve as potent adjuvants in the immunotherapy of cancer and infectious diseases.

Sphingomyelin--Structural activity relationships concerning specificity of sphingomyelinase were studied [38]. Four analogues of sphingomyelin were synthesized by Bittman's laboratory in which the C-3 hydroxyl group was replaced by a hydrogen atom (to produce a deoxy-sphingomyelin analogue), or with O-methyl, O-ethyl or O-tetrahydropyranyl groups. The deoxy-sphingomyelin analogue failed to compete with a substrate of sphingomyelinase and was not hydrolysed by the enzyme, suggesting that the hydroxyl group is essential for activity. The size of the alkyl group on the ether moiety was also found to be important. Thus, the 3-

O-tetrahydropyranyl-sphingomyelin failed to compete with substrate of neutral pH-optimum sphingomyelinase.

Ceramide 1-phosphate--Bittman in collaboration with Gomez-Muñoz and Brindley demonstrated that C2- and C8-Ceramide-1-phosphates (N-acetylsphingosine-1-phosphate and N-octanoylsphingosine-1-phosphate, respectively) stimulated DNA synthesis and promoted division of fibroblasts [39]. Recent studies by Gomez-Muñoz and Bittman demonstrated that C1P stimulates reactive oxygen species (ROS) formation in primary bone marrow-derived macrophages and that C1P induced ROS formation promotes mitogenesis of macrophages [40]. This requires activation of NADPH oxidase, which is down-stream of C1P-dependent activation of cytosolic calcium-dependent phospholipase A_2 and PKCα. Bittman contributed to this study by synthesising BHNB (4-bromo-5-hydroxy-2-nitrobenzhydryl)-C1P (a photolabile caged-C1P analogue), which was critical to the study because it allowed demonstration that C1P elicits these effect via an intracellular action. This was an important advance as it distinguished the actions from those in which C1P can elicit extracellular effects via a putative unidentified cell surface receptor.

Sphingosine--Contributions to this area of research is evident by studies showing that sphingoid bases synthesised in Bittman's laboratory induced apoptosis in HL-60 and U937 cells via a mechanism that did not involve their acylation to ceramides by ceramide synthases. Thus, Fumonisin B1 (FB1, ceramide synthase inhibitor) failed to abrogate the effects of the sphingoid bases [41]. These sphingoid bases inhibited PKC and potentiated the lethal effects of ceramide, thereby providing evidence that PKC, which is inhibited by sphingoid bases, has a cytoprotective role in leukemic cells. This was followed by studies using phenethylisothiocyanate derivatives of sphinganine and sphingosine (PEITC-Sa and PEITC-

So) that were synthesised in Bittman's laboratory [42]. The PEITC-sphingoid base derivatives were shown to exhibit higher cytotoxicity (by PKC inhibition) than their natural counterparts and the clinically tested sphingoid base, safingol. Such studies, highlighted the potential use of synthetic lipids as conventional PKC/novel PKC chemotherapeutic agents.

Bittman also collaborated with Hla's group to identify novel intracellular targets of sphingosine [43]. Using affinity chromatography and proteomics, they identified acidic leucine-rich nuclear phosphoprotein-32A (ANP32A; an inhibitor of PP2A) as a direct binding target of sphingosine and *N,N′*-dimethylsphingosine (DMS). DMS relieved the ANP32A-mediated inhibition of PP2A and induced stimulation of the p38 MAPK pathway and expression of cyclooxygenase (COX)-2 in HUVEC cells. These studies therefore advanced the field by identifying signalling pathways in which sphingosine is involved. The sphingosine mimetic, FTY720 was also known to activate PP2A, a tumour suppressor. Indeed, certain myeloproliferative neoplasms are characterized by the expression of the Jak2(V617F) oncogene, which inactivates PP2A. Bittman synthesised FTY720, which was used to reactivate the PP2A and reduce Jak2(V617F) activity and clonogenic potential [47]. Likewise, FTY720 decreases leukemic allelic burden and splenomegaly and increases Jak2(V617F) leukemic mice survival. The effects of FTY720 require a PP2A interacting protein SET K209. In contrast, FTY720-P, acting as a $S1P_1$ receptor agonist, promotes Jak2-SET-mediated PP2A inhibition. These findings were important not least in defining the interplay between the S1P signalling pathway, SET2-PP2A and Jak2, but also in identifying novel strategies for eliminating the oncogenic drive in myeloproliferative neoplasm [44].

Previous studies had demonstrated that sphingosine and FTY720 could disrupt the proliferative signalling of the scaffold and adapter protein 14-3-3 [45] by enabling its protein kinase A

(PKA)-catalysed phosphorylation of Ser58 which destabilizes 14-3-3 dimerization. Dimeric 14-3-3 is functional, and the phosphorylation prevents the pro-survival action of 14-3-3 in cells. In a study published after Bittman's death, Woodcock and colleagues used FTY720 mimetics (quaternary ammonium analogues of FTY720) to stimulate phosphorylation of 14-3-3 at Ser58, which is deep in the interface of the dimer, resulting in mitochondrial-mediated apoptosis. Bittman laboratory synthesised RB-011 and RB-012, which are poor inhibitors of sphingosine kinase [46] and these were used to disrupt 14-3-3 dimers at low micromolar concentrations and to induce abrogation of Raf-ERK-1/2 and PI3K/Akt signalling in Jurkat cells [47]. RB-011 and RB-012 were also shown to induce apoptosis of human A549 lung cancer cells and RB-012 reduced xenograft growth of these tumours in mice. When mutant phosphorylation deficient 14-3-3 proteins are expressed in cells, they function in a dominant negative manner forming dimers with endogenous 14-3-3 that disrupts ERK-1/2 signalling. The phenotype was recapitulated by RB-012. These compounds were therefore used not only to demonstrate potential therapeutic utility in targeting 14-3-3 as an anti-cancer strategy, but also in delineating novel pathways by which sphingosine can regulate cell death.

Sphingosine 1-phosphate--The first suggestion that S1P might function as a bioactive lipid came from studies showing that sphingosine mobilised calcium from intracellular stores mediated from a product of sphingosine and believed to be S1P [48]. Subsequent studies demonstrated that a number of effects attributed to sphingosine, were mediated by S1P including the stimulation of DNA synthesis of Swiss3T3 fibroblasts [49]. Others demonstrated that S1P inhibits cell motility and tumour invasiveness [50]. Additional studies characterised the enzymes involved in the metabolism of S1P including sphingosine kinase, S1P lyase and lipid phosphate phosphatases. By the mid 1990's there was compelling evidence that S1P could also induce pertussis toxin-dependent cell signalling responses, such as ERK-1/2

activation that suggested some of the S1P responses were mediated by specific G protein-coupled receptors (GPCR) [51, 52]; this was later confirmed by the identification of five S1P-specific GPCR, now termed $S1P_{1-5}$. In addition, there were several studies that attempted to decipher and distinguish the bioactive functions of ceramide, sphingosine and S1P. Due to the nature of their inter-conversion, delineation of their individual roles was not a trivial exercise. Several studies lead to the concept of the sphingolipid rheostat. This was deduced from assessment of the effect of ceramides, sphingosine and S1P on phospholipase D activity and mitogenesis in fibroblasts [53] and on ERK-1/2, JNK/SAPK, p70S6K and cAMP signalling in airway smooth muscle cells [54]. Later, S1P was shown to inhibit ceramide-induced cell death, which also blocked ceramide-induced stress activated signalling (JNK/SAPK) in U937 cells and Jurkat cells [55]. These studies provided evidence that elucidated different biological effectors of ceramide, sphingosine and S1P, thereby separating the function of these sphingolipids.

Bittman's interest in S1P started in the mid 1990's when the controversy and debate concerning whether this lipid induced specific cell biological responses was at its height. Spiegel and colleagues had shown that S1P and sphingosylphosphorylcholine (SPC) stimulate DNA binding activity of activator protein-1 (AP-1) in Swiss 3T3 fibroblasts [56]. The collaboration with Bittman demonstrated that the free 2-amino group and the 4E double bond of SPC and S1P were important for AP1 transactivation [57]. A reduction in sphingoid backbone chain length, substitution of the 3-hydroxyl group or saturation of the 4E bond significantly reduced the mitogenic/AP-1 transactivation induced by S1P, but not as significantly for SPC. These early studies highlighted the power of structural-activity relationships in defining functionality to S1P over other sphingolipids, identified S1P as a *bona fide* bioactive lipid and suggested applications in wound healing and inhibition of metastasis.

Studies on sphingosine 1-phosphate receptors

In addition to the regulation of sphingolipid signalling in cells mediated through changes in dynamic flux through the sphingolipid rheostat, it is now clear that S1P can exert biological actions through a variety of G-protein coupled receptors whose natural ligand is S1P. The identification and cloning of five S1P receptors opened avenues of research in which Bittman could utilise his chemical synthetic expertise to expand our knowledge of the structural activity relationship of receptor function and the role of each S1P receptor type in cell biology. His first contribution in this area was the synthesis of two photoreactive analogues of S1P; [^{32}P]-labelled (*2S,3R*)-14-O-(4'-benzoylphenyl)- and (*2S,3R*)-14-O-((4'-trifluoromethyldiazirinyl)phenyl)-(4E)-tetradecenyl-2-amino-3-hydroxy-1-phosphate [58]. The $S1P_1$ receptor bound the benzophenone-containing ligand, while no specific binding was found with the diazirine-containing ligand. In contrast both ligand bound to plasma carrier proteins (presumably albumin). These studies revealed that different parts of the S1P pharmacophore of S1P interact with its receptor and plasma carrier proteins.

Bittman also explored the activity of FTY720 and FTY720-phosphate in a number of biological systems. For instance, the effect of S1P and FTY720-phosphate on constrictor responses were investigated in bladder smooth muscle (detrusor) in collaboration with Spiegel's group [59]. S1P-induced a phasic and tonic contraction of detrusor muscle that was dependent $S1P_2$. The phasic response required phospholipase C (PLC). FTY720-phosphate, an agonist for all S1P receptors except $S1P_2$ induced a slow and sustained contraction, suggesting that this contraction phase was dependent on a receptor independent mechanism (possibly calcium Trp channel and/or voltage-operated calcium channels). Detrusor tone is deregulated in overactive bladder

syndrome, suggesting that S1P signalling might represent a therapeutic target in this disease. Bittman and colleagues also achieved the first enantio-selective synthesis of chiral isosteric phosphonate analogues of FTY720 and demonstrated, in collaboration with Tigyi's group, that the (*R*)-FTY720-vinylphosphonate was a full agonist of $S1P_1$ while the (*S*) enantiomer was a pan-antagonist of $S1P_{1-5}$ receptors [60, 61]. Both enantiomers inhibited autotaxin, induced transient peripheral lymphopenia and produced an anti-apoptotic effect in camptothecin-treated IEC-6 intestinal epithelial cells. At concentrations above 1 μM, (*S*)-FTY720 vinylphosphonate no longer exhibited an anti-apoptotic effect. This was consistent with other studies involving collaboration with the Pyne group which demonstrated that (*S*)-FTY720 vinylphosphonate (> 1 μM) inhibited sphingosine kinase 1 (SK1) activity and promoted its proteasomal degradation and this was associated with an apoptotic effect in prostate and breast cancer cells [62]. Such studies highlighted the complexity of the pharmacological intervention of the S1P signalling pathway.

The biological action of FTY720-phosphate at $S1P_1$ receptors in T-lymphocytes was initially considered to underlie the major effect of this compound in alleviating symptoms of multiple sclerosis. However, there is now a general agreement that FTY720-phosphate can also exert direct effects on the central nervous system. Indeed, there was demonstration of a direct protective effect of FTY720-phosphate on oligodendrocyte progenitors that maintain myelination of neurons in the brain [63]. Treatment of these cells with FTY720 causes activation of ERK-1/2 and Akt, and protection from apoptosis. However, FTY720 also arrested oligodendrocyte differentiation counteracted by neurotrophin-3, which not only enhanced the survival of oligodendrocyte progenitors induced by FTY720 but also stimulated their maturation. These studies raised the possibility that multiple sclerosis therapies with FTY720 should include the use of differentiation-enhancing factors such as neurotrophin-3.

Bittman in collaboration with Garcia, Dudek and Natarajan explored the potential effect of FTY720 analogues on vascular endothelial cell barrier integrity in terms of inflammatory diseases such as acute lung injury. It had been demonstrated that S1P and FTY720 exhibit potent barrier-enhancing activity. Bittman's laboratory synthesised a number of FTY720 analogues that were used in collaboration with Dudek and colleagues to assess effect on endothelial barrier integrity [64]. They demonstrated that the (*R*)- and (*S*)-enantiomers of FTY720-phosphonate induced a sustained endothelial cell barrier enhancement that was effective in a lower concentration range and with an earlier onset than either S1P or FTY720. Indeed, the (*S*)-FTY720-phosphonate reduced alveolar and vascular permeability in a lipopolysaccharide-stimulated murine model of ALI without induction of lymphopenia. These findings suggest therapeutic utility for FTY720 analogues in reducing pulmonary vascular leakage and inflammation and which might have implication for future treatments of acute lung injury. Indeed, these studies were extended in collaboration with Garcia to demonstrate that radiation-induced lung injury, which is a common toxicity in patients administered thoracic radiotherapy, is reduced by (*S*)-FTY720-phosphonate, along with decreased radiation-induced gene dysregulation [65]. These results were supported by findings which demonstrated that mice with deletion of SK1 ($SK1^{-/-}$) or with reduced expression of certain S1P receptors ($S1P_1^{+/-}$, $S1P_2^{-/-}$, and $S1P_3^{-/-}$) exhibited radiation-induced lung injury susceptibility. (*S*)-FTY720-phosphonate was also assessed in a bleomycin-induced lung injury model and was shown to reduce lung leak and inflammation, while maintaining $S1P_1$ receptor expression. Indeed, (*S*)-FTY720-phosphonate does not induce β-arrestin recruitment and does not promote $S1P_1$ ubiquitination and proteasomal degradation. This contrasted with FTY720, which did not protect against bleomycin-induced acute lung injury in mice and significantly down-regulated $S1P_1$ receptor expression. The significant advance here was the identification of a compound

that promotes barrier integrity but lacks the potential barrier disrupting effect of down-regulating $S1P_1$ [66].

Tigyi and Bittman were also involved in a collaboration to map the binding site of FTY720-phosphate in S1P receptors [67]. To achieve this they exploited the fact that FTY720-phosphate binds to the $S1P_1$ receptor but not the $S1P_2$ receptor. A constructed $S1P_2$ chimera with $S1P_1$ sequence from the N terminus to transmembrane domain 2 was activated by FTY720-phosphate and exhibited an $S1P_1$-like activation. Twelve residues in this inserted domain, distributed in four motifs (a-d), differ between $S1P_1$ and $S1P_2$. Insertion of RPMYY in motif b alone or simultaneous swapping of five other residues in motifs c and d from $S1P_1$ into $S1P_2$ introduced responsiveness to FTY720-phosphate. Thus, the use of FTY720-phosphate enabled insight into specific receptor-ligand interactions.

Studies on sphingosine kinases

The modulation of the sphingolipid rheostat to shift the balance to formation of apoptotic ceramide provides a potential means to abrogate hyper-proliferative diseases such as vascular remodelling in pulmonary hypertension and both solid and haematological cancers. Sphingosine kinase (SK) is a logical target for therapeutic intervention since inhibition of this enzyme functions to prevent conversion of sphingosine into S1P, with concomitant back conversion of sphingosine into apoptotic ceramide. With this rationale, a collaboration was established between Bittman and the Pyne group to synthesise and test sphingoid base and FTY720 analogue inhibitors of SK1 and SK2. The demonstration that FTY720 inhibits SK1 activity [62] provided a strong rationale for development of FTY720 analogues as SK inhibitors. Moreover, (*S*)-FTY720 vinylphosphonate was shown to be an allosteric inhibitor of SK1 activity [68, 69] and function to stabilise a conformation of SK1 that enhances auto-

inhibition of the catalytic site. In addition, two new FTY720 analogues (a conjugate of sphingosine with a fluorophore and (*S*)-FTY720 regioisomer) stimulated SK1 activity, suggesting relief of the auto-inhibition of SK1 activity [68]. These studies were extended by demonstration that replacement of the amino group in (*S*)-FTY720 vinylphosphonate with an azido group converted the (*S*)-FTY720 vinylphosphonate from an allosteric inhibitor to an allosteric activator of SK1 at low μM concentrations [70]. (*S*)-FTY720 vinylphosphonate also induced the proteasomal degradation of SK1 in androgen-independent prostate cancer cells and promoted the apoptosis of these cells, which are highly resistant to chemotherapeutic agents [68].

Bittman's laboratory also undertook the stereospecific synthesis of an analogue of FTY720 called (*R*)-FTY720-OMe (ROMe), which was demonstrated, in collaboration with the Pyne group, to be a competitive inhibitor of SK2. (*S*)-FTY720-OMe failed to inhibit SK1 activity, thereby demonstrating stereospecific inhibition of SK2 [71]. Prolonged treatment of HEK293 cells with ROMe also induced a reduction in SK2 expression, inhibited DNA synthesis and prevented S1P-stimulated rearrangement of actin in MCF-7 breast cancer cells, indicative of an application to prevent metastasis. ROMe also induced autophagic death of T-cell acute lymphoblastic leukemia cells [72] and promoted growth arrest in MDA-MB-231 breast cancer cells [73]. The latter involves the accumulation of the $S1P_2$ receptor in the nucleus of cells treated with ROMe; a process which is recapitulated by knockdown of SK2 with siRNA [73].

Bittman's laboratory also synthesized inhibitors that were selective for SK1 and SK2. For example, the thiourea adduct of sphinganine (F-02) was selective for SK2 whereas the 1-deoxysphinganines 55-21 and 77-7 were selective for SK1. (*2S,3R*)-1-deoxysphinganine (55-21) induced the proteasomal degradation of SK1 in human pulmonary arterial smooth muscle

cells and inhibited DNA synthesis, while the more potent SK1 inhibitors PF-543 and VPC96091 failed to inhibit DNA synthesis [74]. These findings indicated that moderate potency inhibitors such as 55-21 are likely to have utility. There was also the design, synthesis, and evaluation of the potency of new isoform-selective inhibitors of SK1 such as 1-(4-octylphenethyl)piperidin-4-ol) (RB-005) [46, 75]. Structural activity relationship analysis was performed in which the lipophilic tail, polar head group, and linker region were modified. Using modelling studies with the recently published crystal structure of SK1, the basis for the key residues targeted by this profiled series was achieved along with identification of determinants that provide a rationale for the development of inhibitors with better selectivity for SK2 over SK1 [75].

Studies on sphingosine 1-phosphate lyase

Collaboration between Bittman and Saba's group identified temporal and spatial accumulation of delta(4,6)-sphingadienes in Sply Drosophilia mutants that were linked with muscle degeneration [76]. Further work established that dietary sphingadienes induce colon cancer cell apoptosis and reduce intestinal tumourigenesis. This is achieved by inhibition of the translocation and activation of Akt to the plasma-membrane (without affecting PI3K) and subsequent stimulation of 4-EBP-1 phosphorylation thereby preventing proteins synthesis. Over-expression of constitutively activated Akt reduced sphingadiene-induced cell death, autophagy and apoptosis [77]. Byun and Bittman [78] then published the synthesis of 4,6- and 4,8-sphingadienes on a scale of gram quantities. The sphingadienes were also shown to reduce the levels of β-catenin and Wnt targets such as c-Myc and cyclin D1 in Apc(Min/+) mouse intestinal tissues [79]. These effects are mediated via reduced GSK3β levels, and are ablated by over-expression of constitutively active Akt. Moreover, combined treatment with sphingadienes and rapamycin decreased polyps in Apc(Min/+) mice. The study also

demonstrated that sphingadienes inhibit Wnt signalling through a PP2A/Akt/GSK3β-dependent mechanism that promotes anti-cancer activity. More recent findings have demonstrated that dietary sphingadienes induce the up-regulation of S1P lyase expression and this reduces S1P levels and abrogates colon carcinogenesis. The mechanism involves a sphingadiene-induced reduction in phosphorylated STAT3 levels and pro-inflammatory mediator production, such as TNFαand the interleukins, IL-6, IL-21 and IL-23 [80]. The work elegantly demonstrated that sphingodienes prevent tumourigenesis driven by STAT3 activated miRNAs that suppress anti-oncogenes.

The collaboration between Saba and Bittman also explored whether *trans*-2-hexadecenal (produced upon the degradation of S1P by S1P lyase) has signalling functions in cells [81]. The findings demonstrated that *trans*-2-hexadecenal induces cytoskeletal reorganization and an oxidative stress-dependent activation of JNK-mediated apoptosis. This was evidenced by cytochrome c release, Bax activation, Bid cleavage and increased translocation of Bim into mitochondria. In addition, Saba and Bittman showed that *trans*-2-hexadecenal reacts with deoxyguanosine and DNA to produce the diastereomeric cyclic 1,N(2)-deoxyguanosine adducts 3-(2-deoxy-β-d-erythro-pentofuranosyl)-5,6,7,8-tetrahydro-8R-hydroxy-6R-tridecylpyrimido[1,2-a]purine-10(3H)one and 3-(2-deoxy-β-*d*-erythro-pentofuranosyl)-5,6,7,8-tetrahydro-8S-hydroxy-6S-tridecylpyrimido[1,2-a]purine-10(3H)one with potentially mutagenic consequences [82].

Lysophosphatidic acid

The biological activity of LPA was first identified in the late 1970's with the demonstration that LPA can induce pressor responses [83], platelet aggregation [84] and neutrophil chemotaxis [85]. In addition, LPA was shown to markedly inhibit synaptic membrane

(Na^+/K^+)-ATPase activity in rat brain suggesting a role in depolarization and/or increase in intracellular calcium concentrations [86]. In the early 1990's LPA was identified as a mitogen that acted through a pertussis sensitive G-protein coupled receptor(s) and p21Ras in fibroblasts [87-89]. Tigyi et al. [90] demonstrated that LPA bound to serum albumin activated membrane currents in oocytes and induced neurite retraction in PC12 cells. Numerous studies confirmed LPA to be a bioactive lipid and there has been extensive characterization of the receptors that mediate its action. There are multiple species of LPA that account for its pleiotropic actions, including both mitogenic and anti-mitogenic effects in mammalian cellular systems [91]. Different molecular species of LPA are found in biological fluids as saturated, and mono and poly-unsaturated variants of *sn*-1 or *sn*-2 regioisomers. The majority of LPA species in serum are unsaturated and these have higher potency at LPA receptors and intracellular targets, such as the peroxisome proliferator-activated receptor γ [92]. Phospholipase A1 enzymes catalyse formation of unsaturated *sn*-2 LPA. Cyclic phosphatidic acid (1-acyl-*sn*-2,3 cyclic phosphate) a naturally occurring analogue of LPA from the slime mould *Physarum polycephalum* to human blood [93, 94], alkyl-ether [95, 96] and alkenyl-ether analogues [97] of LPA have also been identified and are weak agonists of LPA-specific GPCR [98, 99]. In contrast, LPA_5 exhibits preference for 1-O-alkyl glycerophosphate compared with acyl analogues with the same chain length. LPA binds to two classes of GPCR, namely the EDG family cluster (LPA_{1-3}) and the P2Y cluster ($LPA_{4,5}$). Additional members of the P2Y class include P2Y5 (LPA_6), and potentially GPR35. The related GPCRs [100-102] and P2Y10 [103], in spite of initial claims of being activated by LPA, are no longer classified as LPA receptors. LPS1/GPR34, LPS2/P2Y10 and LPS3/GPR174 are now established as lysophosphatidylserine receptors [104, 105]

Bittman extensively synthesised chemical tools to interrogate the structural functional aspects of LPA receptors. His work also involved development of LPA agonists and antagonists that facilitated understanding of the pharmacology of ligands acting at LPA receptors. In collaboration with Tigyi, N-palmitoyl-serine phosphate and N-palmitoyl-tyrosine phosphate were synthesized and shown to inhibit LPA-stimulated Cl^- currents with nM potency in oocytes, thus rendering them as potential antagonists [106, 107]. The D- and L-stereoisomers were equally effective, consistent with the finding that both enantiomers of LPA bind to LPA receptors. Both of these LPA antagonists lacked activity when microinjected into the oocyte and were not effective against acetylcholine, serotonin, and glutamate receptors that had been over-expressed in the oocyte. In this 1996 publication that preceded the molecular cloning of the first LPA GPCR, the differential sensitivity to N-palmitoyl-serine and N-palmitoyl-tyrosine phosphates provided pharmacological evidence for multiple LPA receptor subtypes, which were named low- and high-affinity LPA receptors. However, it took 13 years before N-palmitoyl-serine phosphate was finally re-examined in cells transfected with individual LPA receptors to find that LPA_1 was inhibited by this compound with an IC_{50}= 3.45 μM whereas it was a weak agonist of LPA_2 (GPR92) [108]. Interestingly, when platelets are pre-incubated with N-palmitoyl-serine phosphate prior to LPA exposure, their response is fully blocked [109, 110]. Platelet activation is mediated by LPA_5 [111]. The question arises as to why N-palmitoyl-serine phosphate is inhibitory to this response? One potential explanation lies in the pre-incubation of platelets used in the study where prolonged N-palmitoyl-serine phosphate exposure prior to LPA desensitizes LPA_5 in the platelet. Clearly, these acylated amino acid analogues represent a promising scaffold for lead development and deserve more investigation to develop new receptor-specific probes.

The pharmacological characterisation of LPA receptors was further advanced by the synthesis and characterisation of the LPA analogues, 1-O-hexadecyl-*sn*-glycero-3-phosphate (1-C16-GP) and 3-O-hexadecyl-*sn*-glycero-1-phosphate (3-C16-GP). These were shown to induce calcium mobilisation in RH7777 cells over-expressing either LPA_1 or LPA_2 or LPA_3 indicating a lack of stereo-specificity in their action [112]. The enantiomers also induced Cl^- transients in oocytes, which express LPA_1 and PSP24 and heterologous desensitisation was evident in NIH3T3 cells (which express LPA_1 and LPA_2) stimulated with oleoyl-LPA and 1-C16-GP or 3-C16-GP. These findings indicated that these ligands bind to the same LPA receptors. Finally, both enantiomers were dephosphorylated by the lipid phosphate phosphatase, LPP1. This study importantly contributed to the notion that LPA binds to LPA receptor in a non-stereospecific manner, while other unnatural analogues, such as N-acyl-serine phosphate and N-acyl-ethanolamine phosphate (which contain a serine and an ethanolamine backbone, respectively, in place of glycerol) bind to LPA receptors in a stereospecific manner. There are also subtle differences in the binding of these ligands to different LPA receptor sub-types. This provided a strong rationale and belief that it is possible to develop sub-type specific antagonists of LPA receptors that might exhibit therapeutic value. Bittman's compound with the glycerol backbone replaced with amino acid analogues had a profound impact on the development of LPA GPCR probes. The realization that the glycerol backbone was not required for the ligand to activate or inhibit the receptors has fuelled the synthesis of fatty alcohol phosphates that completely lack the glycerol moiety yet depend on their hydrocarbon chain-length [113-115]. One of these analogues, octadecyl thiophosphate is an effective radioprotective agent that mitigates the acute radiation syndrome in mice and non-human primates via activation of the LPA_2 receptor [114, 116, 117].

The collaboration with Tigyi also lead to the finding that LPA is formed during mild oxidation of LDL leading to the stimulation of platelet activation and endothelial stress-fibre formation [110]. These effects were blocked by N-palmitoyl-tyrosine phosphate that had been synthesised and characterised in earlier work. The significance of these findings was the identification of LPA in LDL where it is enriched in the lipid core and which is highly atherothrombogenic. The effects on cardiovascular pathology were followed by studies on LPA targets distinct from the LPA GPCR. The basis of these studies was that products formed during mild oxidation of LDL induce neointimal formation in the coronary artery; this is an important feature of the pathology of atherosclerosis. Bittman and Tigyi demonstrated that ethyl or acyl analogues of LPA also induce neointima formation. These effects were blocked by an antagonist (GW9662) of the peroxisome proliferator-activated receptor gamma (PPARγ) and mimicked by agonists of PPARγ (e.g. rosiglitizone). This contrasted with a lack of effect of the PPARα agonist, stearoyl-oxovaleryl phosphatidylcholine, which failed to induce neo-intimal formation [118]. Moreover, extensive structural activity relationship analysis placed the effects of the LPA analogues with PPARγ rather than with LPA GPCR and highlighted a role in programming de-differentiation of vascular smooth muscle cells, an essential step in allowing these cells to enter the cell-cycle. These seminal findings identified LPA as a natural ligand for the transcription factor, PPARγ and underscored the importance of this biological interaction by demonstrating an important role of LPA in the pathophysiology of coronary atherosclerosis. This work was followed by demonstration that a naturally occurring ether analogue of LPA, 1-O-octadecenyl-2-hydroxy-*sn*-glycero-3-phosphate (AGP), is a potent partial agonist of PPARγ [119]. AGP, which binds to PPARγ with a Kd = 60 nM, was demonstrated to displace 40% of the bound rosiglitizone from PPARγ and activated a PPARγ reporter gene expression by ~ 40% of that induced by rosiglitizone. These findings categorised AGP as partial agonist of PPARγ. A key piece of evidence in support of the role of PPARγ in

neointimal growth came from the stereo-selective activation of the nuclear receptor by AGP. Bittman synthesized the 3-O-octadecenyl-2-hydroxy-*sn*-glycero-1-phosphate stereoisomer of AGP that failed to activate PPARγ unlike the naturally occurring AGP isomer [118]. This type of stereochemical selectivity distinguishes LPA GPCR from PPARγ. Bittman's synthesis of the LPA stereoisomeric compounds has been a prerequisite to delineating this essential difference between the two types of LPA targets. Molecular modelling of the ligands based on the PPARγ crystal structure suggested that AGP and rosiglitazone occupy partially overlapping positions but use different hydrogen bonding and ion pairing interactions. Site-directed mutagenesis demonstrated that H323 and H449 in PPARγ are essential for binding rosiglitizone, while R228 is essential for binding AGP. The different modes of binding provide a molecular explanation for the full and partial agonism of rosiglitizone and AGP respectively. Information from such studies could conceivably be used to produce LPA analogues with full agonism properties and therefore of high therapeutic value in the treatment of atherosclerosis.

Bittman's interest in LPA also extended to the development of chemical reagents used to assess autotaxin (ATX) activity. ATX is a lysophospholipase D which catalyses the formation of LPA from lysophosphatidylcholine and has a role in cancer invasion, metastasis, tumour progression, neuropathic pain, fibrotic disease, cholestatic pruritus, lymphocyte homing and thrombotic diseases. Bittman and colleagues synthesised a lysophosphatidylcholine-like fluorogenic substrate ADMAN-LPC [120], which was used by Tigyi's group to screen a compound library, in order to find novel inhibitors of ATX [121]. Three compounds were identified, two of which (918013, (2,4-dichloro-N-(3-fluorophenyl)-5-(4-morpholinylsulfonyl) benzamide) and 931126, (4-oxo-4-{2-[(5-phenoxy-1H-indol-2-yl)carbonyl]hydrazino}-N-(4-phenylbutan-2-yl)butanamide) were competitive inhibitors of ATX activity whereas another (966791, N-(2,6-dimethylphenyl)-2-[N-(2-furylmethyl)(4-(1,2,3,4-

tetraazolyl)phenyl)carbonylamino]-2-(4-hydroxy-3 methoxyphenyl) acetamide) which was a competitive inhibitor of an alternative lyosphospholipase D fluorogenic substrate FS-3 and the phosphodiesterase substrate p-nitrophenyl thymidine 5'-monophosphate. Computational docking and mutagenesis demonstrated that the two competitive and selective ATX activity inhibitors target the hydrophobic pocket and block accessibility of the substrate to the active site of ATX. The compounds also exhibited biological activity in blocking the invasion of A2058 human melanoma cells and B16-F10 murine melanoma cells colonisation of the lung in C57BL/6 mice. The lasting legacy of these studies might be the longer term development of novel therapeutics targeting ATX in cancer metastasis.

Ether linked lipids

Bittman had a long lasting interest in the chemical synthesis and biology of ether linked lipids, and developed several productive collaborations which brought to the attention of the scientific community important applications for this class of lipids, particular as anti-cancer agents. Bittman's group synthesised ether-linked glycero-alpha- and beta-D-glucopyranosides and glycero-1-thio-alpha- and beta-D-glucopyranosides. In collaboration with Salari and colleagues, alpha-D-thioglucopyranoside [1-O-hexadecyl-2-O-methyl-3-S-(alpha-D-1'-thioglucopyranosyl-*sn*-glycerol)] was reported to be cytotoxic against cancer cells and lacked platelet aggregation activity [122]. 3-S-(alpha-thioglycopyranosyl)-*sn*- glycerols bearing a long-chain O-alkyl group at the *sn*-1 position and a methoxy group at the *sn*-2 position of glycerol were therefore identified as anti-neoplastic agents with low risk of inducing thrombosis. Bittman also synthesised the ether linked lipid, 1-O-hexadecyl-2-O-methyl-*sn*-glycero-3-phosphocholine (ET16-OCH_3-GPC) and demonstrated its ability to inhibit DNA synthesis of HL60 cells [123]. A non-hydrolysable analogue of ET16-OCH_3-GPC was active, thereby suggesting that biological activity was attributable to ET16-OCH_3-GPC rather than a

bio-transformed activity [123]. The ether-linked lipids were shown to compete with diacylglycerol/phosphatidylserine for binding sites on PKC, but this was not linked with cytotoxicity [124]. These workers also demonstrated that ET16-OCH_3-GPC was not a platelet activating factor receptor modulator [125]. Thus, comparison with platelet activating factor agonists demonstrated opposing rank order potency for platelet aggregation activity *versus* cytotoxicity against WEHI-3B leukemic cells.

In collaboration with Arthur, the anti-proliferative effect of ET18-OCH_3 was demonstrated to involve blockade of Raf-1 association with Ras at the plasma-membrane resulting in ablation of the down-stream activation of the ERK pathway [126, 127]. ET-18-OCH_3 was also shown to inhibit the phosphorylation and activation of p70 S6 kinase in MCF-7 Cells [128]. Subsequently, Bittman synthesised enantiomeric unsaturated phosphonocholine analogues of ET-16-OCH_3 which displayed differential cytotoxicity towards two neuroblastoma cell lines, SK-N-SH and SK-N-MC [129]. This was the first report of enanatioselectivity of AELs on antiproliferative activity and cytotoxicity. The differential cytotoxic effects of the enantiomers were demonstrated to be due to the differential activation of the JNK signalling pathway in the cells [129].

Bittman with Arthur reported the synthesis of a GAEL, 1-O-hexadecyl-2-O-methyl-3-O-(2'-amino-2'-deoxy-beta-D-glucopyranosyl)-*sn*-glycerol (Gln) that had comparable or better cytotoxic effects against human epithelial cancer cell lines than ET-18-OCH_3 [130]. They demonstrated the mechanism by which glycosylated anti-tumour ether lipids (GAELs) exhibit anti-cancer activity. Gln activates autophagy but does not kill cells by this mechanism or through apoptosis [131]. Later studies demonstrated that Gln neutralises lysosomal pH, increases autolysosome formation and promotes lysosomal membrane permeabilization with

the release of hydrolases into the cytoplasm, which then promote cell death [132]. Gln was also shown to diffuse across cell membranes into lysosomes, where protonation of the amine results in the formation of vacuoles [133]. Such studies highlighted the distinct killing mechanisms perpetrated by phospholipids and sphingolipids.

The collaboration also demonstrated that ether-linked glucosyl diglycerides represent a distinct group of anti-tumour ether lipids from alkylphosphocholines and alkyllysophospholipids. The studies demonstrated that 4-O-hexadecyl-3(S)-O-methoxybutanephosphonate was more active than 1-O-octadecyl-2-O-methyl-glycero-3-phosphocholine in epithelial cancer cell lines. Since LPA was non-mitogenic in all the cell lines except the neuroblastoma line SK-N-SH, it is unlikely that the inhibition of cell proliferation by 4-O-hexadecyl-3(S)-O-methoxybutanephosphonate is due to antagonism of the action of LPA at its GPCRs [130].

These studies and others described here clearly defined different classes of ether lipids as having unique biological activities that could be distinguished from other phospholipids. This was further extended to consider glycosylated analogues of ET-18-OCH3. Stereospecific glycosylation was achieved with analogues differing from ET-18-OCH3 by the replacement of the *sn*-3-phosphocholine residue with either beta- and alpha-2-deoxy-D-arabino-hexopyranosyl, alpha-D-mannopyranosyl or 2-O-methyl-beta-D-glucopyranosyl, and 2-O-methyl-alpha-D-mannopyranosyl. The value of these studies was exemplified by the finding that 1-O-Hexadecyl-2-O-methyl-3-O-(2'-deoxy-beta-D-arabino-hexopyranosyl)-*sn*-glycerol was more effective than ET-18-OCH3 in inhibiting the growth of cancer cells [134].

Bittman and Arthur also studied the effects of D-myo-inositol 4-(hexadecyloxy)-3(S)-methoxybutanephosphonate (C4-PI), an isosteric phosphonate analogue of

phosphatidylinositol. C4-PI was shown to inhibit PI-phospholipase C-beta (PLC-β) while activating PI-PLCγ and PI-PLCδ at low concentrations and inhibiting these enzymes at higher concentrations. C4-PI had little effect on PI4-kinase activity and blocked the proliferation of MCF-7 and MDA-MB-468 cell lines [135].

Synopsis

In the present review we compiled a partial account of the contributions made by Robert Bittman to select areas of lipid research. His legacy will live on not only in over 340 publications he authored but also in the concepts that evolved from research enabled by the compounds he made available to lipid researchers. The compounds he synthesized not only had a profound impact on the pursuit of the biological function of several important lipid mediators but also accelerated research toward their therapeutic exploitation.

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Figure Legends

Fig. 1 Photograph of Robert Bittman (1942-2014).