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Original article

Synthesis and bioactivity of sphingosine kinase inhibitors and their novel aspirinyl conjugated analogs

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ABSTRACT

Sphingosine kinase (SphK) is a lipid kinase with oncogenic activity, and SphK inhibitors (SKIs) are known for their anti-cancer activity. Here, we report highly efficient syntheses of SKIs and their aspirinyl (Asp) analogs. Both SKIs and their Asp analogs were highly cytotoxic towards multiple human cancer cell lines; in several cases the Asp analogs were up to three times more effective. Furthermore, they were equally potent inhibitors of SphK. The pharmacokinetic study indicated that SKI-I-Asp cleaved efficiently to form SKI-I and the half-life of SKI-I was increased from ~7 h in SKI-I to ~10 h in SKI-I-Asp injected mice, thereby prolonging its effect. In summary, the Asp-conjugated SKIs seem to be promising prodrugs of SKIs where delivery *in vivo* remains a problem.

1. Introduction

Sphingosine kinase 1 (SphK1) is an oncogenic lipid kinase closely linked to signaling pathways associated with cancer cell proliferation/survival, metastasis and multi-drug resistance (MDR) (for review [1–6]). SphK1 regulates the fate of cells by catalyzing the formation of sphingosine-1-phosphate (S1P) at the expense of ceramide. Over-expression of SphK1 is commonly observed in many tumor tissues relative to patient matched normal mammary tissues [7,8]. Importantly, studies showed that high SphK1 expression levels were a prognostic factor for decreased metastasis-free survival and overall poor outcome of patients with hormone-independent (i.e. ER^-) tumors [9,10]. Based on these and many other studies [11–24], it has been proposed that targeting SphK1 is a novel approach for the treatment of cancers including metastatic and/or MDR. In this regard, we have previously identified several "drug-like" compounds that potently inhibited SphK1 activity, and showed that these novel SphK inhibitors (SKIs) are potent inducers of cytotoxicity against various human cancer cell lines [7]. Among these, two compounds SKI-I and SKI-II (Fig. 1) were the most effective and most studied agents.

SKI-II has been shown to be an excellent agent that inhibits endogenous S1P formation in intact cells, SKI-II has a high degree of specificity towards SphK1 with low cross-reactivity towards other kinases, SKI-II treatment effectively inhibits tumor growth in a syngenic mouse model of breast cancer [7,25]. In contrast, although our ongoing studies show that SKI-I is more potent than SKI-II, the poor solubility and minimal bioavailability of these compounds have become the main problems in their efficacy as anti-cancer agents in *in vivo* animal studies. To overcome this problem, we designed and synthesized prodrug conjugates of SKI-I and SKI-II. Since both these inhibitors have a phenolic hydroxyl group, esterification was considered to be the method of choice for developing their prodrug conjugates. In this regard, we made use of 2-(acetoxy)benzoate (aspirinyl) conjugation. The rational for making the aspirinyl derivatives of SKI-I and SKI-II as prodrug conjugates was that the 2-(acetoxy)benzoic acid (aspirin), released upon hydrolysis by esterase (Fig. 1), is already a well established non-steroidal anti-inflammatory drug. It has shown chemopreventive effects against the risk of developing various cancers [26–30] and heart diseases [31]. US Preventive Services Task Force (USPSTF, March, 2009) has recommended the use of aspirin for the primary prevention of coronary heart disease. Regular low dose (~81 mg), amount in a baby aspirin is actually recommended for

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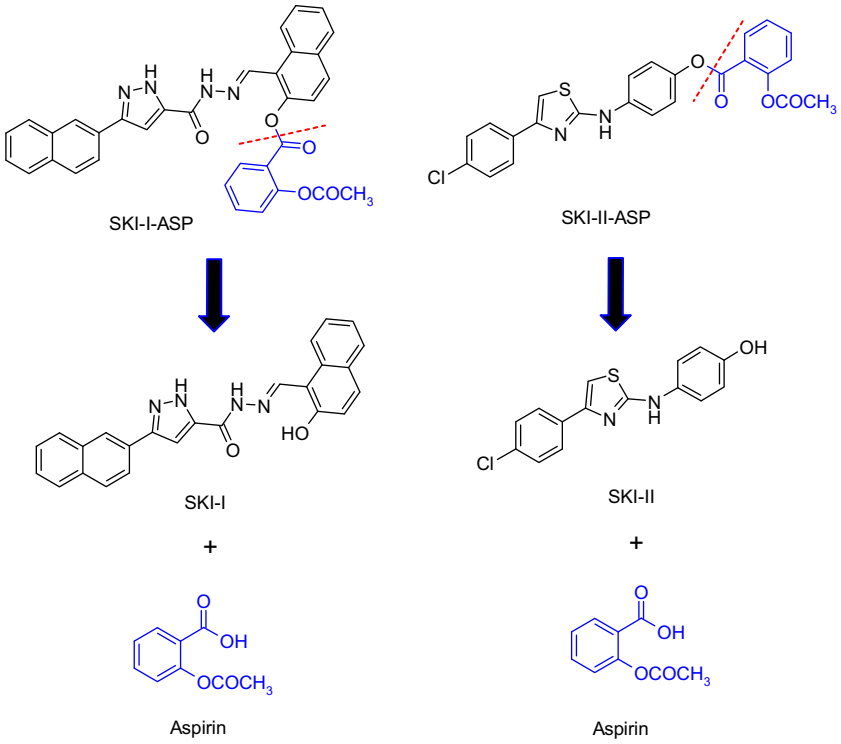


Fig. 1. Formation of SKI-I and SKI-II from corresponding aspirinyl derivatives.

people who are at a high risk of having heart diseases. At high doses aspirin may cause undesirable side effects e.g. gastrointestinal ulcers. However, in a cancer therapy setting, as is the case for SKIs, the release of aspirin outweighs the risks. The release of aspirin is therefore not expected to cause side effects and could potentially add its anti-inflammatory properties.

In this study, we describe simple and novel synthetic method for SKI-I and SKI-II and their aspirinyl substituted prodrugs SKI-I-Asp (**10**) and SKI-II-Asp (**14**). The cytotoxicity and activity of aspirinyl derivatives were compared with the corresponding parent compounds and the suitability of the aspirinyl analogs was determined by evaluating their degradation pattern in *in vitro* culture conditions and in a mouse model.

2. Results

2.1. Chemistry

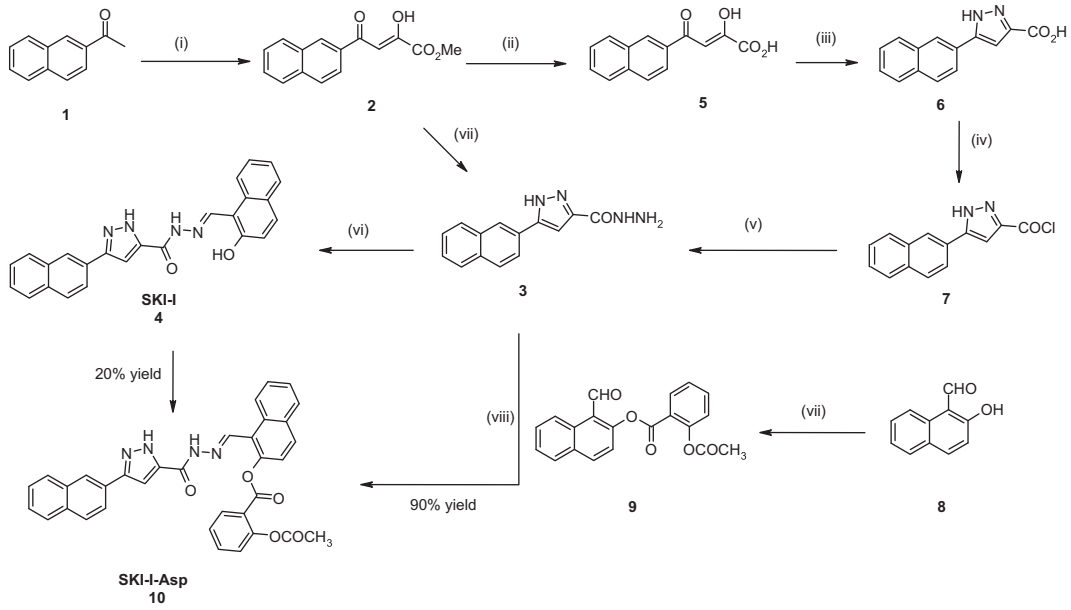
The synthesis of SKI-I was accomplished in just three steps as outlined in Scheme 1. The synthesis of ester **2** was carried out using a literature method [32], by reacting commercially available 2′-acenaphthone (**1**) and dimethyl oxalate in the presence of sodium hydride with excellent yield. Treatment of **2** with anhydrous hydrazine [33] led to the formation of pyrazole hydrazide **3** which on condensation with 2-hydroxynaphthaldehyde under standard conditions [34] furnished the desired SKI-I (**4**) in good yield. Alternatively, the synthesis of SKI-I can also be achieved by first hydrolyzing the ester **2** using 1 N HCl following a literature procedure [35] to yield the corresponding acid **5** followed by treatment with hydrazine [32] to the form the pyrazole derivative **6** as a white solid. Acid chloride derivative **7** was obtained by reaction of **6** with thionyl chloride. Refluxing of **7** with anhydrous hydrazine [33] in ethanol yielded hydrazide **3**, which was then converted to SKI-I (**4**) as described above.

The solubility/bioavailability of SKI-I is the main hurdle in its delivery to animals. The compound tends to precipitate in the body and is thus not readily bioavailable. To address this problem, we synthesized an aspirinyl conjugate of SKI-I. First approach was to treat SKI-I with 2-acetoxybenzoyl chloride in the presence of a base. However, this reaction led to multiple products resulting in lower yields. Several attempts to enhance the yield of this step failed. We then changed the strategy and treated 2-hydroxy-1-naphthaldehyde (**9**) with 2-acetoxybenzoyl chloride in the presence of potassium carbonate to first synthesize intermediate **9** (Scheme 1). The use of triethylamine as a base instead of potassium carbonate in this alkylation resulted in multiple products leading to lower yield of **9**. Condensation of **9** with hydrazide **3** finally gave the desired SKI-I-Asp (**10**) in excellent yield (Scheme 1). The aspirinyl linkage provided facile solubility in organic solvents and easy purification of this compound.

To generalize the feasibility of aspirinyl prodrugs, we also synthesized 2-(acetoxy)benzoate derivative of SKI-II (Scheme 2). SKI-II (**13**) was prepared by condensing 2-bromoketone (**11**) with thiourea **12** following a literature method [36]. In contrast to SKI-I, SKI-II reacted efficiently with 2-acetoxybenzoyl chloride in the presence of triethylamine to yield the desired product SKI-II-Asp (**14**). All the intermediates and the final products were purified by silica gel column chromatography and characterized on the basis of NMR and high-resolution mass spectra.

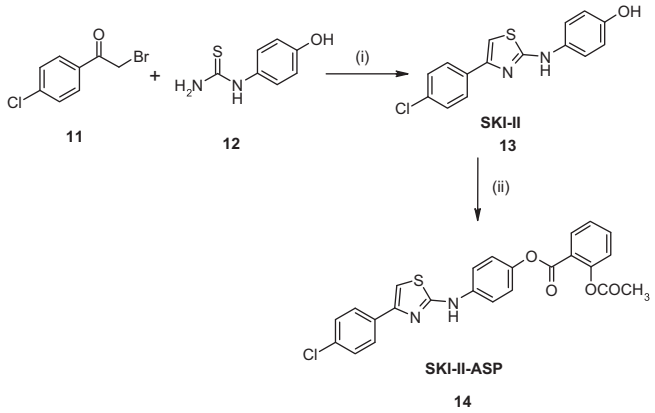
2.2. SKIs and their aspirinyl analogs induce human cancer cell cytotoxicity

We have previously demonstrated that both SKI-I and SKI-II compounds are potent inducers of cancer cell cytotoxicity [7]. To determine whether aspirinyl linkage to SKI-I or SKI-II would alter the cytotoxic potency, we performed the Sulforhodamine B (SRB) cytotoxicity assays on human cancer cell lines which include brain (U78MG), ovarian (HeLa), lung (H460, H226, H441, and A549),



Scheme 1. Synthesis of SKI-I (**4**) and SKI-I-Asp (**10**). *Reagents and conditions:* (i) NaH, dimethyl oxalate, C_6H_6 and MeOH, RT, overnight (ii) 1 N HCl, 1,4-dioxane, reflux, 2 h (iii) hydrazine hydrate, EtOH, reflux, 30 min (iv) $SOCl_2$, C_6H_6, reflux, 5 h (v) hydrazine hydrate, EtOH, reflux, overnight (vi) 2-hydroxynaphthaldehyde, AcOH (cat.), DMSO (vii) K_2CO_3, THF, 16 h, RT, (viii) AcOH (cat.), DMSO, RT, 3 h.

breast (MDA-MB-231), and pancreatic (Mia-PaCa2), and immortalized normal human mammary epithelial cell line, MCF-10A, and mouse embryonic fibroblast (MEF) cell line (cancer cell lines). Cancer cells (5×10^3) were cultured in a 96 well plate for 24 h, and then treated with various concentrations of SKIs and their aspirinyl conjugates or control DMSO for 72 h. As shown in Table 1, the cytotoxic potency (indicated by IC_{50} values) of aspirinyl-linked SKI analogs was similar or better than their parental SKI compounds. Specifically, the IC_{50} values of SKI-I and its prodrug SKI-I-Asp remained essentially the same towards brain and ovarian cancer cell lines, whereas SKI-I-Asp was approximately ~3 times more potent than the parent SKI-I compound towards breast, pancreatic and all the four lung cancer cell lines. Additionally, consistent with our previous findings [7], the SKI-I and its aspirin derivative SKI-I-Asp showed lower IC_{50} values compared to the SKI-II or its aspirin derivative SKI-II-Asp. Similar results were observed in normal human mammary epithelial cell line, MCF-10A. Together, these results indicate that aspirinyl linkage of SKIs may improve the cytotoxic potency of parental SKI compounds [7].



Scheme 2. Synthesis of SKI-II (**13**) and SKI-II-Asp (**14**). *Reagents and conditions:* (i) EtOH, reflux, 1.5 h, NH_4OH (ii) Et_3N, THF, overnight, RT.

2.3. Metabolism of aspirinyl prodrugs of SKIs in cell culture media

As indicated above in cytotoxicity studies, the parent compound SKI-II and its aspirinyl derivative **14**, were equally cytotoxic in various cancer cell lines (Table 1). On the other hand, aspirinyl derivative **10** of SKI-I was more potent than the parent compound SKI-I. This could be due to fact that aspirinyl-linked SKIs are more stable and resistant to spontaneous rapid cleavage of the ester bond in 10% FBS containing culture media. To test this potential possibility of degradation, we subjected SKIs and their aspirinyl-linked analogs to same culture condition (10% FBS). Specifically, SKI-I and SKI-II were incubated in DMEM culture medium at 37 °C for 2, 16, and 72 h time points. Subsequently, samples were extracted with ethyl acetate and analyzed for decomposition using HPLC. We determined the ratio of asprinyl derivatives **10** and **14** to their corresponding parent compounds **4** and **13**, respectively (Fig. 2A). In case of SKI-I-Asp (**10**), ~40% of the aspirinyl derivative was decomposed to SKI-I in just 2 h. After 16 h, ~80% of the compound was converted. After 72 h, the formation of SKI-I remained constant at ~82%. The SKI-II-Asp (**14**) derivative degraded to a nearly similar extent in 72 h (Fig. 2B) but a little more slowly and steadily. About 15% SKI-II was formed in 2 h, ~50% in 16 h and ~75% at the end of 72 h i.e. about 25% SKI-II-Asp (**14**) was still remaining after 72 h. To

Table 1
IC_{50} (μM) of SKIs and their aspirinyl analogs using SRB assay[a] (72 h treatment).

Cancer/normal cell lines	Tissue	SKI-I	SKI-I-Asp	SKI-II	SKI-II-Asp
U87MG	Brain	1.0 ± 0.2	1.5 ± 0.5	10.7 ± 1.1	8.1 ± 1.6
HeLa	Ovary	1.0 ± 0.3	1.0 ± 0.1	9.1 ± 1.5	11.2 ± 2.9
H460	Lung	3.2 ± 1.0	1.2 ± 0.5	9.2 ± 2.3	9.1 ± 0.8
H226	Lung	5.7 ± 2.1	1.7 ± 0.3	8.6 ± 1.7	6.7 ± 1.6
H441	Lung	2.0 ± 1.2	0.6 ± 0.2	8.0 ± 0.9	5.7 ± 1.9
A549	Lung	3.8 ± 1.6	0.9 ± 0.1	7.8 ± 0.8	5.9 ± 2.1
MDA-MB-231	Breast	4.4 ± 1.7	1.3 ± 0.9	8.2 ± 1.0	8.4 ± 1.4
Mia-PaCa2	Pancreas	4.8 ± 2.6	1.6 ± 0.5	7.7 ± 1.4	8.7 ± 1.4
MCF-10A	Breast	4.1 ± 1.5	2.6 ± 1.2	14.5 ± 2.2	11.3 ± 2.0
MEF	Fibroblast	5.0 ± 1.3	5.5 ± 0.7	8.9 ± 1.2	9.4 ± 1.8

[a] All values represent the average from three independent experiments.

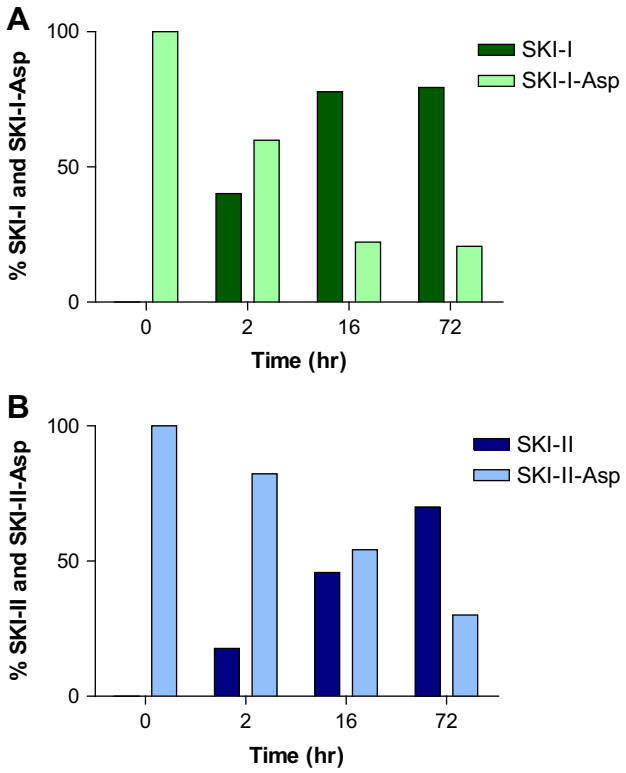


Fig. 2. Serum metabolism of (A) SKI-I-Asp and (B) SKI-II-Asp *in vitro:* 1 mg/ml of each compound in DMSO was dissolved in 5 mL 10% FBS supplemented DMEM or serum free DMEM media in 15 mL conical tubes. The tubes were placed in a shaking incubator at 37 °C. At 0, 2, 16 and 72 h samples were taken out, filtered, and analyzed using HPLC. The percentage of SKIs and SKI-Asp peaks was calculated for each time point and plotted.

determine if the degradation is due to an enzymatic reaction or solely due to the pH dependent hydrolysis in the media, we incubated SKI-I-Asp and SKI-II-Asp with serum free media. Interestingly, both SKI-I-Asp and SKI-II-Asp remained unchanged even after 72 h of incubation at 37 °C. This suggested that the degradation is due to some enzymatic hydrolysis.

Taken together with the cytotoxicity data, this suggests that the effect seen for aspirinyl derivatives in the *in vitro* studies may not solely be due to the decomposition to the parent compounds, but that the aspirin derivatives themselves may also have some contribution towards the overall observed cytotoxicity. This argument is strengthened by the fact that the cells absorbed most of the compound in the initial 1–2 h when 60–85% of aspirinyl derivatives of both SKI-I and SKI-II were still present within the cell.

2.4. Metabolism of aspirinyl prodrug of SKI-I in mice

To determine the decomposition patterns of SKI-Asp analogs *in vivo,* mice were injected with SKI-I and its prodrug SKI-I-Asp (60 mg/kg body weight) and the blood was collected at 1, 2, 4, 8, and 16 h time points. Each sample was subjected to HPLC analysis and compared with SKI-I and SKI-I-Asp standards. One hour after injection, the HPLC profile of blood from SKI-I-Asp treated mice revealed the presence of only the SKI-I peak and did not show any SKI-I-Asp signal. The maximum serum concentration (C_{max}) of SKI-I was observed 2 h after injection of both SKI-I and its corresponding aspirinyl analog. Interestingly, the C_{max} of SKI-I from SKI-I-Asp mice was ~50% of SKI-I injected mice, instead of being ~70% as expected from the molar ratio of SKI-I (MW 407) fragment in SKI-I-Asp (MW 591) structure (Fig. 3), suggesting that the dose of SKI-I-Asp could be increased by nearly 40% without any additional systemic toxicity. Furthermore, the concentration of SKI-I in SKI-I-Asp injected mice decreased more slowly over time as compared to SKI-I (Fig. 3). Therefore, the half-life of SKI-I in SKI-I injected mice was ~7 h which was increased in SKI-I-Asp injected mice to ~10 h.

2.5. Sphingosine kinase activity assay

The data from the cytotoxicity assays, presented above, indicates that the SKI lead compounds and their aspirinyl derivatives have essentially equal cytotoxicity across a panel of cancer cell lines. This finding suggests that the aspirinyl derivatives also have roughly equal inhibitory activity towards SphK1. To confirm this, we treated HeLa cells with increasing concentrations of the SKIs and their aspirinyl derivatives for 24 h prior to performing *in vitro* SphK1 activity assays. As shown in Fig. 4, at lower doses (i.e., <1.25 μM), the SKI-I-Asp and SKI-II-Asp derivative were equally effective at inhibiting SphK1 as the parent SKI compounds. At higher doses (≥5 μM), the aspirinyl derivatives were less effective than the parent SKI compounds.

3. Discussion

The aspirinyl analogs of SKIs were designed to take advantage of the fact that apart from an easy release of the parent compound by the hydrolysis of the ester linkage by esterases on administration *in vivo,* aspirin will be released as a side product. Aspirin is a well established anti-inflammatory drug and does not require any additional study on determining its toxicity or ADME profile. Therefore, its release in the process is not expected to cause any additional systemic toxicity and in fact could potentially add its anti-inflammatory effect in addition to the anti-cancer effect of SKIs. The synthesis of SKI-I was accomplished in just three steps in a good overall yield. The aspirinyl analog of SKI-I could not be synthesized from SKI-I treatment with 2-acetoxybenzoyl chloride, however pretreatment of 2-acetoxybenzoyl chloride with 2-hydroxynapthaldehyde followed by condensation led to a very good yield of SKI-I-Asp (**10**) (Scheme 1). The synthesis of SKI-II-Asp (**14**) on the other hand was efficiently obtained from SKI-II, synthesized in just one step (Scheme 2), by treatment with 2-acetoxybenzoyl chloride.

The comparison of the cytotoxicity of SKIs and their corresponding aspirinyl analogs showed that SKI-I-Asp was ~3 times more effective than SKI-I in killing breast, pancreas and all the lung cancer cells tested (Table 1), while being equally cytotoxic to brain

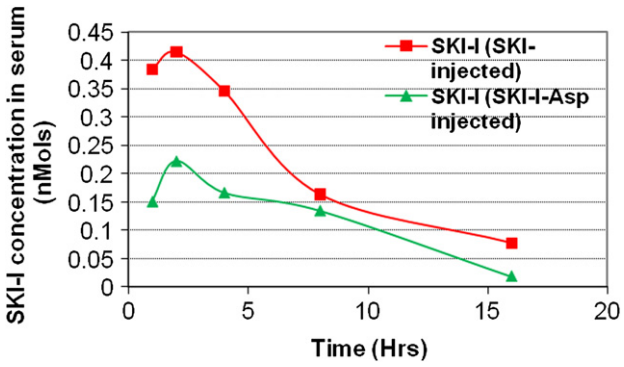


Fig. 3. Pharmacokinetics in mice (C3H-HEJ strain): Mice were injected with con-genic mouse AML cell line called p210-GFP. GFP is fluores-tag. Both the SKI-I and SKI-I-Asp were injected 60 mg/kg (ip). Serum was collected at 1, 2, 4, 8, and 16 h time points and analyzed by HPLC.

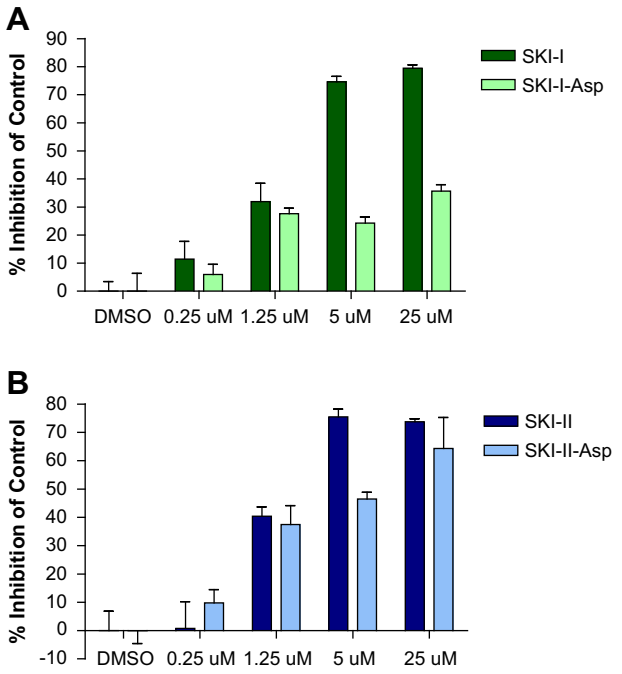


Fig. 4. SphK1 activity assay: HeLa cells were treated for 24 h with either DMSO (vehicle) or increasing concentrations of SKIs or their aspirinyl derivatives. *In vitro* SphK1 activity assays were performed on 10 μg of cytosol in triplicate ($n = 3$).

and ovarian cancer cells. However, SKI-II and SKI-II-Asp (**14**) were equally effective in killing various cancer cells e.g. brain, ovarian, lung, breast, and pancreatic cancer cells. This suggested that the observed activity of aspirinyl derivatives may actually be the activity of parent SKIs that get cleaved during the cells treatment. On the other hand equal cytotoxicity could as well be the result of a coincidental similar activity of the SKI-Asp derivatives. To determine if the activity was due to the aspirinyl derivatives themselves or due to the release of parent SKI inhibitors, metabolism studies of the aspirinyl derivatives SKI-I-Asp (**10**) and SKI-II-Asp (**14**) were conducted in culture conditions in the presence as well as in the absence of serum. Incubation of **10** and **14** in cell culture media (DMEM supplemented with 10% FBS, without cells) indicated that both the aspirinyl analogs cleaved over time to release the corresponding parent compounds but about 18–25% of the Asp analogs still remained at the end of 72 h treatment (the time used for the treatment of cells in SRB assay). This suggested that similar cytotoxicity observed for aspirinyl conjugates **10** and **14** and corresponding parent compounds in SRB assay, may either be due to an enzyme secreted from the cells to the culture medium that catalyzed the cleavage further to release the parent SKIs or SKI-Asp analogs themselves contribute to the overall cytotoxicity of these compounds. It is important to note that no degradation of aspirinyl analogs was observed in the serum free media (DMEM, without 10% FBS or cells) indicating that degradation in media with serum is due to some secreted cellular enzymatic activity.

The degradation of aspirinyl analogs was not complete under *in vitro* culture conditions. An ideal prodrug, once administered, is metabolized *in vivo* to an active metabolite. Therefore, to establish the cleaving ability of aspirinyl group from the SKI-Asp prodrugs *in vivo,* we evaluated this phenomenon in mice with SKI-I-Asp. Interestingly, no trace of SKI-I-Asp was observed just after 1 h of administration. The concentration of SKI-I in the serum in SKI-I-Asp injected mice, peaked at the same time (2 h after injection) but was lower as compared to SKI-I injected mice. The lower than expected concentration of SKI-I, the lack of presence of SKI-I-Asp in the blood, and a slow decline in SKI-I concentration in SKI-I-Asp injected mice suggests that a part of SKI-I-Asp may be absorbed in tissues and release into blood stream overtime, keeping blood levels of the SKI-I from decreasing rapidly and thereby prolonging the effect of the drug.

The systemic toxicity and potency of a drug is generally decided by its concentration in blood at a given time. The lower concentration of SKI-I at a given time in SKI-I-Asp treated mice also suggests that SKI-1-Asp dose may be increased to match comparable C_{max} of SKI-I in the blood after 2 h, as observed for SKI-I treated mice, without causing additional systemic toxicity, thus lowering the dosing frequency.

At lower doses (i.e., <1.25 μM), aspirinyl conjugates **10** and **14** were equally effective at inhibiting SphK1 as the parent SKI compounds, while at higher doses (≥5 μM), the aspirinyl derivatives were less effective than the parent SKI compounds (Fig. 4). Whether this is due to the fact that either aspirinyl-linked SKI results in less affinity for SphK1 or less availability of the active SKI (as indicated in Fig. 2) due to slow conversion of aspirinyl derivatives to active SKI by cleavage of the ester bond, is not known at this time. However, the fact that the SKI-I-Asp (**10**) readily cleaves to SKI-I *in vivo,* a little lower SphK1 activity shown by aspirinyl analogs at higher concentrations would not be of much importance. In either case, it is clear that aspirinyl-linked SKI compounds are potent inhibitors of SphK1 demonstrating the feasibility of parent SKI compounds modifications to improve their cytotoxic potency without the loss of specificity towards SphK1.

4. Conclusion

In summary, practical syntheses of SKI-I and SKI-II and their aspirinyl derivatives were accomplished in high overall yields. The syntheses are not only short and efficient but also provide a strategy to procure additional analogs for further structure–activity studies. Both the parent SKIs and their aspirinyl derivatives showed similar cytotoxicity in SRB assays in most cases except that in lung, breast and pancreatic cancer cell lines, SKI-I-Asp was ~3 times more effective as compared to SKI-I. The SphK1 activity of parent SKIs and their corresponding aspirinyl conjugates was comparable at lower doses while at higher doses of 5 and 25 μM, the aspirin analogs showed lower activity. The degradation pattern under the cell culture media conditions indicated that ~75–82% of the aspirinyl derivatives were cleaved to the corresponding parent compounds by the end of the assays (72 h). *In vivo* however, the SKI-I-Asp was completely metabolized to SKI-I within 1 h. Collectively, the results suggest that aspirinyl prodrug conjugates of the SKI-I and SKI-II may be effective surrogates for the parent compounds. Further studies are, however, warranted to determine the efficacy of these compounds.

5. Experimental section

5.1. Chemistry

Melting points were recorded on a Fischer-Johns melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker Avance 500 MHz spectrometer. Chemical shifts (δ) were reported in parts per million (ppm) downfield from the internal standard. The signals are quoted as s (singlet), d (doublet), t (triplet), m (multiplet) and dt (doublet of triplet). High-resolution MS (EI) were determined at the Chemistry Instrumentation Center, State University of New York at Buffalo, NY. Thin-layer chromatography (TLC) was developed on aluminum-supported pre-coated silica gel plates (EM industries, Gibbstown, NJ). Column chromatography was conducted on silica gel (60–200

mesh). The decomposition of aspirinyl derivatives was analyzed by HPLC (Waters Breeze System) on a 4.6 × 250 mm (5 μM) Bondclone C18 reverse-phase column (Phenomonex) using solvent system 1 [solvent A (0.1% TFA in water) and solvent B (acetonitrile), using a linear gradient program from A:B (70:30) to 50% B over 20 min, to 65% B next 5 min, to 70% B next 20 min, to 100% B next 5 min] or solvent system 2 (A: Water, B: Methanol; 50% B to 75% B in 0–10 min, 75% B to 100% B in 10–45 min, 100% B in 45–50 min).

5.1.1. Methyl-(2-hydroxy-4-naphthalen-2-yl-4-oxo-but-2-enoic) acetate (**2**)

To a suspension of sodium hydride (4.17 g, 116.2 mmol, 60% oil dispersion) in benzene (150 mL) at 0 °C was added methanol (5 mL) dropwise. To this cold mixture was added a solution of 2-acetonaphthone (10 g, 58.7 mmol) and dimethyl oxalate (13.88 g, 117.5 mmol) in benzene (150 mL) dropwise. The mixture was allowed to warm to room temperature, stirred overnight, quenched with 1 N HCl solution, and filtered. The residue was purified through silica gel column chromatography using methylene chloride:hexanes (7:3) as an eluent to yield 7.5 g (50%) of **4** as a yellow solid; mp 104–106 °C; ^{1}H NMR (Acetone-d_6): δ 8.83 (1H, s), 8.21 (1H, d, J = 8.0 Hz), 8.15–8.06 (2H, m), 8.04 (1H, d, J = 2.0 Hz), 7.73 (1H, t, J = 6.4 Hz), 7.68 (1H, t, J = 8.0 Hz), 7.35 (1H, s), 3.95 (3H, s); HRMS (ESI) calcd for $C_{15}H_{12}O_4.H^+$, 257.0811; found, 257.0815.

5.1.2. 2-Hydroxy-4-naphthalen-2-yl-4-oxo-but-2-enoic acid (**5**)

To a suspension of **2** (13.0 g, 50.7 mmol) in 1,4-dioxane (200 mL) at room temperature was added 1 M HCl (200 mL) and the mixture was refluxed for 4 h. The reaction mixture was cooled to room temperature and the solvent was removed under reduced pressure. The crude yellow solid thus precipitated was filtered, and washed with water and diethyl ether. The crude solid was crystallized from toluene and ethyl acetate to yield 12.0 g (97%) of acid **5** as a yellow solid; mp 170–171 °C; ^{1}H NMR (Acetone-d_6): δ 8.85 (1H, s), 8.21 (1H, d, J = 7.0 Hz), 8.16–8.14 (2H, m), 8.06 (1H, d, J = 7.0 Hz), 7.73 (1H, t, J = 6.5 Hz), 7.68 (1H, t, J = 7.0 Hz), 7.35 (1H, s); MS (ESI) 243 ($M^+ + 1$).

5.1.3. 5-Naphthalen-2-yl-1H-pyrazole-3-carboxylic acid (**6**)

A mixture of **5** (12.3 g, 49.6 mmol), anhydrous hydrazine (3.97 g, 124.0 mmol) and absolute EtOH (250 mL) was heated to reflux for 30 min. The precipitated hydrazide was filtered, washed with diethyl ether and dried under vacuum to give **6** (8.7 g, 74%) as a white solid; mp 265–267 °C; ^{1}H NMR (DMSO-d_6): δ 8.4 (1H, s), 8.03–7.93 (4H, m), 7.51–7.57 (2H, m), 7.35 (1H, s); HRMS (ESI) calcd for $C_{14}H_{10}N_2O_2.Na^+$, 261.0634; found, 261.0648.

5.1.4. 5-Naphthalen-2-yl-1H-pyrazole-3-carbonyl chloride (**7**)

To a suspension of **6** (8.0 g, 33.6 mmol) in dry benzene (150 mL) under nitrogen was added thionyl chloride (4.9 mL, 67.2 mmol) dropwise with stirring at room temperature. The reaction mixture was refluxed for 5 h and the volatiles were removed under reduced pressure. The crude solid thus obtained was triturated with diethyl ether and filtered to yield **7** (7.8 g, 91%) as a yellow solid; mp 185 °C (dec.); ^{1}H NMR (DMSO-d_6): δ 8.42 (1H, s), 8.03–7.93 (4H, m), 7.59–7.51 (2H, m), 7.36 (1H, s); HRMS (EI) calcd for $C_{14}H_9ClN_2O$, 256.0398; found, 256.0406.

5.1.5. 5-Naphthalen-2-yl-1H-pyrazol-3-carboxylic acid hydrazide (**3**)

Method 1: Anhydrous hydrazine (1.69 mL, 54.7 mmol) was added to a suspension of **7** (7.0 g, 27.3 mmol) in EtOH (250 mL) and the mixture was refluxed overnight. The precipitated white solid was filtered, washed with EtOH and dried under vacuum to yield 6.3 g (91%) of **3** as a white solid.

Method 2: To a solution of methyl ester **2** (0.45 g, 1.76 mmol) in EtOH (20 mL) was added hydrazine hydrate (0.055 mL, 12.3 mmol) and the reaction mixture was refluxed for 6 h under nitrogen. The reaction mixture was cooled to room temperature and the precipitated white solid was filtered and washed with mixture of ethanol:hexanes (1:9) to yield 0.35 g (79%) of **3** as a white solid; mp >270 °C; ^{1}H NMR (MeOH-d_4): δ 9.31 (1H, s), 8.82–8.74 (4H, m), 8.32–8.38 (2H, m), 8.1 (1H, s); HRMS (ESI) calcd for $C_{14}H_{12}N_4O.Na^+$, 275.0903; found, 275.0904.

5.1.6. 5-Naphthalen-2-yl-1H-pyrazol-3-carboxylic acid (2-hydroxynaphthalen-1-ylmethylene)-hydrazide (**4**)

To a solution of hydrazide **3** (1.0 g, 4.0 mmol) in DMSO (50 mL) was added 2-hydroxy-1-naphthaldehyde (1.02 g, 5.9 mmol) in DMSO (10 mL) followed by a catalytic amount of acetic acid. The reaction mixture was stirred at RT overnight, poured in water (100 mL), and the solid thus formed was filtered and washed with methylene chloride:hexanes (7:3). The crude product was purified by crystallization from THF–haxenes mixture to afford 1.5 g (94%) of **1** as a pale yellow solid; mp 182–183 °C; ^{1}H NMR (DMSO-d_6): δ 14.1 (1H, s), 12.9 (1H, s), 12.3 (1H, s), 9.65 (1H, s), 8.25 (1H, s), 8.07–7.9 (7H, m), 7.64–7.56 (3H, m), 7.45–7.41 (2H, m), 7.25(1H, d, J = 7.5 Hz); ^{13}C NMR (DMSO-d_6): δ 158.4, 147.5, 133.5, 133.2, 132.2, 129.5, 129.2, 128.6, 128.3, 128.2, 127.3, 127.1, 124.6, 124.1, 124.0, 121.4, 119.4, 109.1; HRMS (ESI) calcd for $C_{25}H_{18}N_4O_2.H^+$, 407.1503; found, 407.1510.

5.1.7. 1-Formyl-2-(2-acetoxybenzoyloxy)naphthalene (**9**)

To a solution of acetylsalicyloyl chloride (1.5 g, 8.7 mmol) in dry THF (10 mL) was added dropwise to a mixture of 2-hydroxynaphthaldehyde (1.0 g, 5.81 mmol) and K_2CO_3 (1.2 g, 8.7 mmol) in THF (40 mL) at 0 °C under nitrogen atmosphere. The reaction mixture was warmed to room temperature and stirred vigorously for 16 h. The mixture was poured into water (40 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic phases were washed with brine (100 mL), dried ($MgSO_4$), and evaporated in vacuo. The crude product thus obtained was purified through column chromatography over silica using methylene chloride: hexanes (1:1) mixture to afford 1.3 g (72%) of **9** as a white solid; mp 117–119 °C; ^{1}H NMR (DMSO-d_6): δ 10.66 (1H, s), 9.06 (1H, d, J = 8.5 Hz), 8.41 (1H, d, J = 8.5 Hz), 8.33 (1H, d, J = 7.5 Hz), 8.12 (1H, d, J = 8.5 Hz), 7.84 (1H, t, J = 8.0 Hz), 7.8–7.74 (m, 1H), 7.69 (1H, t, J = 8.0 Hz), 7.57–7.54 (m, 2H), 7.39 (1H, d, J = 8.0 Hz), 2.25 (3H, s); MS (ESI) 357 (M^+ + Na).

5.1.8. SKI-I-Asp (**10**)

To a solution of **3** (0.396 g, 1.57 mmol) in DMSO (20 mL) was added salicyloyl derivative **9** (0.5 g, 1.57 mmol) followed by a catalytic amount of acetic acid. The mixture was stirred overnight at room temperature, poured into water (30 mL) and extracted with ethyl acetate. The organic layer was washed with brine (3 × 30 mL), dried ($MgSO_4$), and concentrated in vacuo. The residue was purified by silica gel column chromatography using a mixture of methylene chloride and hexanes (3:7) to afford 0.6 g (67%) of **10** as a white solid; mp 183–185 °C; ^{1}H NMR (DMSO-d_6): δ 9.34 (1H, s), 9.11 (1H, s), 8.41 (2H, d, J = 7.5 Hz), 8.26–7.96 (6H, m), 7.84 (1H, t, J = 7.5 Hz), 7.74 (1H, t, J = 7.0 Hz), 7.66 (1H, t, J = 7.0 Hz), 7.60–7.57 (3H, m), 7.44–7.39 (3H, m), 2.25 (3H, s); ^{13}C NMR (DMSO-d_6): δ 169.8, 163.1, 158.7, 151.3, 149.1, 147.3, 144.3, 143.7, 135.7, 133.4, 133.2, 132.9, 132.3, 131.0, 129.2, 129.1, 128.6, 128.5, 128.2, 127.4, 127.2, 127.0, 126.8, 126.7, 126.4, 124.7, 124.6, 124.1, 122.4, 122.2, 121.5, 104.5, 21.2; HRMS (ESI) calcd for $C_{34}H_{24}N_4O_5.Na^+$, 591.1639; found, 591.1624.

5.1.9. 4-[4-(4-Chlorophenyl)thiazol-2-ylamino]phenol (**13**)

To a solution of haloketone (1.4 g, 5.99 mmol) in ethanol (20 mL) was added 1-(4-hydroxyphenyl)-2-thiourea (1.0 g, 5.99 mmol) and

the reaction mixture was refluxed for 2 h. The hydrochloride formed was filtered and washed with ether and crystallized from aqueous ethanol. The hydrochloride was boiled with excess ammonium hydroxide. The solid thus obtained was filtered, washed with water and crystallized from 95% alcohol to yield 1.14 g (60%) of **13** as a white crystalline solid; mp 179–180 °C; ^{1}H NMR (DMSO-d_6): δ 9.94 (1H, s), 9.14 (1H, s), 7.91 (2H, d, J = 9.0 Hz), 7.48–7.45 (4H, m), 7.29 (1H, s), 6.75 (2H, d, J = 9.0 Hz); ^{13}C NMR (DMSO-d_6): δ 164.9, 152.9, 149.3, 134.0, 133.7, 132.3, 129.1 (2× CH_{arom}), 127.8 (2× CH_{arom}), 119.8 (2× CH_{arom}), 116.0 (2× CH_{arom}), 103.1; HRMS (ESI) calcd for $C_{15}H_{11}ClN_2OS.H^+$, 303.0353; found, 303.0355.

5.1.10. SKI-II-Asp (14)

A solution of acetylsalicyloyl chloride (0.98 g, 4.5 mmol) in dry THF (5 mL) was added dropwise to a mixture of **2** (1.0 g, 3.3 mmol) and Et_3N (0.45 g, 4.5 mmol) in THF (20 mL) at 0 °C under nitrogen atmosphere. The reaction mixture was warmed to room temperature and stirred vigorously overnight. The mixture was poured into water (50 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic phases were washed with brine (100 mL), dried ($MgSO_4$), and evaporated in vacuo. The crude product thus obtained was purified through silica gel column chromatography using methylene chloride:hexanes (3:1) mixture to afford 1.2 g (70.7%) of **14** as a white solid; mp 150–152 °C; ^{1}H NMR (DMSO-d_6): δ 8.25 (1H, dd, J = 8.0 and 2.0 Hz), 7.81 (2 H, d, J = 9.0 Hz), 7.68 (1H, dt, J = 8.0 and 2.0 Hz), 7.50 (1H, d, J = 9.0 Hz), 7.44 (1H, d, J = 7.5 Hz), 7.40 (1H, d, J = 8.5 Hz), 7.21 (3 H, d, J = 8.5 Hz), 76.84 (1 H, s), 2.35 (3H, s); ^{13}C NMR (DMSO-d_6): δ 169.7, 163.7, 163.5, 150.8, 149.3, 144.6, 139.6, 135.5, 133.8, 132.5, 132.2, 129.1 (2× CH_{arom}), 127.9 (2× CH_{arom}), 127.0, 124.6, 122.9, 122.6 (2× CH_{arom}), 118.2 (2× CH_{arom}), 104.4, 21.2; HRMS (ESI) calcd for $C_{24}H_{17}ClN_2O_4S.H^+$, 465.0670; found, 465.0680.

5.2. Cell lines and culture conditions

The following cell lines were employed for cytotoxicity and in vivo inhibitory studies: HeLa (ATCC: CCL2), U87MG (ATCC: HTB-14), H460 (ATCC: HTB-177), H226 (ATCC: CRL-5826), H441 (ATCC: HTB-174), A549 (ATCC: CCL-185), MDA-MB-231 (ATCC: HTB-26), and MiaPaCa2 (ATCC: CRL-1420). These cell lines were cultured in DMEM supplemented with 10% FBS at 37 °C in a 5% CO_2 atmosphere. Additionally, immortalized normal human mammary epithelial cell line MCF-10A (ATCC: CRL-10317), and mouse embryonic fibroblast, MEF (Dr. H.G. Wang, Pennsylvania State College of Medicine) were used as normal control cell lines.

5.2.1. Cytotoxicity assays

Sulforhodamine B (SRB) colorimetric assay was used to determine the cytotoxic effects of SKIs and their aspirinyl-linked SKI compounds according to the methods described by Vichai et al., [37] with minor modifications. Briefly, all cancer cell lines (5×10^3) were plated in a 96 well plate and 24 h later triplicate wells were exposed to DMSO (vehicle) or increasing concentrations (0.001, 0.005, 0.025, 0.1, 0.5, 1.0, 2.5, 5.0, 25.0, and 50.0 μM) of SKIs and their aspirinyl prodrugs for 72 h. After 72 h of treatments, cells were fixed in 10% trichloroacetic acid (TCA) for 1 h, TCA was discarded and plates were extensively washed with slow-running tap water. After air-drying the plates overnight at room temperature, cells in each well were stained with 100 μL of 0.057% (wt/vol) of SRB solution for 30 min and then the plates were rinsed four times with 1% (vol/vol) acetic acid to remove unbound dye. Plates were then allowed to dry at room temperature, and subsequently cells stained with SRB were incubated with 10 mM Tris base solution. After 1 h of solubilizing SRB stain, the absorbance (O.D.) was measured at 510 nm. Based on the absorbance results, IC_{50} for each SKI compounds were determined using the Prism software (GraphPad Prism 5.1).

5.3. In vitro SphK1 activity assay

HeLa uterine cancer cells, were preincubated for 24 h with increasing concentrations of SKIs or their derivatives with DMSO as a vehicle control. Cells were flash frozen in liquid nitrogen and cell lysates were centrifuged at 100,000*g* for 30 min at 4 °C. Supernatants were removed and 10 μg of total protein was added to a standard *in vitro* SphK1 activity assay as follows. Briefly, samples were combined with 50 μM D-erythro-sphingosine, 200 μM ATP and 2 μCi [γ-^{32}P] ATP in a 100 μL final reaction volume of SKAAB buffer (20 mM Tris pH 7.4, 1 mM β-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100, 1 mM Na_3VO_4, 15 mM NaF, 0.5 mM 4-deoxypyridoxine) for 30 min at 37 °C with shaking. Kinase reactions were terminated by the addition of 10 μL 6 N HCl and the labeled lipids were extracted by the addition of 400 μL of Chloroform/MeOH 100:200 v/v and 125 μL Chloroform and 125 μL 1 M KCl. The organic phase containing lipids was dried down under nitrogen stream. Samples were then resuspended in 30 μL chloroform and applied to a Silica Gel TLC plate (Whatman, Florham Park, NJ) and the lipids were separated using a butanol:water:acetic acid 3:1:1 v:v:v solvent system. The plates were analyzed by X-ray exposure and the region of the TLC plate corresponding to the R_f value (0.32) of sphingosine-1-phosphate (S1P) was scraped and the amount of S1P formed was determined by liquid scintillation counting [38].

5.4. Degradation of SKI-Asp analogs

1 mg/mL of each compound in DMSO was dissolved in 5 mL 10% FBS supplemented DMEM or serum free DMEM media in 15 mL conical tubes. The tubes were placed in a shaking incubator at 37 °C. At 0, 2, 16, and 72 h samples were taken out, filtered, and analyzed using Waters HPLC on Bondclone 10 μm C18 column and solvent system 1 or solvent system 2. The percentage of SKIs and their aspirin conjugates peaks was calculated for each time point and plotted.

5.5. Pharmacokinetic property studies

To determine pharmacokinetic properties of SKIs and their aspirinyl-linked analogs, we intraperitoneally injected mice (12–15 weeks old) with SKI compounds at concentration of 30 mg/kg. Subsequently, whole blood was collected from mice at various time points (1, 2, 4, 8 and 16 h). Collected blood was coagulated and serum was collected by centrifugation. Subsequently, serum was subjected to HPLC using solvent system 1 or 2 to determine the levels of SKIs or their aspirinyl-linked analogs. The amount of SKI compounds in serum was calculated by area under the curve using a standard curve and plotted against time.

5.6. Statistical analysis

Statistical analysis was undertaken using the One-way ANOVA followed by an appropriate *post hoc* test. Results were considered significant at a P-value of <0.05.

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References

[1] P. Signorelli, R. Ghidoni, J. Dairy Res. 72 (2005) 5–13.
[2] A. Huwiler, U. Zangemeister-Wittke, Crit. Rev. Oncol. Hematol. 63 (2007) 150–159.
[3] O. Cuvillier, Anticancer Drugs 18 (2007) 105–110.
[4] T.A. Taha, Y.A. Hannun, L.M. Obeid, J. Biochem. Mol. Biol. 39 (2006) 113–131.
[5] T.M. Leclercq, S.M. Pitson, IUBMB Life 58 (2006) 467–472.
[6] P. Xia, J.R. Gamble, L. Wang, S.M. Pitson, P.A. Moretti, B.W. Wattenberg, R. J. D'Andrea, M.A. Vadas, Curr. Biol. 10 (2000) 1527–1530.
[7] K. French, R.S. Schrecengost, B.D. Lee, Y. Zhuang, S.N. Smith, J.L. Eberly, J. K. Yun, C.D. Smith, Cancer Res. 63 (2003) 5962–5969.
[8] K.R. Johnson, K.Y. Johnson, H.G. Crellin, B. Ogretmen, A.M. Boylan, R.A. Harley, L.M. Obeid, J. Histochem. Cytochem. 53 (2005) 1159–1166.
[9] E. Ruckhaberle, A. Rody, K. Engels, R. Gaetje, G. von Minckwitz, S. Schiffmann, S. Grosch, G. Geisslinger, U. Holtrich, T. Karn, M. Kaufmann, Breast Cancer Res. Treat. 112 (2008) 41–52.
[10] J.R. Van Brocklyn, C.A. Jackson, D.K. Pearl, M.S. Kotur, P.J. Snyder, T.W. Prior, J. Neuropathol. Exp. Neurol. 64 (2005) 695–705.
[11] S. Spiegel, S. Milstien, Nat. Rev. Mol. Cell Biol. 4 (2003) 397–407.
[12] S.E. Alvarez, S. Milstien, S. Spiegel, Trends Endocrinol. Metab. 18 (2007) 300–307.
[13] Y. Baran, A. Salas, C.E. Senkal, U. Gunduz, J. Bielawski, L.M. Obeid, B. Ogretmen, J. Biol. Chem. 282 (2007) 10922–10934.
[14] D. Pchejetski, M. Golzio, E. Bonhoure, C. Calvet, N. Doumerc, V. Garcia, C. Mazerolles, P. Rischmann, J. Teissie, B. Malavaud, O. Cuvillier, Cancer Res. 65 (2005) 11667–11675.
[15] A. Pilorget, M. Demeule, S. Barakat, J. Marvaldi, J. Luis, R. Beliveau, J. Neurochem. 100 (2007) 1203–1210.
[16] F. Doll, J. Pfeilschifter, A. Huwiler, Endocr. Relat. Cancer 14 (2007) 325–335.
[17] F. Doll, J. Pfeilschifter, A. Huwiler, Biochim. Biophys. Acta 1738 (2005) 72–81.
[18] L. Qiu, C. Zhou, Y. Sun, W. Di, E. Scheffler, S. Healey, H. Wanebo, N. Kouttab, W. Chu, Y. Wan, Oncol. Rep. 16 (2006) 907–913.
[19] V. Gouaze-Andersson, J.Y. Yu, A.J. Kreitenberg, A. Bielawska, A.E. Giuliano, M. C. Cabot, Biochim. Biophys. Acta 1771 (2007) 1407–1417.
[20] H. Devalapally, Z. Duan, M.V. Seiden, M.M. Amiji, Int. J. Cancer 121 (2007) 1830–1838.
[21] R. Kolesnick, Z. Fuks, Oncogene 22 (2003) 5897–5906.
[22] A. Haimovitz-Friedman, R.N. Kolesnick, Z. Fuks, Br. Med. Bull. 53 (1997) 539–553.
[23] O.A. Sukocheva, L. Wang, N. Albanese, M.A. Vadas, P. Xia, Mol. Endocrinol. 17 (2003) 2002–2012.
[24] O. Sukocheva, C. Wadham, A. Holmes, N. Albanese, E. Verrier, F. Feng, A. Bernal, C.K. Derian, A. Ullrich, M.A. Vadas, P. Xia, J. Cell Biol. 173 (2006) 301–310.
[25] K.J. French, J.J. Upson, S.N. Keller, Y. Zhuang, J.K. Yun, C.D. Smith, J. Pharmacol. Exp. Ther. 318 (2006) 596–603.
[26] K.B. Moysich, R.J. Menezes, A. Ronsani, H. Swede, M.E. Reid, K.M. Cummings, K. L. Falkner, G.M. Loewen, G. Bepler, BMC Cancer 2 (2002) 31–37.
[27] D.M. Schreinemachers, R.B. Everson, Epidemiology 5 (1994) 138–146.
[28] L.D. Ratnasinghe, B.I. Graubard, L. Kahle, J.A. Tangrea, P.R. Taylor, E. Hawk, Anticancer Res. 24 (2004) 3177–3184.
[29] E.J. Jacobs, M.J. Thun, E.B. Bain, C. Rodriguez, S.J. Henley, E.E. Calle, J. Natl. Cancer Inst. 99 (2007) 608–615.
[30] N.R. Cook, I.-M. Lee, J.M. Gaziano, D. Gordon, P.M. Ridker, J.E. Manson, C. H. Hennekens, J.E. Buring, JAMA 294 (2007) 47–55.
[31] J. Hung, Med. J. Aust. 179 (2003) 147–152.
[32] K.L. Kees, T.J. Caggiano, K.E. Steiner Jr., J.J. Fitzgerald, M.J. Kates, T. E. Christos, J.M. Kulishoff Jr., R.D. Moore, M.L. McCaleb, J. Med. Chem. 38 (1995) 617–628.
[33] M.C. Davis, Synth. Commun. 37 (2007) 1457–1462.
[34] P. Madsen, A. Ling, M. Plewe, C.K. Sams, L.B. Knudsen, U.G. Sidelmann, L. Ynddal, C.L. Brand, B. Andersen, D. Murphy, M. Teng, L. Truesdale, D. Kiel, J. May, A. Kuki, S. Shi, M.D. Johnson, K.A. Teston, J. Feng, J. Lakis, K. Anderes, V. Gregor, J. Lau, J. Med. Chem. 45 (2002) 5755–5775.
[35] C. Maurin, F. Bailly, G. Mbemba, J.F. Mouscadet, P. Cotelle, Bioorg. Med. Chem. 14 (2006) 2978–2984.
[36] K.A. Thakkar, D.D. Goswami, S.R. Choudhari, J. Pharm. Sci. 67 (1978) 587–589.
[37] V. Vichai, K. Kirtikara, Nat. Protoc. 1 (2006) 1112–1116.
[38] S.F. Kralik, X. Du, C. Patel, J.P. Walsh, Anal. Biochem. 294 (2001) 190–193.