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Research Paper

Lysosomes Contribute to Anomalous Pharmacokinetic Behavior of Melanocortin-4 Receptor Agonists

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Purpose. A series of melanocortin-4 receptor (MC4R) agonists, developed for use as anti-obesity agents, were found to have unusual pharmacokinetic behavior arising from excessive retention in the liver, with nearly undetectable levels in plasma following oral administration in mice. This work investigates the molecular basis of the prolonged liver retention that provided a rational basis for the design of an analog with improved behavior.

Materials and Methods. The livers of mice were harvested and techniques were utilized to fractionate them into pools differentially enriched in organelles. The distribution of organelles in the fractions was determined using organelle-specific enzymatic assays. Livers from mice dosed with drug were fractionated and comparisons with organelle distributions assisted in determining the subcellular localization of the drug. Further analysis in cell culture systems was used to confirm results from liver fractionation studies and also allowed for more extensive evaluations to examine the mechanism for organelle compartmentalization

Results. Fractionation of livers following oral administration of the agonist showed sequestration in lysosomes. Subsequent evaluations in a cell culture system confirmed this finding. Agents used to disrupt acidification of lysosomes led to decreased lysosomal accumulation of the drug, which implicated a pH-partitioning type sequestration mechanism. These findings led to the rational synthesis of an analog of the parent compound with properties that reduced lysosomal sequestration. When this compound was examined in mice, the liver retention was found to be greatly reduced and plasma levels were significantly elevated relative to the parent compound.

Conclusions. Weakly basic drugs with optimal physicochemical properties can be extensively sequestered into lysosomes according to a pH-partitioning type mechanism. When administered orally in animals, this particular sequestration event can manifest itself in long term retention in the liver and negligible levels in blood. This work revealed the mechanism for liver retention and provided a rational platform for the design of a new analog with decreased liver accumulation and better opportunity for pharmacokinetic analysis and therapeutic activity.

KEY WORDS: cell fractionation; lysosomes; pharmacokinetics; pH-partitioning.

INTRODUCTION

Important decisions in drug development often occur during preclinical pharmacokinetic studies in laboratory animals. When therapeutically promising melanocortin-4 receptor (MC4R) agonist anti-obesity agents were administered to mice, the compounds were virtually undetectable in the plasma, urine or feces following oral administration. Likewise, metabolites were not readily detectable in these samples. The inability to monitor blood levels and/or metabolic products is typically detrimental to the successful development of any drug. Considering this, it was desirable to understand the anomalous behavior on a molecular level, which would in turn allow for rational development of structurally related derivatives without the aforementioned problems. Two lead compounds (GSK045A and GSK047A, see Fig. 1 for partial structures), are weakly basic with pKa values above neutrality. Generally, such compounds are known to have large volume of distributions relative to other weakly acidic or neutral drugs (1), however, the degree of the apparent volume of distribution was unusually large for these compounds. In attempts to determine where the drug was accumulating, several organs were examined post-dosing. The liver, in particular was identified as an organ containing large amounts of drug following oral administration. A plot showing the levels of GSK047A in the liver and in plasma

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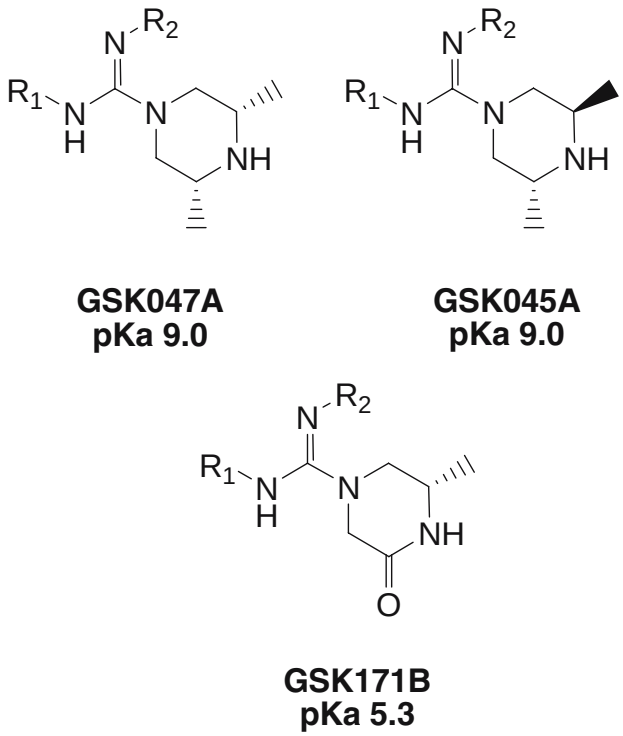

Fig. 1. Partial structures of a series of melanocortin-4 receptor (MC4R) agonists, revealing the basic portion of the drug molecule relevant to the current study. R2 is the same for all structures shown. R1 is different than R2 but is identical between molecules GSK045A and GSK047A. R1 in GSK171B is very similar in structure with the R1 of other structures but not exactly the same. The calculated pKa of the molecules is shown below the name of each compound.

following a single oral dose are shown in Fig. 2. As can be seen, the levels in plasma were consistently low and hovered near the limit of detection (5 ng/mL) and fell below it at the 4 and 24 h time points. Alternatively, the amounts in the liver were consistently high throughout the two day sampling time. To investigate the precise site of drug accumulation in the liver, liver samples were fractionated in order to obtain pools enriched in organelles, which were subsequently assayed for drug. Generally speaking, the subcellular fractionation of organelles from tissue sources has been done in the past but it is unrealistic to obtain clean separations of organelles between fractions. More realistically one typically obtains fractions that are merely enriched in particular organelles.

Our laboratory specializes in evaluating the subcellular distribution of drugs in cell culture systems and has developed several techniques which allow for much cleaner and efficient organelle isolations compared to traditional methods (2,3). Therefore, the subcellular distribution of the agonists was additionally evaluated in a human leukemic cell line HL-60, for which we have previously characterized organelle isolation methods. In addition to enhancement of organelle purity, working with cells in culture offers advantages in that it is possible to utilize reagents to modulate cell functions to elucidate the mechanisms of sequestration. Such approaches are typically not practical in studies with live animals. The combined approaches allowed us to establish the involvement of lysosomes in the sequestration of GSK045A. Moreover, we established the mechanism for sequestration to be consistent with pH partitioning, which relies on the large pH gradient between the intra-lysosomal environment and the cell cytosol. Understanding the mechanism provided a rational basis for the synthesis of a lower pKa derivative (see GSK171B, Fig. 1) that was shown to accumulate to a much lower degree in lysosomes in experiments with cultured cells. Subsequent administrations in mice likewise demonstrated that the lower pKa derivative had dramatically reduced hepatic sequestration and greater plasma concentrations. This work highlights the importance of understanding the subcellular distribution of drugs and how it can significantly impact macroscopic pharmacokinetic behavior. Moreover, this work exemplifies how such mechanistic information can be used in the design/selection of derivatives of drugs with improved intracellular distribution and therefore improved likelihood for therapeutic success.

MATERIALS AND METHODS

Reagents and Chemicals

All compounds with partial structures shown in Fig. 1, along with a close structural analog for use as an internal standard were synthesized in house at GlaxoSmithKline by members of the Department of Medicinal Chemistry. The full structures and synthetic details are to be disclosed in a publication that is in preparation for submission elsewhere. Acetonitrile and methanol were purchased from EMD Chemicals. In-house water purified by MilliQ filtration (Millipore, Billerica, MA) was used. Control mouse plasma was purchased from Bioreclamation Inc. (Hicksville, NY). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

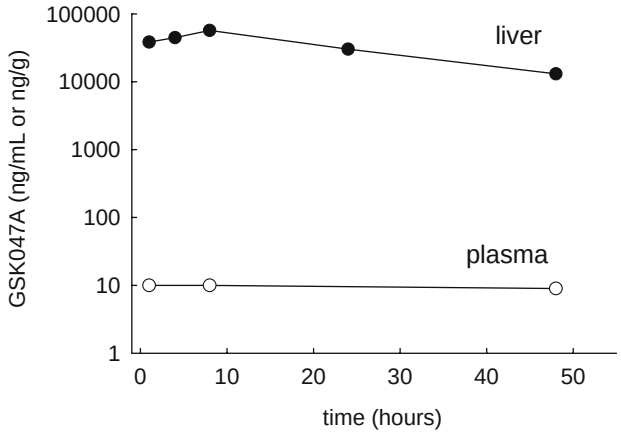

Fig. 2. Representative concentration versus time profile of GSK047A demonstrating the anomalous distribution behavior following oral dosing. The concentrations in the liver (closed circle) and in the plasma (open circle) following a single oral dose of 30 mg/kg to individual mice at indicated time points are shown. The drug is essentially entrapped within the liver for an extended period of time resulting in very low, often undetectable, levels in the plasma. The levels in the plasma were consistently low and were below the limit of detection (5 ng/ml) in mice harvested and sampled at the 4 and 24 h time points. This behavior is representative of three trials with this compound as well as from structurally similar drugs dosed in multiple mice.

Mouse Liver Fractionation

CD-1 mice (ranging in weight from 20 to 25 g) were obtained from Charles River Laboratories and were fasted overnight and given water freely prior to oral dosing and subsequent studies. For purposes of fractionation, freshly isolated livers were minced with a razor blade and suspended in 10 mL of ice cold homogenization buffer (0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) per gram of liver. The suspension was homogenized with two up-and-down strokes of the "tight" teflon pestle of a Potter-Elvehjem homogenizer rotating at a power setting of 35. All remaining centrifuge steps were done in a SW38 swinging bucket rotor at 4°C. The resulting homogenate was first centrifuged at 600×g for 10 min. to pellet nuclei and unbroken cell and tissue pieces. The supernatant was spun at 3000×g for 10 min. and the pellet (P1) was collected. The resulting supernatant was centrifuged at 25,000×g for 10 min. and the pellet was collected (P2). The final supernatant was spun at 100,000×g for 10 min and the pellet (P3) was separated from the supernatant which was designated as cell cytosol (CYT). The fractions were assayed for both drug and enzymes specific to organelles as described in following sections.

For analysis of drug distribution in whole liver for purposes of liver half-life estimates, a slightly different procedure was employed. After oral dosing of GSK compounds, the animals were sacrificed by decapitation at indicated time points following the administration. Livers were excised surgically, weighed on an analytical balance and then transferred into 15 mL centrifuge tubes. The liver was suspended in acetonitrile (4 mL per 1 g of liver) containing 200 ng/mL internal standard. The tissue was homogenized using a HandiShear homogenizer (Virtis, Gardiner, NY) and then centrifuged at 1,500×g for 20 min. The supernatant was transferred to a clean 96 well plate and analyzed as described in the quantification of compounds section.

Enzyme Activity Assays

The presence of lysosomes was assayed for by measuring the activity of acid phosphatase. One hundred μL of both 16 mM *p*-nitrophenyl phosphate and 180 mM sodium acetate-acetic acid (pH 5), where added to 25 μL of designated suspended pellet (in homogenization buffer) or supernatant (without additional buffer) and the mixture was incubated at 37°C for 20 min. The reaction was stopped by the addition of 0.6 mL of 0.25 M NaOH. The mixture was centrifuged in a microcentrifuge at 15,000 RPM for 2 min. The absorbance of *p*-nitrophenol in the supernatant was measured at 410 nm. The presence of mitochondria in fractions was assessed by succinate dehydrogenase activity. The pellet (with homogenization buffer) or supernatant (25 μL total volume of each) was combined with 0.3 mL of 0.01 M sodium succinate in 0.05 M phosphate buffer (pH 7.5) and incubated at 37°C for 15 min. Subsequently, 0.1 mL of 2.5 mg/mL *p*-iodonitrotetrazolium in 0.5 M phosphate buffer (pH 7.5) was added and the incubation continued for an additional 10 min. The reaction was stopped by adding ethyl acetate: ethanol: trichloroacetic acid 5:5:1 (v/v/w) and microcentrifuged at 15,000 RPM for1 min. The absorbance of the supernatant was read at 490 nm. The presence of peroxisomes in fractions was evaluated by measuring catalase activity. Ten micro liters of sample was mixed with 30 μL of buffer containing one part 2% triton X-100 with two parts 20 mM Tris-HCl, pH 7.0 containing 1 g/L bovine serum albumin. To this was added 0.5 mL buffer containing 2.5 mM H_2O_2 in 20 mM Tris-HCl, pH 7.0 containing 1 g/L bovine serum albumin. After 1 min. 1.0 mL of titanium oxysulfate (2.25 g/L) in 1 M H_2SO_4 was added and the sample was microcentrifuged for 2 min. at 15,000 RPM and the absorbance of the supernatant was measured at 405 nm. The presence of endoplasmic reticulum in fractions was assessed by evaluating uridine 5'-diphosphate (UDP)-glucuronosyltransferase (UGT) activity using a derivative of camptothecin named SN-38 as a substrate for glucuronidation. Fractions (0.5 mg total protein) were solubilized in Brij 35 (0.5 mg/mg protein), D-saccharic acid 1,4 lactone (10 mM), magnesium chloride (10 mM), SN-38 carboxylate (300 μM) in a final volume of 1 mL. The reaction was initiated by the addition of the co-factor UDP-glucuronic acid (2 mM) and samples were incubated at 37°C. Aliquots (150 μL) were removed periodically (from 10 to 50 min.) to assess the rate of glucuronidation and quenched with acetonitrile (600 μL) containing internal standard (irinotecan, 400 ng). Samples were then microcentrifuged at 15,000 RPM for 10 min and the acetonitrile layer was removed and evaporated to dryness under a stream of nitrogen. Samples were reconstituted with 250 μL of an aqueous solution containing 30% methanol and 0.1 M ammonium acetate (pH 4.8). Aliquots of 100 μL were injected onto an HPLC and analyzed for appearance of the glucuronidated parent compound. The HPLC system used to separate and detect SN-38 glucuronide consisted of an LC-600 pump (Shimadzu, Tokyo, Japan), AS 100 autosampler (Bio-Rad, Hercules, CA), RF-551 fluorescence detector (Shimadzu), and an axxi-chrom C-18 column (5 μM, 150×4.6 mM, Axxiom, Moorpark, CA). The mobile phase was composed of 42% methanol/0.1 M ammonium acetate and 10 mM tetrabutylammoniumhydrogensulfate, and run at 1.5 mL/min. Excitation and emission wavelengths were fixed at 375 nm and 421 nm, respectively. Elution times were 3.8 min for SN-38 glucuronide and 15.2 min for irinotecan (the internal standard). The identity of each peak was confirmed with an authentic standard. Data is expressed as peak area of SN-38 glucuronide relative to that of irinotecan.

Bradford analysis, using bovine serum albumin for standards, was used to estimate protein concentration as to report all enzyme activities as per weight of protein in the experimental sample.

Cell Culture, Lysosome Isolation and pH Determinations/ Manipulations

The procedure for culturing cells, isolating lysosomes, purity estimates and conversion of amounts of drug in isolated lysosomes to concentration terms have all been previously published (3). Briefly, in this work, 600 mL of multi-drug resistant (MDR) HL-60 cells were cultured to a density of 0.5×10^6 cells/mL. The total volume of media containing cells was reduced to 180 mL by gentle centrifugation and subsequently incubated with iron coated dextran to allow for endocytic uptake for 1 hour. Cells were washed three times with phosphate buffered saline (PBS) and then re-suspended

in iron dextran free media (600 mL total volume) and separated into two 300 mL fractions. One 300 mL fraction received 20 nM concanamycizn A for 24 h (+ Con A), the other did not (−Con A). Cells were incubated for 24 h without dextran since this amount of time has been previously shown to allow the dextran to specifically accumulate into lysosomes. GSK compounds were subsequently added to each group of cells at a final concentration of 5 μM for 1 h then washed with PBS three times to remove unincorporated drug. Designated cells had an additional 12 h (chase) in drug free media prior to homogenization and lysosome isolation. Cells were homogenized and then passed over a magnetic column to isolate lysosomes as previously described. The isolated lysosomes were analyzed for GSK compounds as described in the following experimental section. The pH values associated with relevant compartments of the MDR HL-60 cell line before and after Con A treatment have been previously published (4) and are noted in the text of the manuscript.

Quantification of GSK Compounds from Liver, Plasma and Cells

Standard and internal standard stock solutions of GSK compounds were prepared in dimethyl sulfoxide at a final concentration of 1 mg/mL. Solutions of GSK compounds used to construct a standard curve were obtained by adding standards to various volumes of methanol. Experimental standards were prepared by adding known amounts (low, intermediate and high) of GSK compounds along with internal standards into blank mouse plasma, liver or cell culture fractions. Extraction procedures were performed using a 96 well plate format.

Aliquots of liver (50 μL), plasma (40 μL) or cellular fractions (40 μL) were combined with 200 μL of acetonitrile (containing internal standard at a concentration of 200 ng/mL) and vortexed, then centrifuged for 10 min at 2,055×g. The acetonitrile supernatant was transferred to a new 96 well plate and 10 μL was injected into an LC/MS/MS system for quantification of GSK compounds relative to the internal standard.

The quantification analyses were performed using a high-performance liquid chromatography/tandem mass spectrometry (LC/MS/MS) method. Chromatography was carried out using reverse phase separation on either a X-terra Phenyl column, 50×2 mm, 3.5 micron particle size (Waters, Milford, MA) or a Luna C8(2) column, 30×2 mm, 3 micron particle size (Phenomenex, Torrance, CA) using a linear gradient. The mobile phases used were water/acetonitrile, both containing an additive of either 0.1% formic acid or 0.1% acetic acid. The triple quadrupole mass spectrometers were operated in the positive electrospray ionization (ESI) mode. Product ions of the analytes and internal standard were detected using multiple reaction monitoring (MRM) mode.

pKa Calculations

In silico estimates for each compound's pKa values were calculated by ACD/pKa Batch software version 8, (Toronto, Canada).

Liver Half-Life Estimates

The tissue concentration data were modeled with WinNonLin version 3.1 (Pharsight Corporation, Mountain View, CA) and the pharmacokinetic parameter estimates (including tissue t1/2) were calculated by noncompartmental analysis of the individual liver concentration-versus-time curves. In most cases, three liver concentration-versus-time points were used to calculate the tissue t1/2 by linear regression of the terminal phase. The area under the plasma versus time curves were estimated using the trapezoidal rule. Statistical analysis was performed using a Student's *t*-test.

RESULTS AND DISCUSSION

We reasoned that in order for a drug to be retained in the liver for an extended period of time it must be binding to some intracellular component or alternatively be trapped within a subcellular compartment where its rate of release is a very slow process. In an attempt to identify the drug binding/sequestering entity we utilized differential centrifugation of livers to separate cellular contents into five fractions. For each of the fractions, we evaluated the activity of enzymes specific to the lysosomes, peroxisomes, mitochondria and endoplasmic reticulum. The distribution of these organelles in designated fractions is illustrated in Fig. 3. As can be seen, such fractionation techniques are quite inefficient and single fractions typically contain more than one organelle. After establishing fractionation procedures, mice were dosed with one of the agonists (GSK045A) and fractionated. The drug was subsequently extracted from each of the fractions and quantified. Individual results obtained from two mice are included in Fig. 3 for comparison with organelle distribution in the fractions. The drug was found at its highest concentration in fractions P2 and P3. Following comparisons with organelle distribution, it appeared that the drug was localized with either lysosomes or the endoplasmic reticulum. There is a great deal of prior literature regarding the accumulation of weakly basic drugs such as GSK045A in lysosomes (4–7), conversely drug trafficking to the ER is not common but has been reported (8). Lysosomes have been shown to participate in the accumulation of weakly basic compounds according to a mechanism termed pH-partitioning or ion trapping. DeDuve *et al.* have published an excellent commentary dealing with the mechanistic basis for this phenomenon (9). Briefly, there are several requirements in order for pH-partitioning driven sequestration to occur to a significant extent. First, the basic compound must have a relatively high pKa value (near or above neutrality) and be adequately membrane permeable in the unionized state and be relatively membrane impermeable when ionized. There must also be a significant pH differential existing between the lysosomal lumen (typically acidic) and the cell cytosol (typically neutral). Under these circumstances the base can exist to a significant extent in its membrane permeable state in the cytosol and penetrate across the lysosomal lipid bilayer and then become trapped due to the low pH and increased ionization. Based on these requirements, one way to confirm the involvement of lysosomes in the sequestration of GSK045A would be to raise the pH of the lysosomes in the liver cells prior to administering the drug, which should result

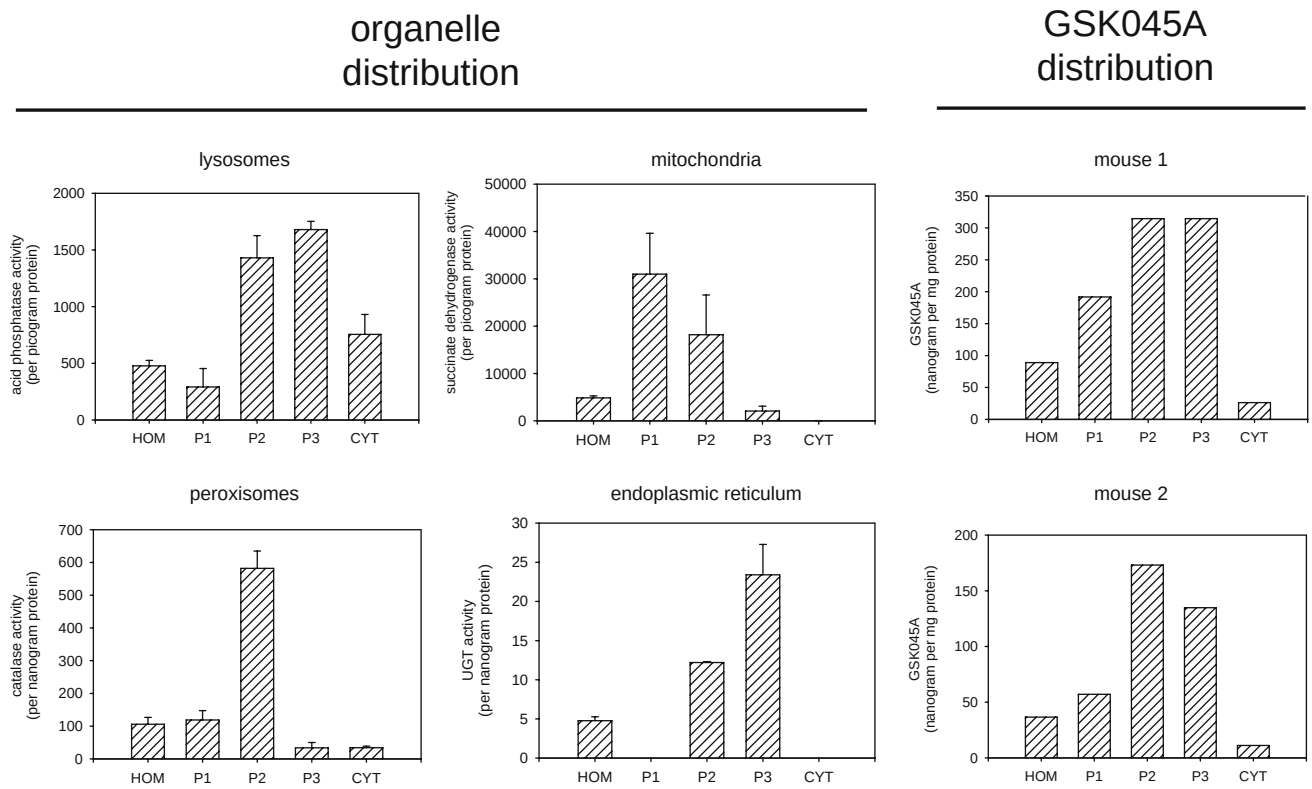

Fig. 3. Graphic illustration of organelle and GSK045A in mouse liver fractions following differential centrifugation described in "MATERIALS AND METHODS". The organelle assayed for is at the heading of each of the graphs and the organelle specific enzyme activity is located on the *y*-axis. Lysosomes were most concentrated in fractions P2 and P3, mitochondria were in P1 and P2, peroxisomes were predominantly in P2 and the endoplasmic reticulum was found mostly in P2 and P3. All organelle enzyme activities are reported as averages of three experiments ± S.D. The distributions of GSK045A in the fractions are reported on a per mg of protein contained within each fraction basis and were most concentrated in fractions P2 and P3. These evaluations were done in two mice and data are presented individually for each mouse.

in decreased accumulation and retention. However, such treatments would be expected to be extremely toxic to the animal, resulting in death or severe impairment, which would most likely confound interpretations of experimental data. On the other hand, there are several known approaches to temporarily increase the pH of lysosomes in cultured cells with no untoward effects (10). Our laboratory routinely utilizes cultured cells for evaluating the subcellular distribution of non-fluorescent drugs. Particularly relevant to this study, we have developed a novel and efficient method for isolating lysosomes and quantifying drugs contained within them (3). The isolation procedure itself, as well as a large number of control studies to confirm the intactness of the organelle during the isolation, have been previously described (2,3). The selection of cell line for this study is an important consideration. Normal primary hepatocytes would obviously be the most appropriate selection for this study, however, for quantification of drugs in subcellular compartments we require substantial quantities of cells and therefore we are limited to cells that grow rapidly, preferably in suspension. We have found the multi-drug resistant variant of the HL-60 human leukemic cell line to be a particularly well suited model cell line for these types of applications. This cell line grows rapidly and in suspension which is conducive for obtaining the quantity of cells required for these evaluations. The reason for selecting the MDR variant of the HL-60 cell line is because we have shown it to have normally acidified lysosomes, which is not the case for many drug naïve cancer cell lines (4,11). Moreover, we have shown that this cell line does not express many of the typical drug transporter

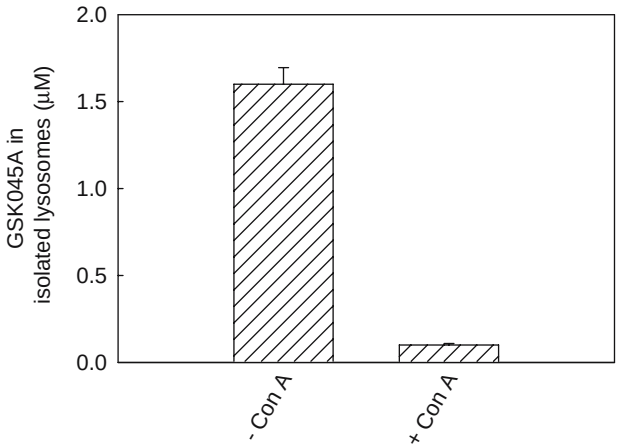

Fig. 4. Concentration of GSK045A in isolated lysosomes from MDR HL-60 cells with or without 20 nM concanamycin A (Con A) pre-incubation for 24 h. Cells were incubated with 5 μM GSK045A for 1 h before isolation of lysosomes and quantitation of the drug associated with them. The results are averages of three independents experiments ± S.D.

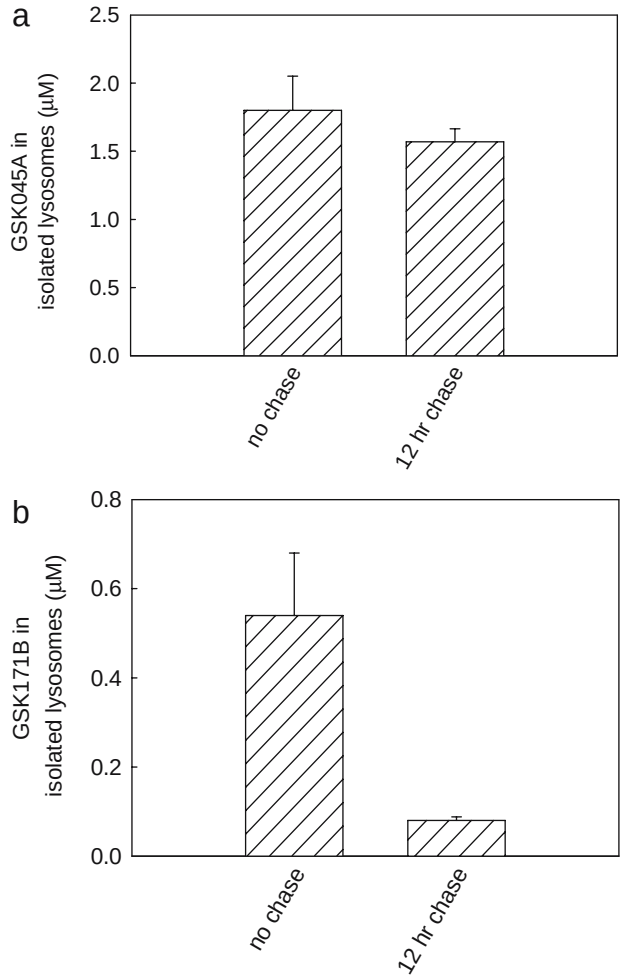

Fig. 5. Graphs illustrating the concentration of drugs in isolated lysosomes before and after a 12 h chase without drug in the media. **a** the concentration GSK045A in isolated lysosomes does not change significantly following a 12 h chase period. **b** The concentration of GSK171B decreases significantly following a 12 h chase. All data are expressed as the average of three independent experiments ± S.D.

proteins at the plasma membrane (i.e., p-glycoprotein, MRP1), which could significantly reduce cellular drug accumulation (4).

The normal pH of lysosomes and cytosol in the MDR HL-60 cell line is 5.2 and 7.1, respectively (4). Therefore, under normal circumstances weakly basic drugs such as GSK045A can be extensively sequestered in the lysosomes of this cell line considering the extensive pH differential between the compartments (~2 units). Theoretically, increasing lysosomal pH to levels near the levels in the cell cytosol would abolish the pH gradient between these spaces and therefore eliminate the driving force for drug sequestration. To evaluate if lysosomal sequestration of GSK045A was occurring by pH-partition, the HL-60 cells were pre-incubated with the vacuolar-ATPase inhibitor concanamycin A (Con A). When treated with Con A we have previously found (4), and reconfirmed again for this study, that lysosomal pH increased to 7.1 and the cell cytosol was also slightly elevated to 7.2, thus abolishing the pH differential. In Fig. 4, it is clear that the accumulation of GSK045A is dramatically reduced in cells treated with Con A, which supports a pH-partitioning type sequestration mechanism in lysosomes.

In previous work in our laboratory we have shown experimentally that at least two parameters are important factors in determining the extent of lysosomal sequestration of weakly basic small molecules. The first is the pKa value of the base (12). In this publication we describe experiments with a series of aminoquinoline structural isomers with variable pKa values and have shown that pKa is proportional to the extent of lysosomal sequestration. Those compounds with pKa values below 5 had virtually no lysosomal sequestration. A second important parameter relates to the delocalization of the positive charge on the molecule (13). Specifically, amines with high pKa values and extensively delocalized positive charges did not accumulate into lysosomes but instead preferentially accumulated into mitochondria. Considering the structure of the agonists (Fig. 1) it is apparent that all have relatively localized positive charges and this characteristic would favor lysosomal sequestration.

In attempts to make derivatives of GSK045A with reduced potential for lysosomal sequestration one could either make changes that lower the pKa or increase the delocalization capacity of the positive charge. The latter would most certainly require dramatic structural changes and was not considered further. The basicity, on the other hand, is easier to manipulate and it was possible to decrease the pKa from 9 (for the parent compound GSK045A) to 5.3 by using a relatively minor chemical addition shown in GSK171B (Fig. 1). According to our previous structure-localization relationship work relating pKa and lysosomal sequestration, a compound with pKa near 5 should not be sequestered (12).

To comparatively examine lysosomal retention we first utilized the HL-60 cells. Cells were incubated with either GSK045A (pKa 9.0) or GSK171B (pKa 5.3) Following drug incubations, the lysosomes were isolated either immediately after the incubation or following a 12 h chase in media devoid of the drugs. As is shown in Fig. 5, the concentration of GSK045A

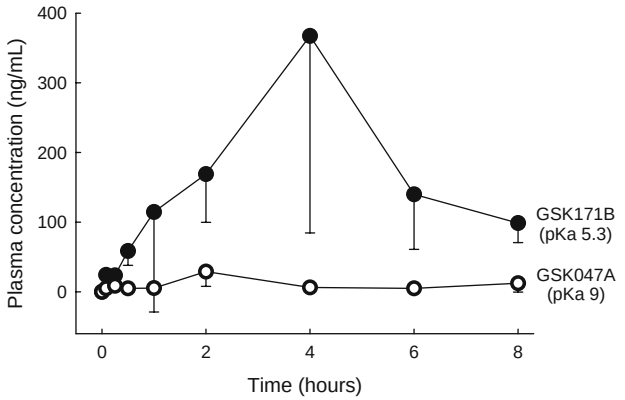

Fig. 6. Plasma versus time profiles for mice dosed orally with 10 mg/kg of GSK171B (pKa 5.3) or GSK047A (pKa 9) to illustrate differences in plasma levels. The data are represented as the average of three independent experiments ± S.D. GSK171B had an area under the plasma concentration vs. time curve (AUC) that was approximately 17 fold-higher compared to GSK0471. Although GSK171B had what appeared to be erratic absorption, reflected by the *large error bars*, the mean area under the curves were statistically different ($P<0.01$).

remains virtually unchanged whereas GSK171B was significantly reduced after a 12 h time period without drug.

The *in vitro* results were encouraging and warranted further evaluations in mice. The half lives were measured after mice received a 30 mg/kg/day oral dose of GSK045A (or a molar equivalent dose of GSK171B) for a 2 week period of time afterwards. Consistent with our results in cells, the half life of GSK171B in the mouse liver was significantly reduced relative to GSK045A. For GSK045A with a pKa 9 the liver half life was 168 h and for GSK171B with a pKa of 5.3 the half life was reduced to 7 h.

The purpose of the work was to reduce liver retention in an effort to increase plasma levels. In order to evaluate this we dosed mice with a high pKa compound GSK047A and compared the plasma levels with the molar equivalent dose of the lower pKa compound GSK171B. Consistent with reduced liver retention observed with GSK171B we observed a significant increase in plasma levels relative to GSK047A (See Fig. 6). The area under the plasma versus time curve (AUC) from 0 to 8 h was calculated for each drug in each mouse and averaged. The AUC was 87±24 ng*hr/mL for GSK047A compared to 1492±567 ng*hr/mL for GSK171B.

CONCLUSIONS

Recently, many investigators have begun to realize that the intracellular distribution of a drug is often an extremely important determinant in drug activity. Despite the fundamental importance, it is not experimentally evaluated in most instances. This is most likely attributed to the difficulty in performing such studies, unless the molecule is fluorescent and its distribution can be easily evaluated using a fluorescence microscope. Despite the difficulties, it is the authors' opinion that this type of investigation represents a new frontier in drug delivery research where much can be learned and applied to improve drug efficacy. For example, evaluations on the intracellular distribution of drugs have led to the elucidation of novel drug resistance mechanisms (4,11,14), the development of novel drug targeting strategies (15) and, as exemplified by this work, the development of drugs with improved distribution. It is anticipated that in the future, new techniques will be developed to facilitate these evaluations, making them more routine. It is far too common that drugs are developed that have good activity *in vitro* with isolated receptors yet fail in *in vivo* evaluations. The reasons for these failures are certainly multi-factorial but it is anticipated that this type of research will provide a step in the right direction in efforts to bring these drugs to market.

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