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Analysis of two matrix metalloproteinase inhibitors and their metabolites for induction of phospholipidosis in rat and human hepatocytes

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Abstract

ABT-770 [(*S*)-*N*-[1-[[4′-trifluoromethoxy-[1,1′-biphenyl]-4-yl]oxy]methyl-2-(4,4-dimethyl-2,5-dioxo-1-imidazolidinyl)ethyl]-*N*-hydroxyformamide], a matrix metalloproteinase inhibitor (MMPI), produced generalized phospholipidosis in rats. Phospholipid accumulation was accompanied by retention of drug-related material and was associated with increased mortality. Generation of a successful drug candidate depended upon understanding the cause of the phospholipidosis and redesigning the chemical structure accordingly. ABT-770 and other MMPIs, plus several metabolites of each, were assayed for their ability to induce phospholipidosis in primary cultured rat and human hepatocytes. Phospholipid accumulation was detected by following the incorporation of a fluorescent phospholipid analogue into intracytoplasmic inclusion bodies characteristic of phospholipid storage disorders. At 24 and 48 hr, none of the parent compounds induced phospholipidosis *in vitro* in rat or human hepatocytes. Phospholipidosis was associated primarily with an amine metabolite of ABT-770. The amine metabolite of another MMPI, ABT-518 ([*S*-(*R**,*R**)]-*N*-[1-(2,2-dimethyl-1,3-dioxol-4-yl)-2-[[4-[4-(trifluoromethoxy)-phenoxy]phenyl]sulfonyl]ethyl]-*N*-hydroxyformamide), produced little phospholipidosis in rat and human hepatocytes even at concentrations up to 100 μM. The presence or absence of phospholipidosis in the *in vitro* assay correlated well with ultrastructural findings and drug accumulation in rat tissues. ABT-770, which produced phospholipidosis associated with its amine metabolite *in vitro* and *in vivo*, also generated a higher tissue to plasma distribution of metabolites particularly in tissues where phospholipidosis was observed. ABT-518 and its amine metabolite, however, produced low tissue to plasma ratios and induced little to no phospholipidosis *in vitro* or *in vivo*. These results demonstrate that the phospholipidosis observed for ABT-770 could be attributed to a cationic metabolite, and that altering the properties of such a metabolite, by modification of the parent compound, alleviated the disorder.

Keywords: Phospholipidosis; Rat; Human; Hepatocytes; Metabolism; Matrix metalloproteinase (MMP)

1. Introduction

MMPs are zinc-dependent proteolytic enzymes capable of degrading components of the extracellular matrix and basement membrane [1]. Deregulated expression of MMPs is associated with a variety of pathological conditions including rheumatoid arthritis and cancer progression [1–4]. Several mechanisms are thought to be involved in the role of MMPs in cancer progression. MMPs mediate invasion and metastasis through degradation of the extracellular ma-

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Abbreviations: MMP, matrix metalloproteinase; MMPI, matrix metalloproteinase inhibitor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; and MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DMSO, dimethylsulfoxide; HPLC, high performance liquid chromatography.

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trix and basement membrane, allowing tumor cells to invade surrounding tissues and enter the blood stream to travel to distant sites [2,3,5]. In addition, MMPs appear to be involved in primary tumor growth, at least in part, by facilitating angiogenesis [6,7]. Gelatinases, both gelatinase A (MMP-2) and gelatinase B (MMP-9), are considered to be particularly good targets for anti-cancer drugs since both enzymes degrade type IV collagen, a major component of the basement membrane, and since expression of both correlates with an aggressive, advanced, invasive, or metastatic tumor phenotype [4,5,8,9]. Knockout mice for either gelatinase have reduced tumor burden, decreased metastasis, as well as reduced tumor angiogenesis without developmental abnormalities[1] [10,11].

Abbott's original lead MMPI compound, ABT-770 [(*S*)-*N*-[1-[[4′-trifluoromethoxy-[1,1′-biphenyl]-4-yloxy]methyl-2-(4,4-dimethyl-2,5-dioxo-1-imidazolidinyl)ethyl]-*N*-hydroxyformamide], exhibited high potency against gelatinase A (MMP-2, IC_{50} = 3.7 nM), lesser potency against gelatinase B (MMP-9, IC_{50} = 120 nM), and only marginal activity against fibroblast collagenase (MMP-1, IC_{50} = 4600 nM) and matrilysin (MMP-7, IC_{50} > 10,000 nM) [12]. Despite its MMP selectivity, ABT-770 was efficacious in several cancer animal models. However, ABT-770 produced several metabolites *in vivo*, some of which exhibited high tissue to plasma distribution ratios in rats. In addition, several toxic changes were observed in rats treated at 100 mg base/kg/day for 28 days, including altered liver enzymes, decreased body weight gain, leukopenia, pulmonary inflammatory foci, multi-organ phospholipidosis, and death. Multi-organ phospholipidosis was observed even at 10 mg/kg/day.

Phospholipidosis is a lipid storage disorder in which phospholipids accumulate in endosomes and then lysosomes as lamellar bodies [13–17]. Cationic amphiphilic drugs can often induce this phenomenon *in vivo.* There are a variety of factors that determine whether or not these drugs induce phospholipidosis. These factors include species differences, tissue affinity, metabolites and the rate of metabolism, pharmacokinetics of the drug and metabolites, and biochemical and structural variations between drugs [17]. In addition, the types of phospholipids that accumulate can vary from one drug to another and can include phosphatidylcholine, bis(monoacylglycero)phosphate, acylphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine [16,18].

Phospholipidosis is associated with increased drug accumulation in affected tissues such as lung and liver. In addition, it is often accompanied by various associated or coincidental toxicities [17,19,20]. While phospholipidosis by itself does not constitute toxicity, it is usually predictive of drug or metabolite accumulation in affected tissues [21] and should be heeded as a warning to investigate possible associated toxicities. In addition, the reversibility of phospholipidosis and drug accumulation within a reasonable time after treatment cessation is a critical parameter for determining the safety margin of compounds that produce this effect.

The current study was undertaken to better understand the mechanism for induction of phospholipidosis by ABT-770 and to identify an MMPI that would not induce this phenomenon *in vivo.* A fluorescent analog of phosphatidylethanolamine was used to determine the ability of ABT-770 and structurally related MMPIs, as well as metabolites of each, to produce phospholipidosis in an *in vitro* assay in rat and human hepatocytes. This assay was predictive for phospholipidosis in previous studies [16,19].

2. Materials and methods

2.1. Synthesis of MMPIs

The synthesis of ABT-770 and several related compounds is described in detail elsewhere (ABT-770 is compound 11) [12]. ABT-518 ([*S*-(*R**,*R**)]-*N*-[1-(2,2-dimethyl-1,3-dioxol-4-yl)-2-[[4-[4-(trifluoromethoxy]-phenoxy]phenyl] sulfonyl]ethyl]-*N*-hydroxyformamide) was prepared in a 5-step synthetic sequence starting with the addition of the anion of 4-(4′-trifluoromethoxyphenoxy)phenyl methyl sulfone to methyl (4*R*)-2,2-dimethyl-1,3-dioxolane-4-carboxylate, followed by reduction with sodium borohydride, alcohol elimination via a mesylate derivative, Michael addition of hydroxylamine, and, finally, formylation with trifluoroethyl formate [22].

2.2. Cell culture

Hepatocytes were isolated from male Sprague–Dawley rats and cultured as previously described [23] as were human hepatocytes from the livers of four Caucasian males ages 34 to 61 [24]. The cells were plated in phenol-red free modified DMEM (Gibco BRL) supplemented with 10% FBS (Gibco BRL). For phospholipidosis assays, hepatocytes were plated at a density of 75,000 rat cells or 200,000 human cells per chamber of a four-chamber coverglass slide (Lab-Tek No. 136420; VWR Scientific Products) coated with rat tail type 1 collagen (Sigma) at 5 $\mu g/cm^2$, and then were incubated overnight at 37° with 5% CO_2. For cytotoxicity assays, rat and human hepatocytes were plated at a density of 30,000 cells/well of a collagen-coated 96-well plate and then were incubated overnight at 37° with 5% CO_2.

2.3. Cytotoxicity assay using MTT

Cytotoxicity in rat hepatocytes was determined by the MTT assay. MTT (0.5 mg/mL; Sigma) in PBS was added to

[1] Bergers G, Coussens L, Werb Z, and Hanahan D. Tumor angiogenesis: mechanisms and therapeutic trials in transgenic mice. In: AACR Special Conference, Cancer Biology and the Mutant Mouse, Keystone, CO, January 1999.

treated monolayers of rat hepatocytes, and then the cells were incubated at 37° for 2 hr. MTT reagent was removed, and DMSO (Sigma) was added while shaking for 10 min. Decreases in mitochondrial reducing activity relative to control were determined by reading the absorbance at 590 nm, using a Spectromax Plus spectrophotometer (Molecular Devices).

2.4. Phospholipidosis assay

Phospholipidosis assays were carried out essentially as described previously [16,19]. Twenty-four hours after plating, lipid vesicles were prepared using NBD-PE (Molecular Probes). NBD-PE was resuspended in ethanol and diluted in DMEM/10% FBS at 50 μM with a final ethanol concentration of 0.46%. The mixture was placed into a sonicating water bath for 30 min and then filtered through a 0.22 μM filter. Stock concentrations of test compounds at 20 mM were prepared in DMSO and then diluted into the DMEM/10% FBS/NBD-PE medium with a final DMSO concentration of 1%. Cells, both rat and human, were treated with two or more concentrations of each parent compound or metabolite at subtoxic concentrations including 12.5 and 50 μM for all compounds tested as well as higher concentrations such as 75 μM for the amine of ABT-770 and 100 μM for the amine of ABT-518. After 24 hr, cells were rinsed with DMEM/10% FBS and then were overlaid with the same, incubated for 30 min at 37° with 5% CO_2, and analyzed using a Bio-Rad MRC 1024 confocal laser scanning microscope (Bio-Rad Laboratories). The gain was set to maximize signal to noise for the positive control and then was kept consistent throughout the analysis. Hepatocytes were imaged using a 488 nm laser line to excite the fluorophore and a 540 nm band pass emission filter. Cells were rated for phospholipidosis as –, +, ++, +++, or ++++ with cells rated ++++ exhibiting the highest levels of phospholipidosis and cells rated as – exhibiting no phospholipidosis. Cells treated with amiodarone (Sigma) at 12.5 and 50 μM were used as a positive control (++++) [14,17] and vehicle-treated (DMSO) and untreated cells as negative controls (–). [Since dead cells (which are always present in primary cultures) are uniformly stained by the lipid probe, the fluorescent inclusions cannot be measured by fluorimetry.]

2.5. In vivo *experiments*

Male Sprague–Dawley rats, 6- to 9-weeks-old and weighing approximately 200 g, were obtained from Charles River Laboratories. Animals were allowed access to food and water for the duration of the study. [^{14}C]ABT-770 (radiolabeled on the biphenyl ring) was administered orally by gavage at a dose of 100 mg free base/kg/day, corresponding to 25 μCi/kg/day and 5 mL/kg/day for up to 18 days. Two additional rats were dosed for 18 days prior to a 7-day drug-free recovery period. Two control rats were administered an appropriate amount of dose vehicle. At selected time points (days 1, 3, 5, 7, 10, 14, and 18, each at 24 hr post-dose and day 24, at 7 days post-dose), two rats were anesthetized with carbon dioxide and exsanguinated by cardiac puncture; selected tissues, including lung and liver, were collected and flash frozen in liquid nitrogen. Blood and tissue were analyzed for determination of drug and analyte concentrations as described below.

In a second study, male and female Sprague–Dawley rats were treated orally with non-radioactive ABT-770 or ABT-518 at 100 mg base/kg/day for 14 or 28 days with a satellite group treated for 28 days with a 1-week recovery. Animals were euthanized under CO_2 (for metabolism studies) or halothane (for pathology and electron microscopy) anesthesia, and lung and liver were again collected at necropsy and treated as described for tissue concentration determinations and electron microscopy.

2.6. Determination of tissue concentrations

The liver and lung were weighed and homogenized in 2 vol. of PBS (i.e. 2 mL PBS/g tissue). Aliquots of tissue homogenates were burned in a Tri-Carb® (Packard Instrument Co.) model 307 Sample Oxidizer. The resultant $^{14}CO_2$ was trapped in Carbosorb® (Packard) and assayed by liquid scintillation counting to calculate the total microgram [^{14}C] drug equivalents. There was insufficient radioactivity for the estimation of metabolite profiles in tissue and plasma samples by HPLC with radioflow detection. Therefore, 250 μL of each plasma or homogenate sample (tissue) was mixed with 500 μL of acetonitrile:methanol (1:1, v/v) and kept on ice for approximately 30 min. Precipitated protein was pelleted by centrifugation at approximately 10,000 *g* at 4° for 10 min. Aliquots (50 μL) of supernatant were transferred to HPLC vials, and 25 μL was analyzed by HPLC as described below. Concentrations of individual analytes were estimated by dividing peak areas of individual drug-dependent analytes by the sum of the area of all drug-dependent peaks and multiplying this fraction by the microgram [^{14}C] equivalents per milliliter of the sample. The estimated mean (N = 17) extraction recoveries of total radioactivity from plasma, liver, and lung were 100.9, 86.4, and 82.9%, respectively.

For the second, nonradioactive study, the same conditions were used except that extracted homogenate was centrifuged, and the supernatant was analyzed by HPLC. For ABT-770 (radioactive and nonradioactive studies), the HPLC system consisted of a Hewlett Packard model 1050 quaternary gradient pump and a photodiode array detector with a model 1100 autosampler. Separations were carried out on a Luna C8 150 × 4.6 mm column (Phenomenex) with gradient elution using parent compound as an external standard. For ABT-518, the HPLC system consisted of a model 1050 quaternary gradient pump and a variable wavelength UV detector with a model 1050 autosampler. Separations were carried out on a Prism RP, 100 × 3 mm column

(Keystone Scientific Inc.) with gradient elution, using parent compound as an external standard.

2.7. Electron microscopy

Samples of liver and lung were collected at necropsy and fixed in modified Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M Sorensen's phosphate buffer, pH 7.3). The tissues were post-fixed in 1% osmium tetroxide, in the same buffer, dehydrated in an ethanol series, and embedded in epoxy resin. Thin sections of the tissues were stained with 1% uranyl acetate followed by Reynolds' lead citrate, and examined using a LEO 910 transmission electron microscope (Leo Electron Microscopy, Inc.). Tissues from two male and two female ABT-518-treated rats and from three male and three female ABT-770-treated rats were examined.

3. Results

3.1. In vivo *drug accumulation of ABT-770*

In a 1-month toxicity study, ABT-770 given at 100 mg base/kg/day produced a multi-organ toxicity in rats and formed several metabolites, including a formamide (Abbott-293517) and an amine (Abbott-292986) metabolite resulting from consecutive reduction and deformylation of the parent drug (Fig. 1A). None of these metabolites, including the formamide and amine, exhibited inhibitory activity against MMPs. ABT-770 also produced high tissue to plasma concentration ratios in rats treated for 18 days, especially in lung and liver (Fig. 2A). Of particular interest was the trend for accumulation in these tissues with no evidence of a plateau at 18 or 28 days of dosing (Fig. 2, A and B). HPLC analysis of rat lung extracts at various points over a 28-day period of daily dosing revealed that the amine metabolite was the most abundant drug-dependent analyte accumulating in lung (Fig. 2B) with a smaller contribution from the parent drug and formamide metabolite. The concentration of the amine metabolite in lung tissue from these animals exceeded 5 mM. The amine metabolite was also the primary form of the drug accumulating in the liver (data not shown).

3.2. Cytotoxicity in rat and human hepatocytes

To identify an MMPI with reduced phospholipidosis and possible associated toxicities including analyte accumulation, *in vitro* phospholipidosis assays were conducted on a limited series of structurally related MMPIs and their metabolites. Individual metabolites were tested to identify the analyte(s) that caused phospholipidosis and because the metabolites were not formed from parent compound *in vitro* (data not shown). Parent compounds and metabolites were analyzed for *in vitro* cytotoxic effects prior to assessment of *in vitro* phospholipidosis. Primary cultured rat and human hepatocytes were treated with concentrations of MMPIs and their metabolites ranging from 12.5 to 200 μM and then were assayed for cytotoxicity using the MTT assay (data not shown). ABT-770 was two to three times more cytotoxic than other structurally related MMPIs in both human and rat hepatocytes. ABT-770 exhibited an IC_{50} of approximately 90 μM in rat hepatocytes and 30 μM in human hepatocytes as opposed to other structurally related MMPIs, which had IC_{50} values in excess of 200 μM (the highest concentration tested) in rat hepatocytes and approximately 90 μM in human hepatocytes. Subtoxic concentrations of ABT-770, a limited series of structurally related MMPIs, and metabolites of each were then used in the *in vitro* phospholipidosis assay.

3.3. Identification of an MMPI, ABT-518, that did not induce phospholipidosis in vitro

None of the parent compounds induced phospholipidosis at any concentration in the *in vitro* phospholipidosis assay. Fig. 3 shows representative confocal images from the *in vitro* phospholipidosis assay in primary cultured rat hepatocytes for negative and positive controls (Fig. 3, A and B, respectively), ABT-770 (Fig. 3C), and one of the structurally related MMPIs, ABT-518 (Fig. 3E). Even at higher concentrations including 100 μM for 24 hr and 50 μM for 48 hr (data not shown), neither of these compounds produced phospholipidosis. By contrast, the amine metabolite of ABT-770 (Abbott-292986) produced high to maximal levels (++++, for a description of the rating system used see "Materials and methods") of phospholipidosis at 12.5 μM (Fig. 3D). Phospholipidosis could be observed even at lower concentrations including 1 μM, although to a lesser degree (++ at 1 μM as opposed to ++++ at 12.5 μM). Concentrations below 1 μM were not tested. Other metabolites of ABT-770 did not produce phospholipidosis *in vitro*. Phospholipidosis analysis of metabolites of the structurally related MMPIs revealed that metabolites of one of these analogs, ABT-518, did not induce phospholipidosis. (The chemical structure of ABT-518 and the metabolic pathway to its amine are shown in Fig. 1B.) This included the amine metabolite of ABT-518 (Abbott-318199), which showed no phospholipidosis in the phospholipidosis assay (Fig. 3F). Even at 100 μM, no phospholipidosis was observed with the amine metabolite of ABT-518 (data not shown).

3.4. No evidence of phospholipidosis in rats treated with ABT-518

Since ABT-518 showed no evidence of phospholipidosis in *in vitro* assays, *in vivo* rat studies were undertaken to confirm that phospholipidosis and associated toxicities were not produced *in vivo*. Sections of lung and liver from rats treated for 28 days with ABT-518 or vehicle control were evaluated by electron microscopy. Fig. 4 demonstrates the

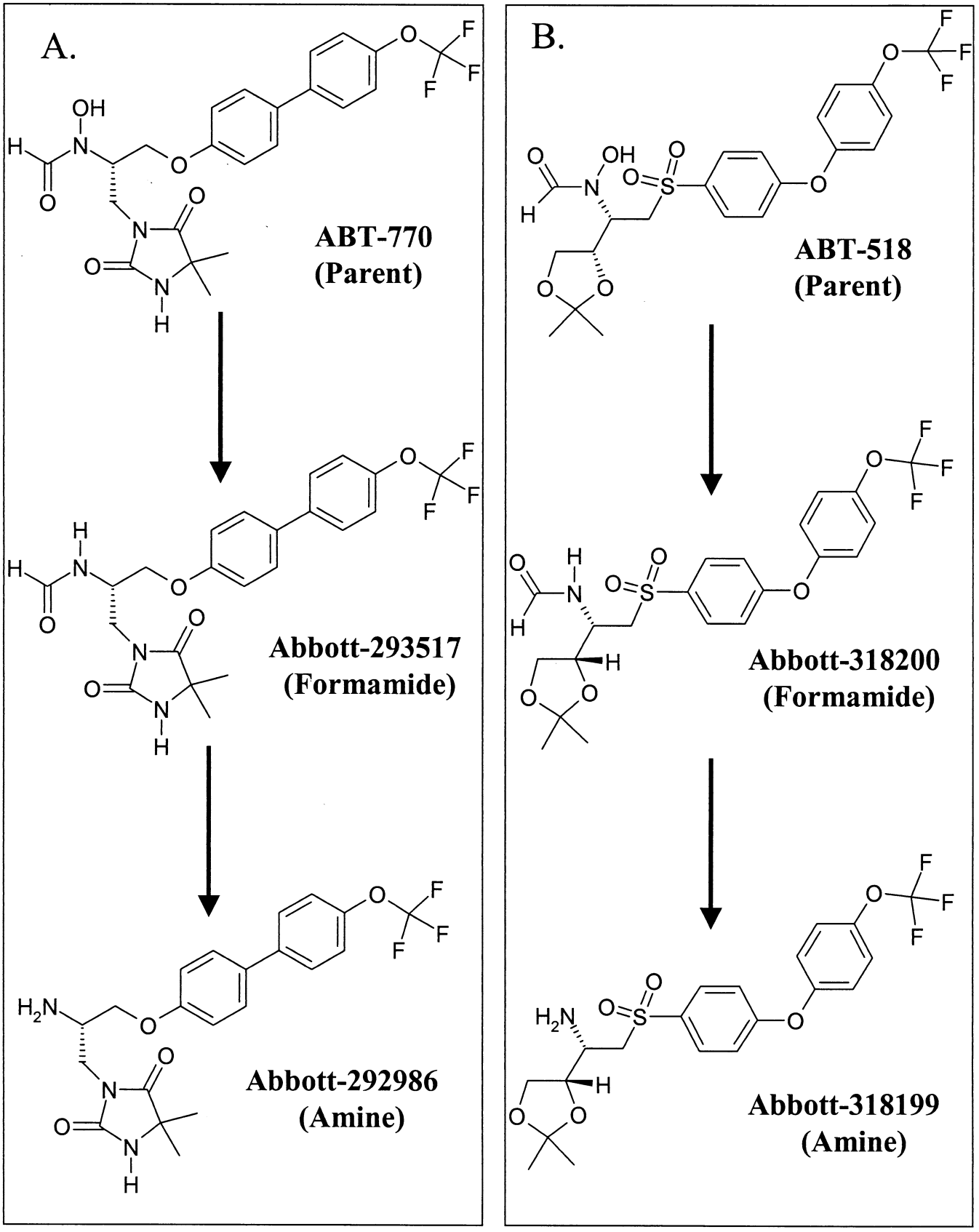

Fig. 1. Chemical structures and metabolic schemes for the metabolism of ABT-770 (panel A) and ABT-518 (panel B) to form their respective amine metabolites in the rat.

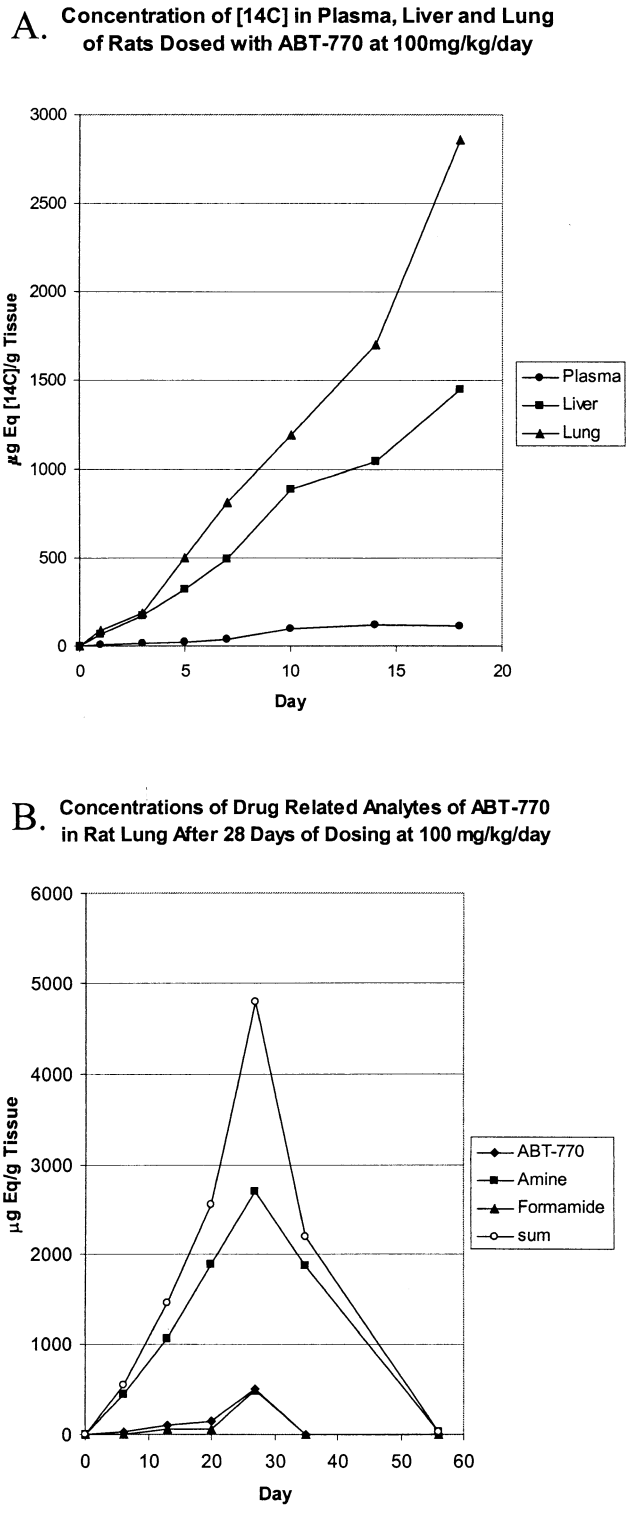

Fig. 2. Concentration of microgram [^{14}C]ABT-770 equivalents/g tissue in plasma, liver, and lung of rats dosed with [^{14}C]-labeled ABT-770 at 100 mg base/kg/day (panel A). Tissue concentrations (μg equivalents/g tissue) of ABT-770, its amine and formamide metabolites, and the sum of all drug-related analytes in rat lung at time intervals ranging from 0 to 28 days of daily dosing at 100 mg base/kg/day followed by a 28-day recovery period (panel B).

difference in ultrastructure observed in macrophages from the lungs of rats treated with ABT-770 (Fig. 4B) as compared with rats treated with vehicle (Fig. 4A) or ABT-518 (Fig. 4C). Lamellar bodies, characteristic of phospholipidosis, were present in the macrophages of ABT-770- but not ABT-518-treated rats. Enlargement of macrophages in the ABT-770-treated rats was also observed. In addition to alveolar macrophages, lamellar bodies were also observed in type I and type II pneumocytes, Clara cells, ciliated bronchiolar epithelial cells, and endothelial cells in the lungs of rats treated with ABT-770 but not those treated with ABT-518. Similarly, in liver, hepatocytes and Kupffer cells of rats treated with ABT-770 (Fig. 5B) contained lamellar bodies, while cells from the livers of vehicle control rats (Fig. 5A) and rats treated with ABT-518 (Fig. 5C) showed no evidence of phospholipidosis. In addition to lung and liver, phospholipidosis was also observed with ABT-770 in proximal and distal convoluted tubular epithelial cells, podocytes, endothelial cells, and pericytes in the kidney, as well as in cells of the stomach, spleen, small and large intestines, and lymphocytes from circulating blood (data not shown). ABT-518 did not produce phospholipidosis in any of these tissues or cell types.

The lack of induction of phospholipidosis by analytes of ABT-518 was not due to decreased activity, decreased efficacy, or lack of bioavailability of ABT-518 relative to ABT-770. ABT-518 exhibited higher potency than ABT-770 against both gelatinase A (MMP-2, IC_{50} = 0.78 nM) and gelatinase B (MMP-9, IC_{50} = 0.5 nM) and only marginal activity against fibroblast collagenase (MMP-1, IC_{50} = 8600 nM) and matrilysin (MMP-7, IC_{50} = 11,000 nM). ABT-518 was efficacious in several animal tumor models and exhibited a wide therapeutic window compared with ABT-770. With the exception of a diol hydroxamate metabolite, analytes of ABT-518, like those of ABT-770, exhibited no inhibitory activity against MMPs.

3.5. Lack of ABT-518 analyte accumulation in tissues

As mentioned above, the amine metabolite of ABT-770 was detected at high concentrations in rat tissues susceptible to ABT-770-induced phospholipidosis such as lung and liver (Fig. 2). To address this issue of metabolite accumulation, lung and liver tissue from rats treated with ABT-770 or ABT-518 at 100 mg/kg/day for 14 days, 28 days, or 28 days with a 1-week recovery were analyzed for all drug-dependent analytes. ABT-518 exhibited relatively little drug/metabolite accumulation in lung (Fig. 6A) or liver (Fig. 6B) at 14 or 28 days or after the 1-week recovery. By contrast, ABT-770 had extremely high levels of analyte accumulation in both lung (Fig. 6A) and liver (Fig. 6B), primarily due to the amine metabolite (Fig. 2). The levels were particularly high at the 28-day time point, and these levels were only partially reversed during the 1-week recovery (Fig. 6, A and B). The lack of drug/metabolite accumulation in tissues of rats treated with ABT-518 com-

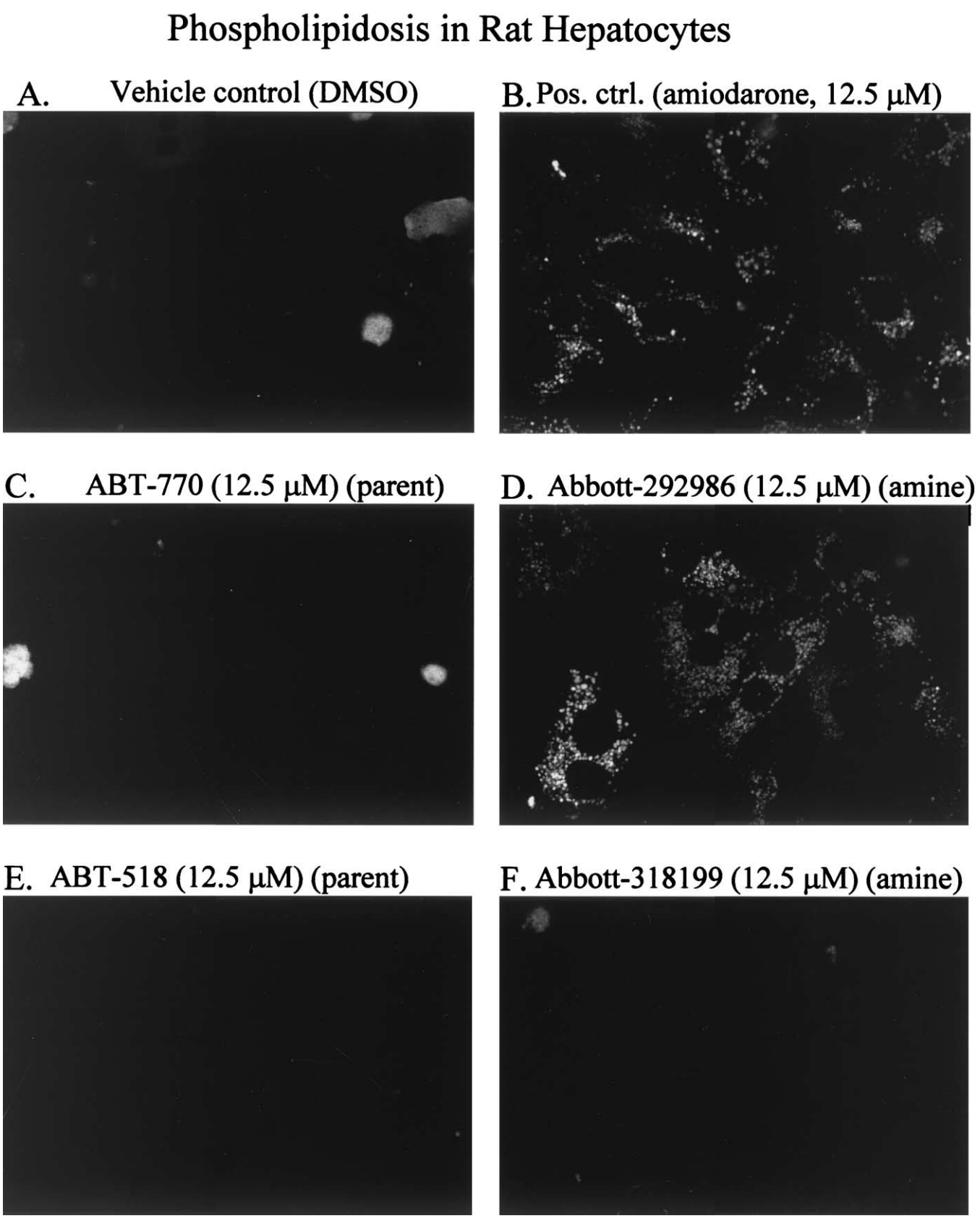

Fig. 3. Confocal laser scanning microscopy images of rat hepatocytes treated for 24 hr with the fluorescent lipid dye NBD-PE and vehicle control (DMSO at 1%) (A), a positive control, amiodarone, at 12.5 μM (B), ABT-770 (12.5 μM) (C), its amine metabolite, Abbott-292986 (12.5 μM) (D), ABT-518 (12.5 μM) (E), and its amine metabolite, Abbott-318199 (12.5 μM) (F). Bright granules within the cells surrounding the nuclei in panel D are intracellular inclusion bodies demonstrating phospholipidosis. Images are representative of three independent rat hepatocyte experiments.

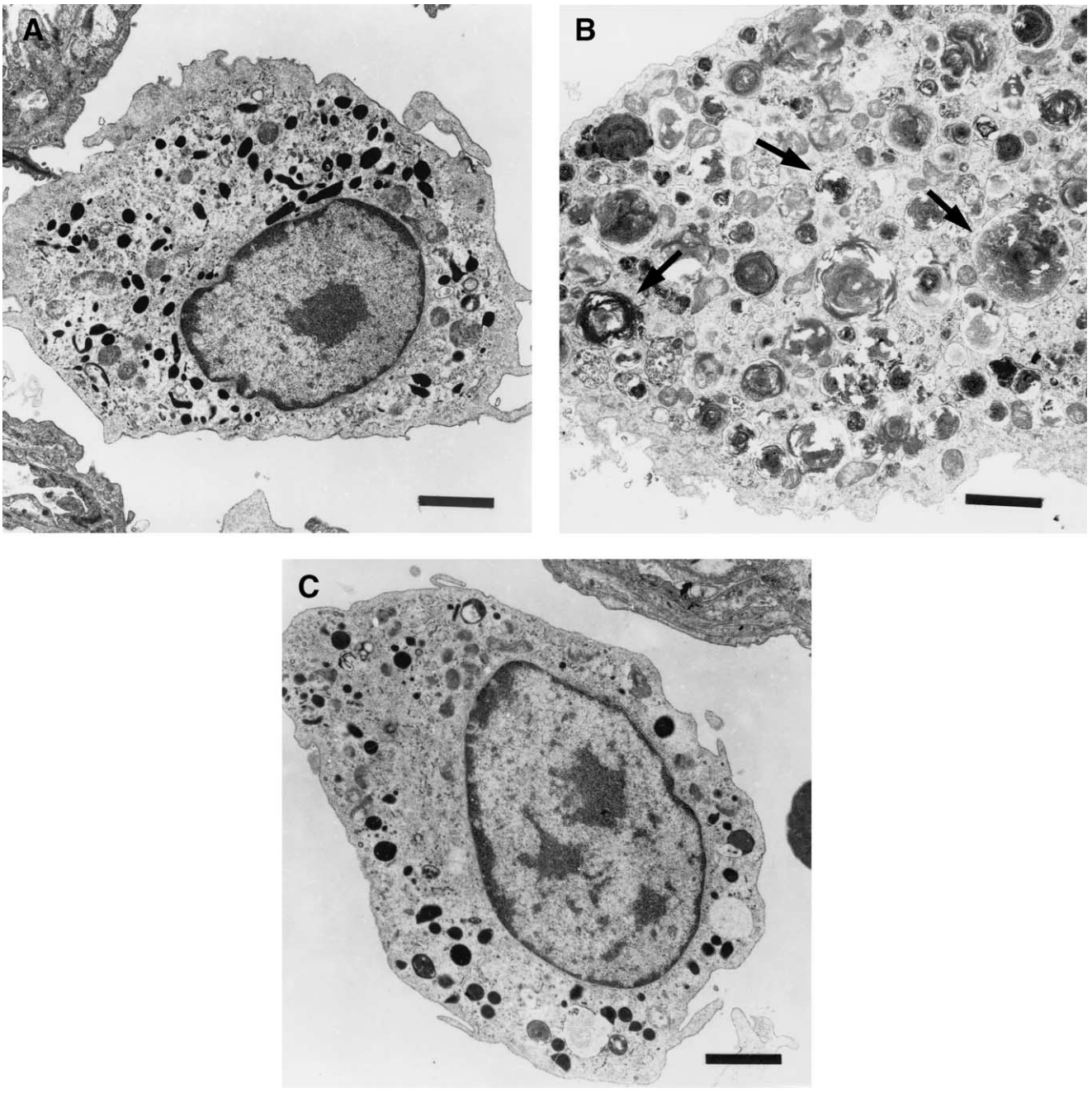

Fig. 4. Electron micrographs of macrophages from lungs of male rats treated for 28 days with vehicle control (A), ABT-770 (100 mg base/kg/day) (B), or ABT-518 (100 mg base/kg/day) (C). Note that the macrophage in panel B contains concentric lamellar whorls (three examples are indicated by arrows) characteristic of phospholipidosis. Bar equals 2 μm.

pared with ABT-770 was consistent with decreased phospholipidosis in the ABT-518-treated animals.

3.6. Phospholipidosis assay results in human hepatocytes

These results confirmed that the phospholipidosis assay had identified *in vitro* in rat hepatocytes a compound that showed no evidence of phospholipidosis *in vivo* in rats. To attempt to predict whether the same results would be observed in humans, hepatocytes were isolated from the livers of four human donors and assayed for phospholipidosis using the *in vitro* assay. The results from the hepatocytes of all four human donors as well as average rat hepatocyte results are summarized in Table 1. Fig. 7 shows representative confocal images from a phospholipidosis analysis of hepatocytes from human donor 1.

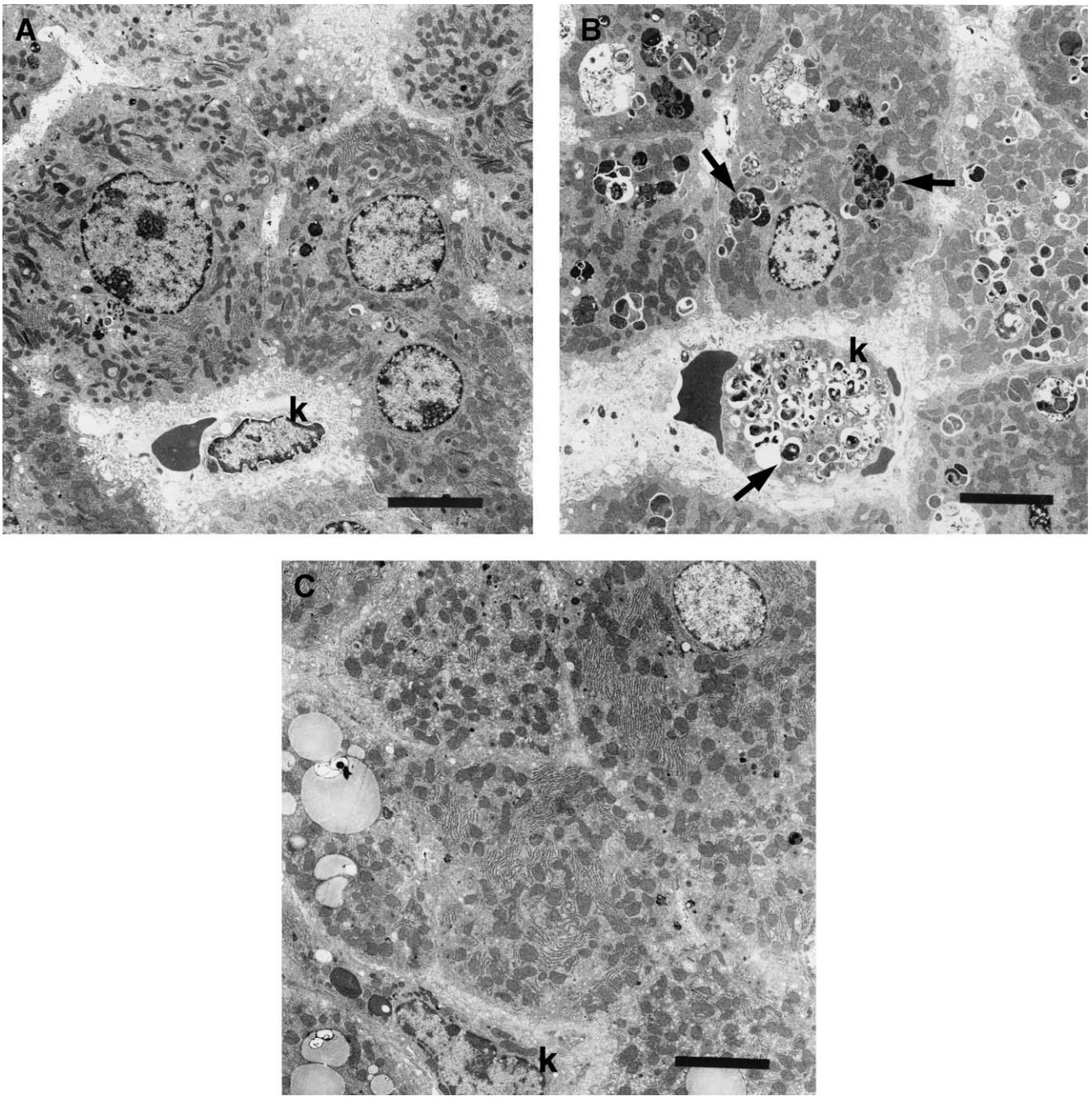

Fig. 5. Electron micrographs from livers of female rats treated for 28 days with vehicle control (A), ABT-770 (100 mg base/kg/day) (B), or ABT-518 (100 mg base/kg/day) (C). The k in each panel indicates a Kuppfer cell; all other cells in the micrographs are hepatocytes. Arrows in panel B indicate some of the lamellar bodies present in Kuppfer cells and hepatocytes. Bar equals 5 μm.

Both the positive control (Fig. 7B) and the amine metabolite of ABT-770 (Fig. 7D) produced moderate (++) to high (++++) levels of phospholipidosis in hepatocytes from all four human donors. By contrast, the negative control (Fig. 7A), both parent compounds (Fig. 7, C and E), and the amine metabolite of ABT-518 (Fig. 7F) produced little to no phospholipidosis in all four human donor hepatocytes. For donors 2, 3, and 4, the amine of ABT-518 was negative for phospholipidosis even at 100 μM. Hepatocytes from donor 1 were clearly negative at 12.5 μM, but at 50 μM some cells were observed to be positive for phospholipidosis. These positive cells had a different morphology and did not appear to be hepatocytes. The hepatocytes that were observed in wells treated with 50 μM Abbott-318199 (the amine metabolite of ABT-518) were negative for phospholipidosis. In

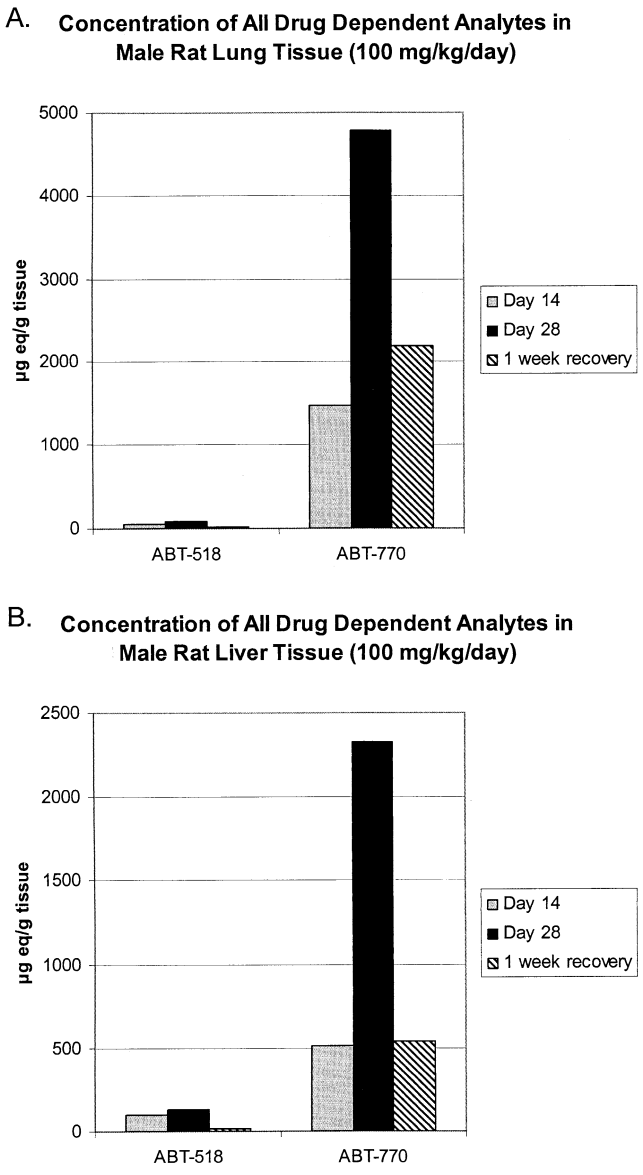

Fig. 6. Concentrations of all drug analytes in male rat lung tissue (A) or liver tissue (B) after treatment with 100 mg base/kg/day of ABT-770 or ABT-518 for 28 days.

hepatocytes from all four human donors, the amine of ABT-518 produced significantly less phospholipidosis than the amine of ABT-770.

4. Discussion

At 24 and 48 hr, none of the parent compounds produced phospholipidosis *in vitro* in human or rat hepatocytes. Phospholipidosis was induced primarily by the amine metabolite of ABT-770 (Abbott-292986). These results directly paralleled *in vivo* results in rats where ABT-770 induced high levels of phospholipidosis presumably through its amine metabolite. This was accompanied by accumulation of the amine metabolite in affected tissues such as lung and liver. In contrast, the amine metabolite of ABT-518 (Abbott-318199) produced little if any phospholipidosis in rat hepatocytes at concentrations up to 100 μM and did not produce phospholipidosis in rats *in vivo.* These results provide evidence that the *in vitro* phospholipidosis assay can be used as a predictor of *in vivo* phospholipidosis.

Data from other studies have suggested that the precursor to the amine metabolite (the formamide, see Fig. 1) is formed by reduction of ABT-770 by gut contents (unpublished results), which may explain why the parent compounds were negative for phospholipidosis *in vitro.* If significant reductive metabolism of ABT-770 could occur in hepatocytes *in vitro,* ABT-770 would have been expected to induce phospholipidosis *in vitro.* Hypoxic conditions do not appear to play a role in the metabolism of ABT-770 to its amine metabolite, at least *in vitro,* since decreased oxygen conditions had no effect in promoting formation of the amine in primary rat hepatocytes (unpublished results).

As expected, there was more variation among human donors than was seen with rats. In addition, all four human donors were male Caucasians, so gender or racial differences would not be reflected in these results. Human hepatocytes also contain more lipid droplets and display more auto-fluorescence, due to the presence of neutral lipid and lipofucsin inclusions, than rat hepatocytes (note the larger

Table 1
Summary of assay results for induction of phospholipidosis by MMP inhibitors

Compound	Form	Rat[a]	Human[b]			
			Donor 1	Donor 2	Donor 3	Donor 4
ABT-770	Parent	−[c]	−	−	−	ND
Abbott-293517	Foramide	−	−	−	−	−
Abbott-292986	Amine	++++ (12.5)[d]	++++ (12.5)	++++ (50)	+/++ (50)	++++(50)
ABT-518	Parent	−	−	−	−	ND
Abbott-318200	Formamide	−	ND	−	−	−
Abbott-318199	Amine	−	+/++ (50)	−	−	−

[a] Data are from three independent rat hepatocyte experiments.
[b] Data are from four hepatocyte experiments, four human donors.
[c] −, no phospholipidosis observed; +, small number of inclusions per cell; ++, intermediate number of inclusions per cell; ++++, maximal number of inclusions per cell; ND, not determined.
[d] Numbers in parentheses indicate the minimum (μM) concentration of drug required to achieve the maximum (indicated), observed effect.

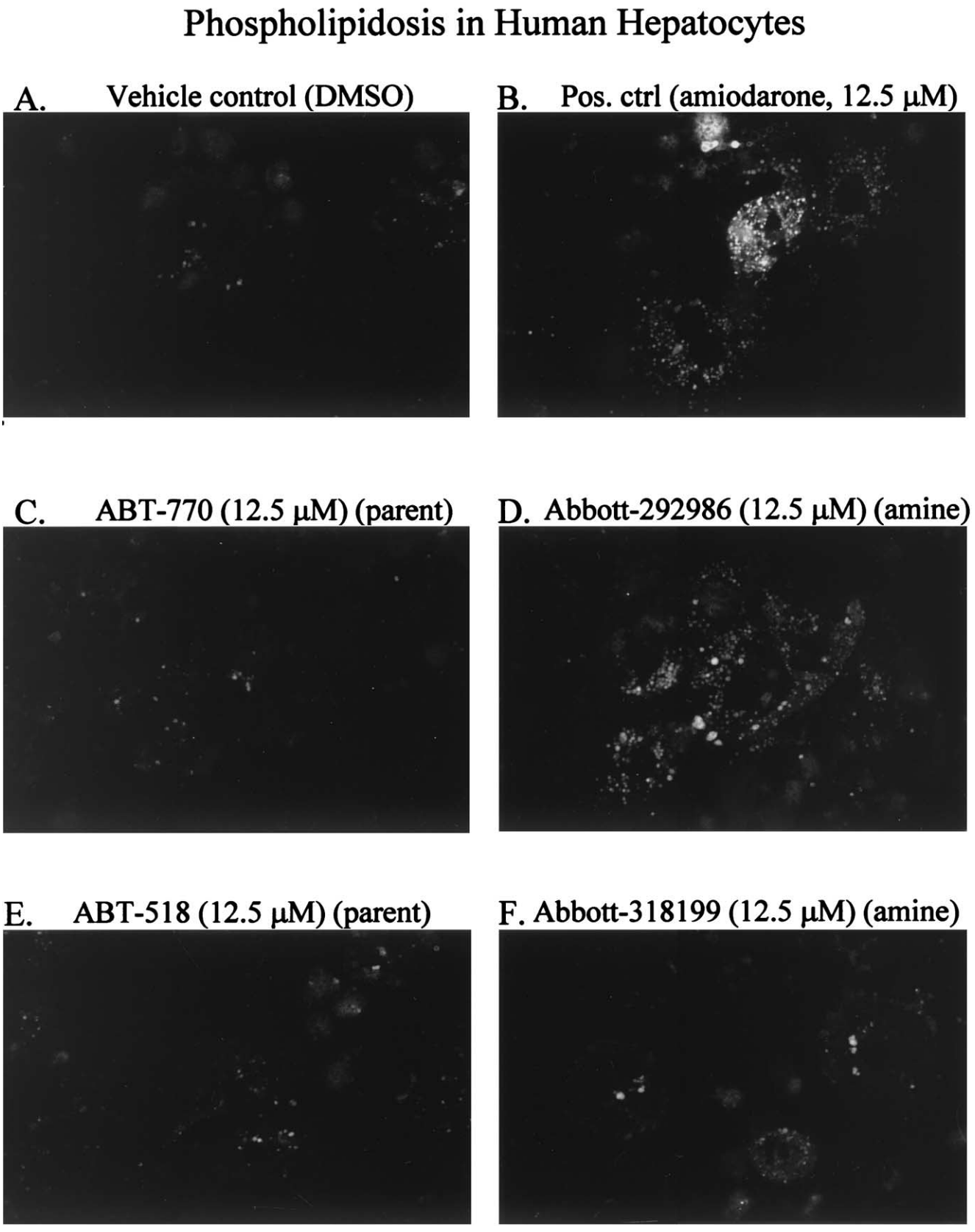

Fig. 7. Confocal laser scanning microscopy images of human hepatocytes treated for 24 hr with the fluorescent lipid dye NBD-PE and vehicle control (DMSO at 1%) (A), a positive control, amiodarone, at 12.5 μM (B), ABT-770 (12.5 μM) (C), its amine metabolite, Abbott-292986 (12.5 μM) (D), ABT-518 (12.5 μM) (E), and its amine metabolite, Abbott-318199 (12.5 μM) (F). Bright granules within the cells surrounding the nuclei in panel D are intracellular inclusion bodies demonstrating phospholipidosis. Note that a few cells in some fields of the negative control have large, non-perinuclear fluorescent granules (panel A). Images are representative of four experiments with human hepatocyte from four independent donors.

granule based fluorescence in the non-perinuclear cytoplasm of the negative control, Fig. 7A). Despite these confounding features, the human hepatocyte phospholipidosis data were highly consistent with the rat data. The amine metabolite of ABT-770 produced intermediate to maximal levels of phospholipidosis in all the human donors, and in all four human donor hepatocytes the amine of ABT-518 (Abbott-318199) produced significantly less phospholipidosis than the amine of ABT-770. In all but one donor, the amine of ABT-518 was completely negative in the phospholipidosis assay, and, in that donor, little to moderate phospholipidosis was observed in a few cells that appeared to be non-hepatocyte in nature. Based on these results, ABT-518 is predicted to pose little to no risk for phospholipidosis in humans. ABT-518 exhibits better efficacy and less toxicity than ABT-770 and is expected to enter clinical trials in late 2001.

While phospholipidosis by itself is not considered a toxic response, it is often associated with target organ toxicity. In addition, the multi-organ and multi-cell-type nature of the observed phospholipidosis was of particular concern from a toxicological perspective. Phospholipidosis is probably responsible for the pulmonary inflammatory foci observed in rats treated with ABT-770. The increased number and size of affected macrophages support this finding. Phospholipidosis is thought to contribute to the observed altered serum levels of liver enzymes in these animals as well. Whether it is the cause of death of rats at higher exposures remains to be determined. Of particular concern from a toxicological perspective was the linear increase in accumulation of the amine metabolite of ABT-770 in tissues also affected by phospholipidosis. Since the MMPIs are cytostatic rather than cytotoxic agents, administration will likely be long-term, potentially over the life-span of the patient. Accumulation of the amine metabolite without an apparent plateau and with a long residual half-life is not an acceptable feature for a drug that is to be administered chronically. By successfully identifying an alternative to ABT-770 with minimal or no propensity for phospholipidosis, any associated or coincidental liabilities to the phospholipidosis, including drug accumulation in tissues, should also have been eliminated.

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