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Review

# 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.



**Contents**



## 1. 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.

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**Fig. 1.** Photograph of Robert Bittman (1942–2014).

## 2. 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.

## 3. Sphingolipid signalling

In the mid 1990s 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.

### 3.1. 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 metabolised 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