

C1 domain-targeted isophthalates as protein kinase C modulators: Structure-based design, structure-activity relationships and biological activities

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Abstract

Protein kinase C (PKC) is a serine/threonine kinase belonging to the AGC family. PKC isoenzymes are activated by phospholipid-derived second messengers, transmit their signal by phosphorylating specific substrates and play a pivotal role in the regulation of various cell functions, including metabolism, growth, differentiation and apoptosis. Therefore, they represent an interesting molecular target for the treatment of several diseases, such as cancer and Alzheimer's disease. Adopting a structure-based approach on the crystal structure of the PKC δ C1B domain, our team has developed isophthalic acid derivatives that are able to modify PKC functions by binding to the C1 domain of the enzyme. The bis[3-(trifluoromethyl)benzyl] 5-(hydroxymethyl)isophthalate (HMI-1a3) and the bis(1-ethylpentyl) 5-(hydroxymethyl)isophthalate (HMI-1b11) were selected among a set of compounds for further studies due to their high affinity for the C1 domains of PKC α and δ . HMI-1a3 showed marked antiproliferative activity in HeLa cells while HMI-1b11 induced differentiation and supported neurite growth in SH-SY5Y cells. Our aim in the future is to improve the selectivity and potency of isophthalate derivatives, to clarify their mechanism of action in cellular environment, and to assess their efficacy in cell-based and *in vivo* disease models. HMI-1a3 has already been selected for a further project and redesigned to function as a probe immobilized on an affinity chromatography column. It will be used to identify cellular target proteins from cell lysates, providing new insights into the mechanism of action of HMI-1a3.

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Abbreviations: [³H]PDBu, [20-³H]phorbol-12,13-dibutyrate; AD, Alzheimer's disease; aPKC, atypical PKC; cPKC, classical/conventional PKC; DAG, 1,2-diacylglycerol; DGK, DAG kinase; ERK, extracellular signal-regulated kinase; MRCK, myotonic dystrophy kinase-related Cdc42-binding kinase; nPKC, novel PKC; PMA, phorbol 12-myristate-13-acetate; PKC, protein kinase C; PKD, protein kinase D; RasGRP, guanyl nucleotide-releasing protein; SAR, structure-activity relationship

Protein kinase C: general concepts

Protein kinase C (PKC) is a family of serine/threonine kinases that belongs to the group of AGC kinases (named AGC after protein kinases A, G and C) [1]. PKC isoforms are ubiquitously expressed and activated by phospholipid-derived second messengers. They phosphorylate specific substrate proteins and play a key role in regulation of a considerable range of cellular responses including cell growth, differentiation, metabolism, and apoptosis [2]. There is evidence that aberrant PKC activity is involved in several human diseases such as diabetes, cardiovascular diseases, cancer, Parkinson's disease and Alzheimer's disease (AD) [3]. Therefore, PKC proves to be a suitable molecular target for drug development and new therapeutic approaches.

The PKC family consists of ten isoenzymes grouped in three classes based on differences in their regulatory domain arrangement and cofactor requirements (reviewed in [4,5]). All of the isoenzymes require the anionic phospholipid Ptd-L-Ser for their activation. The classical/conventional PKCs (cPKCs; isoforms α , β I, β II and γ) require also Ca²⁺ ions and 1,2-diacylglycerol (DAG) whereas only DAG is necessary to activate the novel PKCs (nPKCs; isoforms δ , ϵ , η and θ). Conversely, the activation of the atypical PKCs (aPKCs; isoforms ζ and ι/λ) is neither DAG nor Ca²⁺ dependent. DAG is one of the lipid-derived second messengers, and it is generated in response to hydrolysis of membrane PtdIns(4,5)P₂ by phospholipase C. It was initially found to activate PKC and later on shown to interact selectively with a specialized recognition motif, the so-called "typical" C1 domain [6-8].

The C1 domains are zinc finger structures consisting of a sequence of approximately 50 amino acids [4]. In the regulatory region of cPKCs and nPKCs the C1 domains are DAG-sensitive (thus called typical) and arranged in tandem: C1a and C1b [8]. Conversely, the regulatory region of aPKCs contains a single C1 domain which has no affinity for DAG

(and is therefore called atypical). Besides the PKC family, other six families of proteins contain a DAG-responsive C1 domain: the protein kinase D (PKD), the chimaerins, the Ras guanyl nucleotide-releasing proteins (RasGRPs), the DAG kinases (DGKs) β and γ , myotonic dystrophy kinase-related Cdc42-binding kinases (MRCKs), and the Munc-13 scaffolding proteins [9-11]. Atypical C1 domains are found in numerous protein families [9].

Vast majority of the currently available small molecule drugs acting on protein kinases are inhibitors of kinase activity targeting the ATP-binding site of the kinases in question. Since the ATP binding site is highly conserved among more than 500 kinases in the human genome [12], developing small molecules targeted to the C1 domain of PKC is advantageous considering the much smaller number of C1 domain-containing proteins. Moreover, targeting the regulatory domain enables discovery of PKC agonists, and the C1 domain is thus considered an attractive drug target [13]. Indeed, the first C1 domain ligand ingenol-3-angelate (ingenol mebutate) was approved in 2012 for the topical treatment of actinic keratosis [14,15]. Other natural and semi-synthetic compounds that bind to the C1 domain of PKC are e.g. phorbol esters and bryostatins, which are, however, highly complex and laborious to synthesize (reviewed in [16]). In contrast, the syntheses of rationally designed and highly potent DAG-lactones and benzolactams require significantly fewer reaction steps.

In this short review we describe the design, structure-activity relationship (SAR) and biological activity of a novel class of C1 domain ligands, i.e. small hydrophobic isophthalic acid derivatives.

Design and structure-activity relationship of novel C1 domain-targeted compounds

As a starting point in the development of novel ligands that bind to the C1 domain of PKC we chose to focus on commercially available compounds that are easy to derivatize and that could function as templates in SAR studies. Keeping this in mind, we performed docking studies on the crystal structure of the PKC δ C1b domain complexed with phorbol-13-*O*-acetate [17]. We discovered diethyl 5-(hydroxymethyl)isophthalate as an interesting template [18], since it contains two of the pharmacophores found in the complexed phorbol 13-*O*-acetate and these hydroxyl and carbonyl moieties show the same interatomic distance as found in phorbol (**Figure 1A**) [19]. In addition, because of its two carbonyl groups and C_2 -symmetry, it could bind to the C1 domain similarly to DAG-lactones [20]. Furthermore, diethyl 5-(hydroxymethyl)isophthalate is commercially available and easy to derivatize: novel derivatives could be synthesized in only four steps [18], in contrast to highly complex natural, semi-synthetic or synthetic compounds that bind to the C1 domain of PKC and are usually laborious to synthesize.

The hydroxyl group of the docked diethyl 5-(hydroxymethyl)isophthalate is hydrogen bonded in a similar fashion as the phorbol ester to the bottom of the binding groove of the C1 domain (**Figure 1A**). In addition, one of the carbonyl groups of the template forms a hydrogen bond with a backbone amide proton as the C3 carbonyl of phorbol-13-*O*-acetate. Even though the second carbonyl group of the template did not show any interaction to the protein in this model, possible interactions to charged lipid head groups or to a bridging water molecule between the carbonyl and a backbone carbonyl of the protein might produce binding affinity [21]. Also hydrophobic interactions of ligands are important for high affinity binding to the protein. Hydrophobic side chains of e.g. phorbol esters, DAGs and benzolactams, and derivatives of them are thought to interact with either the hydrophobic amino acids surrounding the ligand binding cleft or with the hydrophobic moiety in the lipid bilayer [17,22,23]. This seemed also to be the case with the dialkyl 5-(hydroxymethyl)isophthalates; while preliminary binding affinity studies of diethyl 5-(hydroxymethyl)isophthalate to the PKC C1 domain did not show any binding, the more hydrophobic dipentyl 5-(hydroxymethyl)isophthalate displaced [3 H]PDBu from PKC (unpublished results).

Encouraged by these results, we set up a synthesis strategy to investigate the pivotal moieties of the template for binding affinity as well as the hydrophobicity needed in the ester side chains. Subsequently, a set of compounds showing different length and shape of the ester groups was synthesized [18]. These included compounds with linear and branched aliphatic as well as aromatic side chains but also ester groups having more hydrophilic moieties. Most of these compounds could be synthesized in only four steps starting from diethyl 5-(hydroxymethyl)isophthalate and needed only a single chromatographic purification step.

Subsequent binding affinity experiments of these compounds showed that dialkyl (3-hydroxymethyl)isophthalates from secondary alcohols with 6-7 carbons, e.g. compounds **HMI-1b10** and **-1b11**, could best displace [3 H]PDBu from PKC (**Figure 1B**). Compounds having a more hydrophilic ester group were not able to displace [3 H]PDBu from PKC. This result is in accordance to the hypothesis that when PKC is activated the ligand binding pocket of the C1 domain is buried into the cell membrane, and hence a hydrophilic group in the ligand would have a negative effect on the binding affinity [17].

In addition to this small library of compounds, we designed and synthesized another set of ligands to study the pharmacophores of the template. Modifications to the template included the conversion of one or both of the ester groups into amide or inverse amide groups, removing one of the ester groups, and changing the 5-hydroxymethyl group of the template to other groups such as methyl, nitro, and amino. [3 H]PDBu displacement experiments of these compounds showed that i) the 5-hydroxymethyl group of the template is needed for binding activity, ii) amide groups were not

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tolerated, iii) inverse amide groups were not tolerated but with a combination of longer and more hydrophobic amide group some binding could be obtained, iv) only one ester group was not enough to produce binding affinity but v) if one of the ester groups was removed and instead a hydrophobic group was inserted next to the other ester group some activity could be seen [18] (partly unpublished results).

Based on the initial screening, we selected three isophthalate derivatives (**HMI-1a3**, **HMI-1b10** and **HMI-1b11**) for more detailed analysis of binding affinity to the C1 domains of PKC α and PKC δ . The three isophthalates displaced [³H]PDBu from PKC α and PKC δ with K_i values in the range of 205–915 nM [18]. No significant differences in affinities between the ligands were detected, but all of them exhibited modestly higher affinities to PKC α than to PKC δ . The K_i values of isophthalates are higher than those of phorbol esters, bryostatins or the best DAG lactones, all of which replace [³H]PDBu with low nanomolar or subnanomolar affinities (for a review, see [16]). Modification of the isophthalate structure would therefore be needed to achieve affinities comparable to the more potent C1 domain ligands.

Since PKCs are not the only proteins with a C1 domain responsive to phorbol esters and DAG, the C1 domain ligands have other targets in the cellular environment as well. Binding to non-PKC DAG effectors has been reported for most classes of C1 domain ligands (see [16]). For the isophthalates, the binding of 15 active and 4 inactive isophthalate derivatives to recombinant full-length human β 2-chimaerin from Sf9 cell lysates was studied to confirm the SAR model created based on the binding experiments with PKC. The results followed the SAR model generated based on binding experiments with PKC (Talman V, unpublished results). The binding properties of **HMI-1a3** were further characterized and it was found to displace [³H]PDBu from the C1 domain of MRCK α produced in *E. coli* and from commercial recombinant PKD1 in a concentration-dependent manner in a similar concentration range as with PKCs (**Table 1**) [24]. Since the K_i values have not been determined for the “non-PKC” DAG effectors, the affinities cannot be directly compared. However, similar IC₅₀ values suggest that there are no major differences in their affinity to these proteins, and the isophthalates can thus be considered non-selective among the DAG-responsive C1 domains. Binding of isophthalates to C1 domains of RasGRPs, DGKs and Munc13s has not been investigated; however, due to high sequence and structural similarity within the C1 domains, it is presumable that the same SAR model holds true with these DAG effectors as well.

Effects on PKC-mediated ERK signalling

Most of the C1 domain ligands reported in the literature function as activators of PKC, and this appears to be the case with isophthalates as well, based on their ability to induce PKC-dependent extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in cells [18,25]. However, the effects of isophthalates on ERK1/2 signalling seem to differ substantially depending on the cell line. In HeLa cervical carcinoma cells **HMI-1a3** induced PKC-dependent ERK1/2 phosphorylation, while **HMI-1b11** had no effect on its own but instead it inhibited phorbol 12-myristate-13-acetate (PMA)-induced phosphorylation of ERK1/2 [18]. However, in SH-SY5Y neuroblastoma cells both **HMI-1a3** and **HMI-1b11** induced prominent increases in the amount of phosphorylated ERK1/2 [25]. A pretreatment with the pan-PKC inhibitor Gö6983 diminished the effects confirming the PKC-dependency of isophthalate-induced activation of ERK1/2 signalling also in the neuroblastoma cell line. The mechanism behind the observed divergence between the cell lines has yet to be clarified. However, a possible explanation may lie in different patterns of PKC isoform expression, although other DAG effectors may also contribute.

Effects on HeLa cell viability, proliferation and morphology

As cancer is considered the best-validated therapeutic indication for compounds targeting PKC and other DAG effectors, many groups of C1 domain ligands have been tested for their ability to inhibit cancer cell proliferation or induce apoptosis (for reviews, see [16,26]). The effects of isophthalates on cell viability and proliferation were investigated using HeLa cervical carcinoma cells [27]. Many of the isophthalate derivatives with high affinity to the C1 domains exhibited an antiproliferative effect and induced non-necrotic cell death at low micromolar concentrations, while their inactive derivatives with poor affinity to the C1 domain had no cytotoxic or antiproliferative effect, suggesting a C1 domain-dependent effect. **HMI-1a3** was the most potent antiproliferative isophthalate: it inhibited HeLa cell proliferation with an IC₅₀ of 2.0 μ M. By contrast, **HMI-1b11** had almost no effect on HeLa cell proliferation or viability. However, the mechanism of the **HMI-1a3**-induced antiproliferative effect remains unclear, since it was not influenced by inhibition or activation of PKC, RNAi-mediated knockdown of PKC α or PKC δ , inhibition of PKD or MRCK; or inhibition of ERK1/2 signalling [24]. It therefore seems probable that the antiproliferative and cytotoxic effects of **HMI-1a3** are not mediated by a single C1 domain-containing protein, but rather through concurrent modulation of several DAG effectors.

In HeLa cells, the antiproliferative isophthalate derivatives induce a distinct change in cell phenotype that is characterized by cell elongation, emergence of cellular protrusions and diminished cell spreading (**Figure 2**) [24,27]. The change in cell phenotype occurs as a result of major reorganization of actin cytoskeleton, including loss of actin stress fibers

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and focal adhesions [24]. Although the exact mechanisms are unclear, the disruption of the contractile stress fibre network may increase the assembly of protrusive arrays of actin filaments thus inducing cellular protrusions or decrease cell contractility resulting in cell elongation [28]. There is some evidence to support a role for MRCK in mediating the **HMI-1a3**-induced morphological change [24], consistent with previous studies showing a crucial role for MRCK in cytoskeletal reorganization [29-31]. However, other mechanisms and DAG effectors most likely also contribute to the observed cell phenotype: PKC, PKD and the chimaerins are also known to participate in the regulation cytoskeleton organization [10,32,33].

Effects on SH-SY5Y cell viability, proliferation and morphology

PKC activity has been shown to control learning and memory through several mechanisms, and therefore PKC has been classified as one of the cognitive kinases [34]. In addition to its beneficial effects on cognition, PKC inhibits several processes linked to AD pathophysiology, such as amyloid precursor protein metabolism and tau hyperphosphorylation [35,36]. Neuroblastoma cell cultures have been widely used as *in vitro* models for neuronal plasticity and differentiation [37], and PKC activation by phorbol esters induces differentiation of neuroblastoma cells *in vitro* (e.g. [38]). In a study performed with SH-SY5Y neuroblastoma cells, we found that the isophthalate **HMI-1b11** inhibits cell proliferation and supports neurite growth without inducing toxicity (**Figure 2**) [25]. This was accompanied with translocation of PKC α into the cytoskeletal fraction and cPKC-dependent upregulation of GAP-43, which is a central mediator of neuronal plasticity and differentiation [39]. **HMI-1a3** on the other hand also inhibited cell proliferation and supported the growth of neurite-like processes, but it also induced toxicity at the highest (20 μ M) concentration [25]. The isophthalates, and especially **HMI-1b11**, thus provide a scaffold for the development of agents that could both inhibit the progression of Alzheimer's disease and improve cognitive functions by enhancing the development of neuronal networks.

Conclusions and future perspectives

An emerging role for PKC-mediated signalling lies in their non-catalytic functions, which are predominantly attributed to protein-protein interactions through the regulatory domains. One of such examples is the induction on neurite outgrowth in neuroblastoma cells, mediated by the C1b domain of PKC ϵ [40,41]. Another example is the key role of PKC α protein but not kinase activity in glioma cell survival and proliferation [42]. These examples, together with the recognized potential of other DAG effectors as drug targets [26] illustrate the potential of the C1 domain as a target in drug development [13]. This is supported by our results with the isophthalate derivatives, as well as studies by other groups with different C1 domain ligands. The C1 domain-containing proteins however regulate numerous interconnecting and convergent signal transduction pathways, which contribute to cell survival, proliferation, apoptosis and morphology [10,43]. This may be an advantage for example in cancer drug development, as targeting several mitogenic signalling routes simultaneously at different levels would decrease the risk of drug resistance. However, the cross-talk between the DAG-mediated signalling routes may hinder rational drug design and determination of exact mechanism of action for the compounds.

Given the interesting results obtained so far with isophthalates **HMI-1a3** and **HMI-1b11**, the simple dialkyl 5-(hydroxymehtyl)isophthalate derivatives delineate a novel promising scaffold for PKC and C1 domain-targeted drug discovery and development. **HMI-1a3** represents a potential lead compound for cancer-related drug discovery due to its marked antiproliferative effects. Because of its non-cytotoxic profile and capability to induce differentiation and support neurite growth in neuroblastoma cells, **HMI-1b11**, on the other hand, may have potential in drug development related to neurodegenerative diseases such as AD. The future work on isophthalates will concentrate on improving the selectivity and potency of isophthalate derivatives on clarifying their mechanisms of action in the above-summarized biological responses, and on assessing their efficacy in cell-based and *in vivo* disease models. In order to investigate the mechanisms of **HMI-1a3**-induced inhibition of cell proliferation, we have designed a derivative of it to be used as a probe immobilized on an affinity chromatography column through an appropriate linker. The new compound will be used as a research tool to fish out its cellular binding partners.

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Tables

Table 1 | Binding affinity of HMI-1a3 to different DAG effectors. The data was analysed with GraphPad Prism 4 with one-site competition equation using mean and SEM values from 3-4 independent experiments. *n.d.*, not determined.

DAG effector	IC ₅₀ (μM)	K _i (μM)	Receptor protein	Purification	Method	Reference
β2-chimaerin	1.2	<i>n.d.</i>	recombinant human β2-chimaerin	crude Sf9 lysate (not purified)	filtr.	[24]
PKD1	6.3	<i>n.d.</i>	recombinant 6His-tagged human PKD1	commercially purified (72% pure)	centrif.	[24]
MRCKα	1.3	<i>n.d.</i>	recombinant GST-tagged C1 domain of human MRCKα	affinity purified (≈80% pure)	filtr.	[24]
PKCα	1.8	0.21	recombinant human PKCα	commercially purified (95% pure)	centrif.	[18]
PKCδ	2.3	0.59	recombinant human PKCδ	commercially purified (95% pure)	centrif.	[18]

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Figures

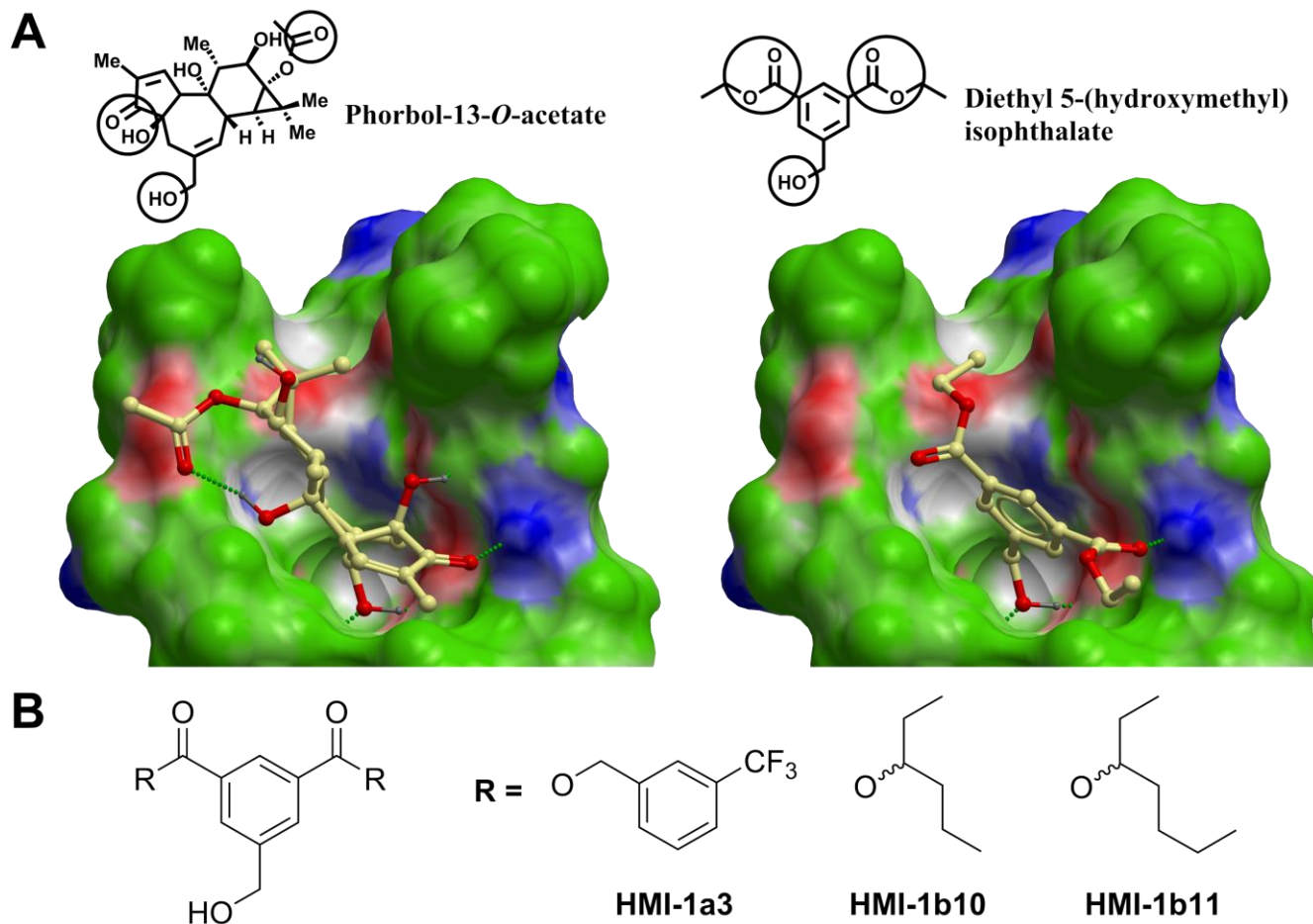


Figure 1 | Design and molecular modelling of dialkyl (3-hydroxymethyl)isophthalates targeted to the C1b domain of PKC δ . **A** Left: Phorbol 13-*O*-acetate in the crystal structure of the C1b domain of PKC δ (PubID: 1PTR) [17]. Right: Diethyl (5-hydroxymethyl) isophthalate docked into the crystal structure of C1b domain of PKC δ . Functional groups of the compounds showing possible interactions to the protein are denoted with circles and hydrogen bonds are denoted with green spheres. The figures were created using ICM-Browser (v.3.7., Molsoft); **B**) The structures of compounds **HMI-1a3**, **-1b10** and **-1b11**.

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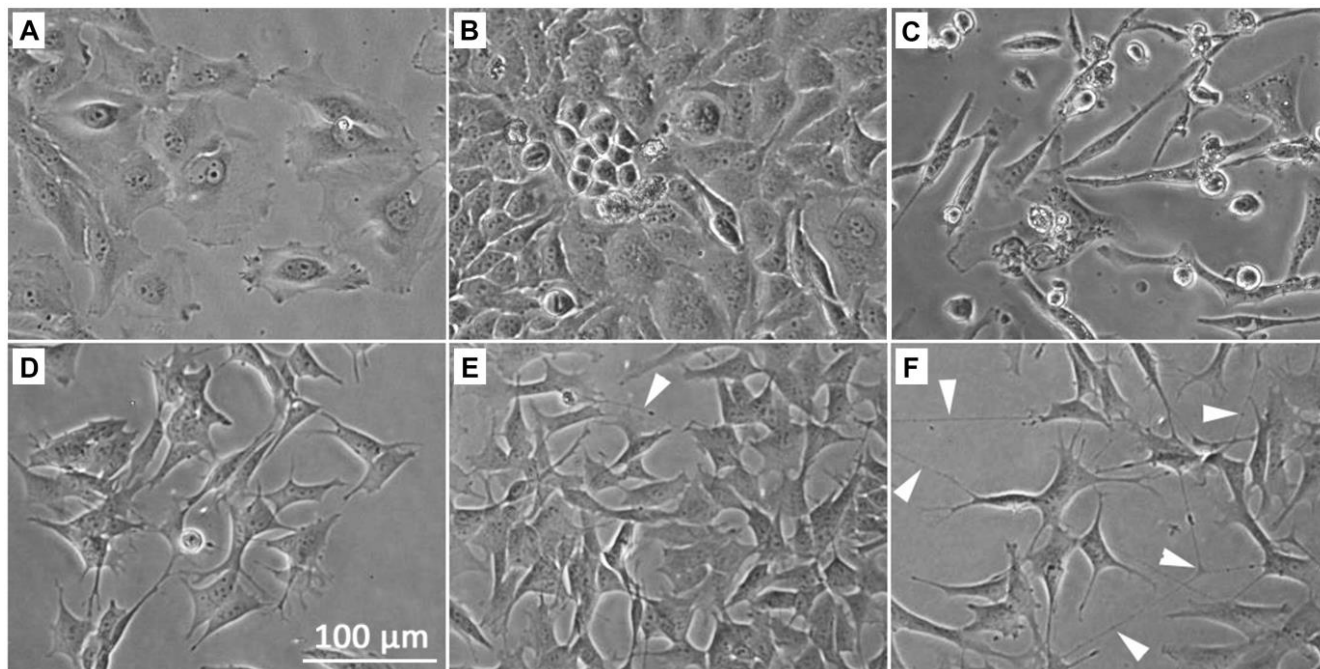


Figure 2 | Effects of isophthalates on proliferation and morphology of HeLa cells and SH-SY5Y cells. Cells were exposed to test compounds, imaged with Cell-IQ® for three days and images were captured at 1-hour intervals (see [25, 27] for methods and quantification results). **HMI-1a3** inhibits HeLa cell proliferation and induces cell elongation (A-C): **A)** Untreated HeLa cells at 1 h; **B)** untreated HeLa cells at 72 h; **C)** HeLa cells exposed to 20 μM **HMI-1a3** at 72 h. **HMI-1b11** inhibits SH-SY5Y cells proliferation and induces neurite growth (D-F): **D)** Untreated SH-SY5Y cells at 1 h; **E)** SH-SY5Y cells exposed to vehicle (0.1 % DMSO) at 70 h; **F)** SH-SY5Y cells exposed to 10 μM **HMI-1b11** at 70 h. Arrowheads indicate examples of neurites.