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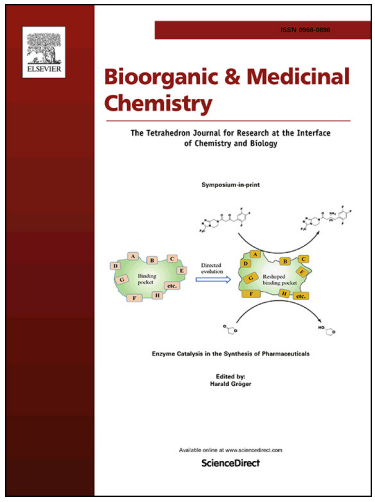

Novel meriolin derivatives as rapid apoptosis inducers

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PII: S0968-0896(19)30526-7
DOI: https://doi.org/10.1016/j.bmc.2019.06.029
Reference: BMC 14966

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 29 March 2019
Revised Date: 28 May 2019
Accepted Date: 18 June 2019

Please cite this article as: Drießen, D., Stuhldreier, F., Frank, A., Stark, H., Wesselborg, S., Stork, B., Müller, T.J.J., Novel meriolin derivatives as rapid apoptosis inducers, *Bioorganic & Medicinal Chemistry* (2019), doi: https://doi.org/10.1016/j.bmc.2019.06.029

Graphical Abstract

Novel meriolin derivatives as rapid apoptosis inducers

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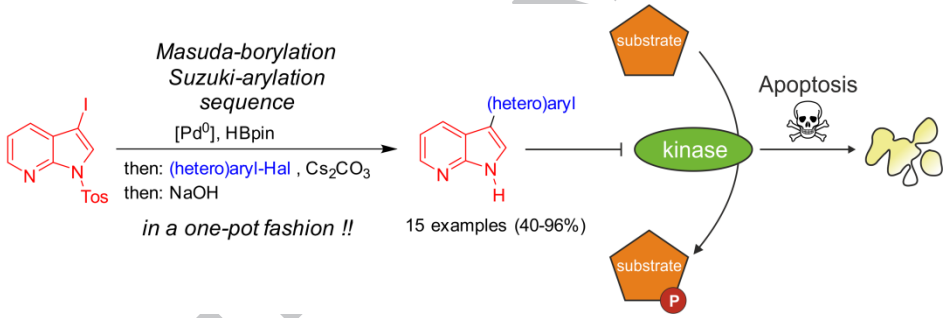

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This work is dedicated to Prof. Dr. Peter Proksch on the occasion of his 65th birthday.

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

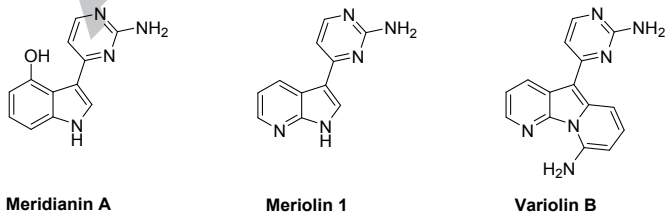
Keywords:
Synthetic methods
One-pot reaction
Masuda borylation-Suzuki coupling
Meriolin
Apoptosis
Sphingosine kinase 2

ABSTRACT

3-(Hetero)aryl substituted 7-azaindoles possessing multikinase inhibitor activity are readily accessed in a one-pot Masuda borylation-Suzuki coupling sequence. Several promising derivatives were identified as apoptosis inducers and, emphasizing the multikinase inhibition potential, as sphingosine kinase 2 inhibitors. Our measurements provide additional insights into the structure-activity-relationship of meriolin derivatives, suggesting derivatives bearing a pyridine moiety with amino groups in 2-position as most active anticancer compounds and thus as highly promising candidates for future *in vivo* studies.

1. Introduction

A variety of marine compounds consists of indole or 7-azaindole core elements[1] and quite some have revealed interesting biological and pharmacological activity. Meridianins originate from the ascidian *Aplidium meridianum*, while variolins were first isolated from the antarctic sponge *Kirkpatrickia variolosa*.[2,3] In comparison to meridianins and variolins (Fig. 1) meriolins can be considered to be truncated hybrid derivatives combining structural motifs of both classes.[4] Quite early 3-(hetero)aryl substituted 7-azaindoles have therefore become a lead structure for the development of kinase inhibitors.



Meridianin A **Meriolin 1** **Variolin B**

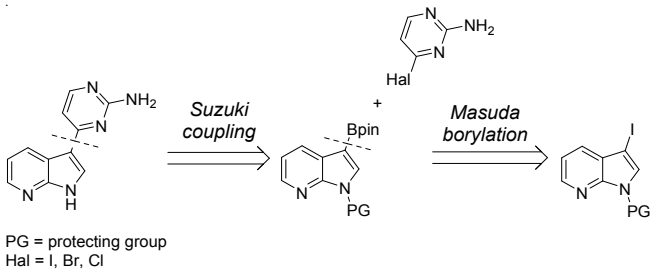
Figure 1. Natural products meridianin A and variolin B with high bioactivity and meriolin 1 as a structural hybrid.

Indeed, meriolins have been reported to strongly inhibit various protein kinases.[5,6] This finding correlates excellently with the ability of 7-azaindoles to bind to the hinge region of kinases. In some cases, also ligands with kinase domains of signaling cascade proteins (e.g. PDK1) were successfully cocrystallized.[7] Further studies also indicated a high potential of meriolins in the treatment of neurodegenerative diseases, bipolar disorders, strokes, cancer, and chronic inflammations.[6]

Multikinase inhibitors are well-known inducers of apoptotic cell death.[8-10] For application in therapeutic context initiating apoptosis is much more favorable than induction of necrosis as apoptosis does not harm the host by causing inflammatory reactions.[11] The major effectors of the highly regulated apoptotic signaling network are cysteinyl-aspartate specific proteases (caspases) including the most potent executioner of apoptosis, caspase-3.[11] Besides caspases several interconnected kinase-driven signaling pathways are involved in the regulation of apoptosis. Since many of these signaling cascades negatively regulate apoptosis in cancer cells, inhibition of kinases is a reasonable approach for anticancer therapy.[12] Particularly in treatment of acute leukemias targeting kinases seems to be a valid strategy as demonstrated by the recent approval of the multitargeted kinase inhibitor midostaurin for acute myeloid leukemia.[13] Furthermore, also lymphomas are reported to be most vulnerable to multikinase inhibition.[14] Interestingly, multiple kinases which were reported to be inhibited by meriolin derivatives[5] are heavily linked to downregulation of apoptosis in cancer cells, for instance GSK3 or CDK7.[15-17] Summing up, meriolin derivatives appear to be promising candidates for induction of apoptosis in leukemia and lymphoma cells.

The sphingosine kinases 1 and 2 (SK1, SK2) are important regulators of the sphingolipid metabolism.[18] By fine-tuning the levels of sphingosine-1-phosphate (through phosphorylation of sphingosine) the enzymes are involved in cell proliferation and apoptosis and hence connected to cancerous diseases. Recent research even reported on the connection between SK2 inhibition and induction of caspase-3 cleavage in TRAIL resistant cancer cells.[19]

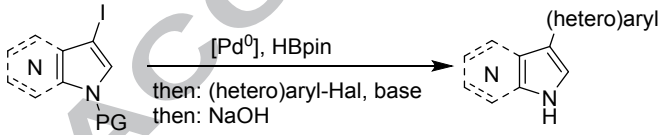
As a consequence, rational design of meriolins as kinase inhibitors by a reliable, modular methodology is highly desirable. First syntheses of meriolins reported longest linear sequences of four steps, however, resulting in low overall yields.[20,21] Based upon the availability and accessibility of (hetero)aryl halides, the retrosynthetic analysis of meriolins suggests Suzuki-Miyaura-coupling[22] as a reliable, versatile terminal key step for the formation of the desired compounds (Scheme 1).



Scheme 1. Retrosynthetic analysis of meriolin 1.

Pinacolyl boronates are stable to moisture, light, can be stored at room temperature and can be obtained by Masuda borylation upon reacting an (hetero)aryl halide in the presence of pinacol borane, triethylamine and a palladium catalyst.[23] In comparison to the related Miyaura borylation[24] with B_2pin_2 the Masuda reaction represents a more atom-economical approach. Most favorably, both catalytic processes, borylation and (hetero)arylation can be concatenated in a one-pot fashion, i.e. in the sense of a one-pot Masuda borylation-Suzuki (hetero)arylation (MBSA) sequence.

First examples of MBSA were reported on the synthesis of biaryls,[25] but it was not before 2011 when we came up with a general MBSA sequence employing heterocyclic halides as substrates, which were successfully applied to concise total syntheses of several marine alkaloids[26-28] and kinase inhibitors (Scheme 2).[7]



Scheme 2. Masuda borylation-Suzuki coupling (MBSA) sequence in a one-pot fashion.

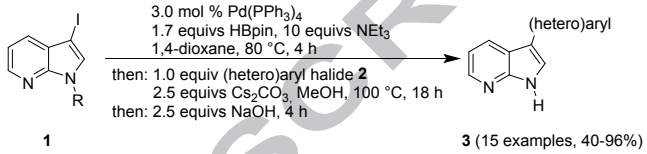
Besides the highly practical nature of one-pot processes, where all transformations are conducted in a consecutive or sequential fashion, the MBSA synthesis of bi(hetero)aryls starting from two different (hetero)aryl halides can be considered as a sequentially Pd-catalyzed process.[29] Most characteristically, the initial catalyst also will catalyze the subsequent reaction(s) without further addition of catalyst loading.

Herein we report an optimized MBSA sequence for the synthesis of novel meriolin derivatives that have previously been identified to be potent inhibitors of PDK1.[7] Various 3-pyrimidyl and 3-pyridyl substituted 7-azaindoles as key pharmacophores, in particular with amino substituents on the azine moieties, were screened with respect to their apoptosis inducing potential and to establish first structure-activity relationships (SAR). In addition, the inhibition of sphingosine kinases 1 and 2 (SK1, SK2) was assessed by initial screening studies.

2. Results and Discussion

2.1. Masuda borylation-Suzuki coupling sequence

Based upon our experience in applying the MBSA sequence to the synthesis of marine natural products and analogues, we first established, starting from *N*-protected 3-iodo-7-azaindole **1** and equistoichiometric amounts of (hetero)aryl halides **2**, an optimized protocol for efficiently preparing meriolin derivatives **3** (Scheme 3, Figure 2).[26,27,28]



Scheme 3. MBSA one-pot synthesis of meriolin derivatives **3**.

The *N*-protected 7-azaindoles **1** were prepared in quantitative yields using a one-pot protocol of iodination followed by subsequent protection in dimethylformamide under basic conditions.[28] A change from *N*-Boc protected 7-azaindoles to a tosyl protection group was chosen to provide a more stable reagent with respect to the reaction conditions. In comparison to our initial protocol by raising the amount of pinacol borane dehydrohalogenation of the starting material can be efficiently suppressed. Also increasing the excess of triethylamine in the Masuda borylation step turned out to be favorable.[23] With a reaction time of 4 h in 1,4-dioxane conversion to the corresponding pinacolyl ester is essentially quantitative, according to its yields, if isolated. Excessive pinacol borane is efficiently scavenged by the addition of methanol after completion of the Masuda borylation. The addition of cesium carbonate and (hetero)aryl halide is sufficient to facilitate the Suzuki coupling. In comparison to *Boc*-protected (7-aza)indoles the tosyl protecting group remains intact during the Suzuki arylation step. The concurrent deprotection by the alcoholic carbonate solution can be suppressed.[30] The catalytic system obviously remains stable to give a full conversion to the protected meriolin derivatives. Finally, by addition of sodium hydroxide to the reaction mixture deprotection of the tosyl group gives rise to the isolation of meriolin derivatives **3** in good to excellent yields. In essence, a single and simple catalyst system sequentially catalyzes both borylation and (hetero)arylation, and finally, the tosyl group can be selectively cleaved by addition of sodium hydroxide. It is noteworthy to mention that all compounds can be uneventfully isolated by a single simple flash chromatography.

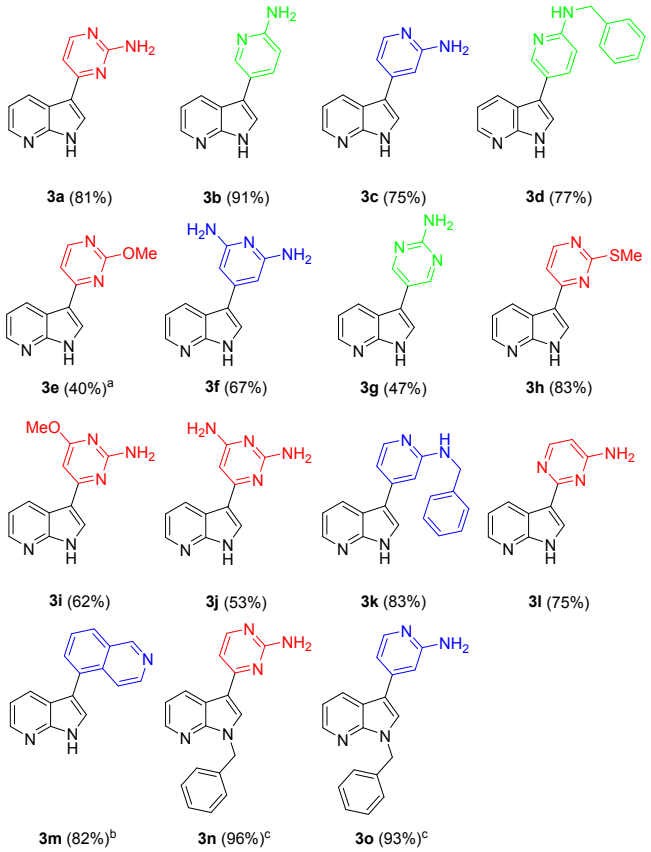


Figure 2. Scope of MBSA one-pot synthesis of meriolin derivatives **3** (isolated yields are given in parentheses; color code for halides **2**: red = chloride, blue = bromide, green = iodide). [a]Second regioisomer was formed. [b]DME/water was used in the Suzuki coupling step. [c]No deprotection after Suzuki coupling step.

2.2. Biological activity

The class of meriolins is known to act highly cytotoxic in cancer cells.[31] In order to characterize their cytotoxicity profile we measured cell viability of leukemia and lymphoma cells after treatment with several novel meriolin derivatives **3** using the resazurin reduction assay. Out of 13 tested meriolins **3**, 6 exhibited pronounced cytotoxicity against the tested cancer cell lines with IC_{50} values ranging from 0.06 to 6 µM (Fig. 3, Table 1). The remaining meriolins **3** possessed low cytotoxicity (IC_{50} > 10 µM) or were inactive in the tested concentration range up to 30 µM (data not shown). Meriolin derivative **3f** demonstrated the highest potency in both cell lines, as reflected by its remarkably low IC_{50} values. Compared to meriolin **1**, compound **3f** showed up to 50-fold increased cytotoxicity. The high quantity of tested meriolin derivatives provides insight into their structure-activity relationship. The amino group in 2-position of the azine moiety seems to be crucial for the cytotoxic efficacy since the vast majority of tested meriolin derivative lacking this group failed to potently induce cytotoxicity (**3e**, **3g**, **3h, 3m**). In addition, meriolin derivatives carrying a pyridine substituent are apparently more cytotoxic than derivatives with a pyrimidine substituent (see **3a/j** vs. **3c/f**). Comparing the cytotoxicity of compounds **3a** and **3j**, a second amino group at the azine in meta-orientation seems to further increase the compound's effect.

In order to determine whether the observed cytotoxicity of compounds **3a**, **3c**, **3d**, **3f**, **3i** and **3j** is related to induction of apoptosis we performed fluorimetric caspase-3 activity assays. Measuring activation of caspase-3 by using two concentrations per selected compound with regard to their cytotoxicity profile, we showed that all six compounds distinctly induce apoptosis within 3-4 h (Fig. 3). Of note, compound **3f** caused even higher activation of caspase-3 in Ramos cells than the multikinase inhibitor staurosporine (STS; used as positive control), although STS was applied in a considerably higher concentration (2.5 µM vs. 0.1 µM). To further verify rapid induction of apoptosis, we detected cleavage of the caspase-3 substrate PARP1 via immunoblotting (Fig. 3). Again, every compound of the mentioned subset induced apoptosis within a few hours, in line with the results of the caspase-3 activity assay.

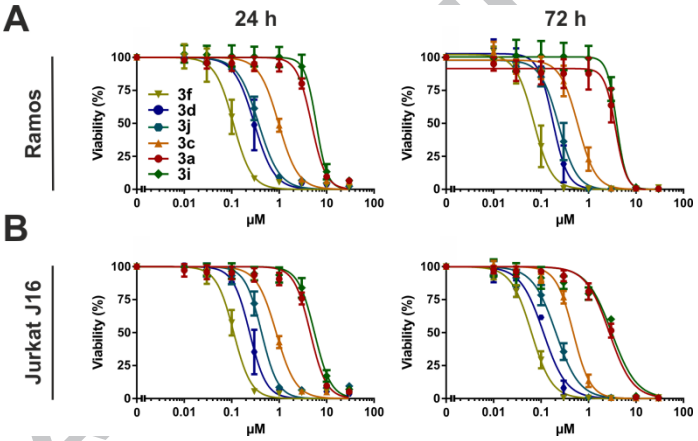


Figure 3. Selected meriolin derivatives **3** exhibit pronounced cytotoxicity on human lymphoma and leukemia cell lines. (A) Ramos (Burkitt's lymphoma) and (B) Jurkat J16 (acute T-cell leukemia) cells were incubated with escalating concentrations of meriolin derivatives **3** for 24 or 72 h. After incubation, cell viability was determined by resazurin reduction assay as described in methods. Relative viability of DMSO (0.1% v/v)-treated control cells was set to 100%. Data points shown are the mean of three independent experiments, error bars = SD. IC_{50} values (IC_{50} = half maximal inhibitory concentration) were calculated via nonlinear regression curve fitting using Prism 6 (GraphPad Software).

Table 1. Cytotoxicity of meriolin derivatives **3** towards leukemia and lymphoma cells as determined by resazurin reduction assay. IC_{50} expressed as mean from three independent experiments. Bracketed values indicate 95% confidence intervals.

compound	Ramos 24h	Ramos 72h	Jurkat J16 24h	Jurkat J16 72h
3a	4.80 µM	3.73 µM	4.53 µM	2.74 µM
	[4.28-5.37]	[2.63-5.28]	[4.00-5.13]	[2.42-3.11]
3c	0.97 µM	0.60 µM	0.87 µM	0.48 µM
	[0.91-1.05]	[0.50-0.71]	[0.81-0.93]	[0.44-0.51]
3d	0.30 µM	0.18 µM	0.24 µM	0.11 µM
	[0.26-0.34]	[0.16-0.20]	[0.21-0.27]	[0.10-0.13]
3f	0.11 µM	0.07 µM	0.11 µM	0.06 µM
	[0.10-0.11]	[0.06-0.08]	[0.10-0.11]	[0.06-0.07]
3i	6.04 µM	3.90 µM	5.56 µM	3.08 µM
	[5.15-7.08]	[2.67-5.72]	[4.93-6.26]	[2.51-3.78]
3j	0.37 µM	0.24 µM	0.43 µM	0.21 µM
	[0.34-0.40]	[0.21-0.27]	[0.37-0.49]	[0.19-0.23]

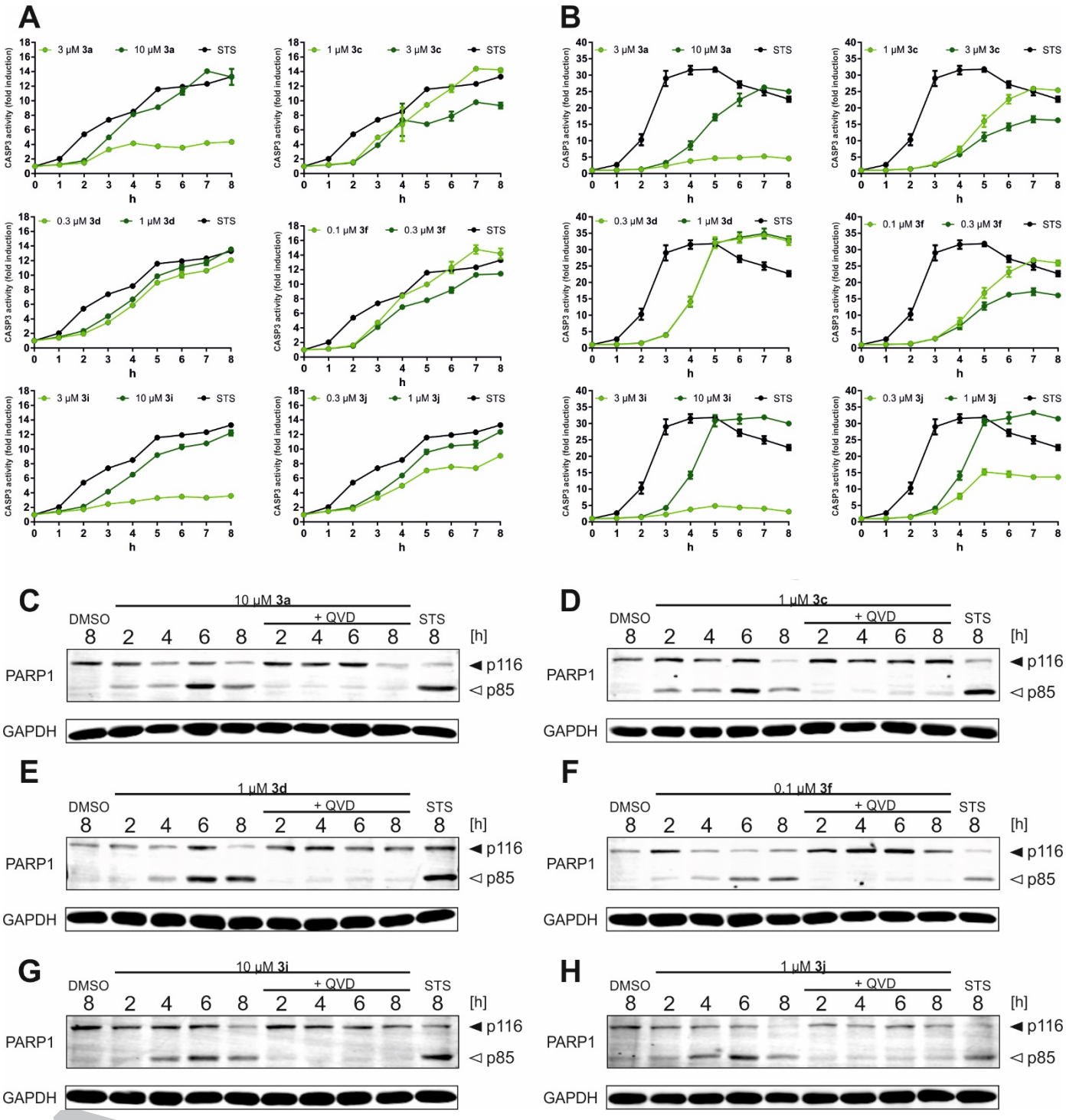


Figure 4. A subset of meriolin derivatives **3** induces apoptosis in rapid kinetics. (A) Ramos or (B) Jurkat J16 cells were treated with the indicated meriolin derivatives **3** for up to 8 h. After a lysis step, the pro-fluorescent caspase-3 substrate Ac-DEVD-AMC was added. Increase in fluorescence of AMC was measured at 37 °C for at least 60 min using a microplate spectrophotometer and reflects caspase-3 activity. The multikinase inhibitor staurosporine (STS, 2.5 µM) served as positive control for caspase-3 activation. Curves for STS are reused in corresponding diagrams. Ramos cells were treated with (C) **3a**, (D) **3c**, (E) **3d**, (F) **3f**, (G) **3i**, or (H) **3j** in defined concentrations (selected on the basis of initial cytotoxicity testings) for 2-8 h. Subsequently, cells were harvested, lysed and deployed for immunoblotting against the caspase-3 substrate PARP1. Expression of GAPDH was used as protein loading control. Co-treatment with the broad-spectrum caspase inhibitor Q-VD-OPh (QVD, 10 µM) was conducted in order to confirm caspase dependency of PARP1 cleavage. The uncleaved form of PARP (p116) is indicated by filled arrowheads, the cleaved form (p85) by open arrowheads. STS (2.5 µM) served as positive control.

In order to improve multikinase inhibitor abilities of the compounds biological activities, selected compounds were evaluated by screening against sphingosine kinase 1 and 2. The selected compounds **3a, 3c** and **3f** showed clear preference for SK2 in a screening at 10 µM. Compound **3c** was the least active compound with 10% ± 5% and 39% ± 5% inhibition of SK1 and SK2, respectively. Compounds **3a** and **3f** showed pronounced inhibition of SK2 with 72% ± 3% and 87% ± 7%, respectively,

while SK1 inhibition was only moderate (10% ± 4% and 18% ± 1%, respectively).

All tested compounds showed a marked inhibition of SK2 and weak action on SK1. It has to be noted that the compounds showed fluorescence quenching that may influence the assay results. Having the similar assay conditions for both isoenzymes, the selectivities on SK2 for **3a** and **3f** are clearly based on biological activity and not on physicochemical properties. The SK2 is known as a target involved in cell proliferation and apoptosis. As recently reported inhibition of SK2 led to TRAIL-dependent caspase-3 cleavage and was able to overcome TRAIL resistance in non-small cell lung cancer cells.[19] Collectively, the presented data support the pharmacological activity observed in cell viability assays and serve as additional argument for the proof of concept of the presented compounds on multi-kinase inhibition.

3. Conclusions

The MBSA sequence is a concise and reliable methodology for easily accessing a broad spectrum of novel active agents in a one-pot fashion. Moreover, a modular diversity oriented synthesis can be readily achieved by simply varying the halides. A particular advantage is the sequentially Pd-catalyzed process, which is highly catalyst economical. This methodological tool is currently applied in various concise total syntheses of natural products and bioactive compounds. Here, we used the methodology for generating several new inducers of apoptotic cell death in cancer cells. In particular, **3f** showed great a great potential as a valuable lead compound for future anticancer studies.

4. Experimental Section

Synthesis of 1a:[28] 7-Azaindole (3.54 g, 30.0 mmol) and potassium hydroxide (4.96 g, 75.0 mmol) were dissolved in DMF (55.0 mL). A solution of iodine (7.63 g, 30.3 mmol) in DMF (55.0 mL) was added dropwise to the solution at room temp and the resulting mixture was stirred for 30 min. After addition of a second portion of potassium hydroxide (4.96 g, 75.0 mmol) the mixture was stirred at room temp for 10 min before a solution of *p*-toluene sulfonyl chloride (12.14 g, 63.0 mmol) in DMF (55.0 mL) was added dropwise. The resulting mixture was stirred at room temp for 3 h. After complete conversion (monitored by TLC) the reaction mixture was poured onto ice water (200 mL) and stored in the refrigerator overnight. The resulting yellow precipitate was filtrated, washed with ice water (50.0 mL) and dried in *vacuo* to give the desired product **1a** (11.74 g, 98%) as a light yellow solid (for full analytical details, see the Supporting Information).

General procedure MBSA (Compound 3f): The 7-azaindole **1a** (1.00 mmol, 398 mg) and tetrakis(triphenyl-phosphane)palladium(0) (35.0 mg, 0.03 mmol) were placed in a dry screw-cap vessel with a magnetic stir bar. After evacuating and refilling with argon for three times dry 1,4-dioxane (5.0 mL) was added and the resulting mixture was degassed with argon for 10 min. Dry triethylamine (1.40 mL, 10.0 mmol) and 4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.25 mL, 1.70 mmol) were successively added. Then the reaction mixture was stirred in a preheated oil bath at 80 °C for 4 h. The mixture was cooled to room temp (water bath). Dry methanol (5.0 mL) was added and the mixture was stirred at room temp for 10 min. After the addition of 2,6-diamino-4-bromopyridine (**2f**) (1.00 mmol, 129 mg) and cesium carbonate (823 mg, 2.50 mmol) the mixture was stirred in a preheated oil bath at 100 °C for 18 h. After the Suzuki coupling was completed the mixture was cooled to room temp (water bath). Sodium hydroxide (100 mg, 2.50 mmol) was added and the reaction was stirred at 100 °C for 4 h. Then, after cooling to room temp (water bath), the solvents were removed in *vacuo* and the residue was absorbed onto Celite®. After purification by chromatography on silica gel (dichloromethane/methanol/ aqueous ammonia, see Supporting Information) and after drying in *vacuo* at 80 °C for 18 h compound **3f** (151 mg, 67%) was obtained as an orange solid. ^{1}H-NMR (DMSO-d_6, 300 MHz): δ = 5.33 (bs, 4 H), 6.05 (s, 2 H), 7.15 (dd, *J* = 4.7, 7.9 Hz, 1 H), 7.74 (d, *J* = 2.5 Hz, 1 H), 8.14-8.44 (m, 2 H), 11.88 (s, 1 H); ^{13}C-NMR (DMSO-d_6, 75 MHz): δ = 93.1, 113.6, 115.9, 117.3, 124.0, 127.7, 142.9, 144.3, 149.0, 159.2; EI MS (m/z (%)): 226 (14) [$M+H^+$], 225 (100) [M^+], 198 (21) [$C_{11}H_{10}N_4^+$], 170 (7) [$C_{10}H_8N_3^+$], 155 (4) [$C_{10}H_7N_2^+$], 118 (5) [$C_7H_6N_2^+$]; Anal. calcd. for $C_{12}H_{11}N_4$ (225.25): C 63.99, H 4.92, N 31.09; Found: C 63.89, H 4.88, N 30.80. (For full experimental and analytical details, see Supporting Information)

Cell lines and reagents: Adult lymphoblastic leukemia T cells (Jurkat J16, no. ACC-282) and Burkitt's lymphoma B cells (Ramos, no. ACC-603) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). Both cell lines were routinely cultured in RPMI 1640 media supplemented with 10% FCS, 120 IU/ml penicillin, and 120 µg/ml streptomycin. Resazurin sodium salt and Q-VD-OPh hydrate (QVD) were purchased from Sigma Aldrich Life Science (Darmstadt, Germany), staurosporine (STS) from LC Laboratories (Woburn, MA, USA) and Ac-DEVD-AMC from Biomol (Hamburg, Germany). The antibodies used for immunoblotting were mouse anti-PARP1 (Enzo Life Sciences, New York, NY, USA; #BML-SA250) and mouse anti-GAPDH (Abcam, Cambridge, United Kingdom; #ab8245).

Resazurin reduction assay: Cell viability was evaluated using the resazurin reduction assay. In viable cells the non-fluorescent dye resazurin gets reduced to highly fluorescent resorufin.[32] Therefore resorufin fluorescence can serve as a measure for metabolic activity. For viability testings, Ramos and Jurkat J16 cells were seeded in a density of 0.5 x 10^6 cells/mL and treated with the compounds of interest for the indicated time. Resazurin (40 µM) was added 3 h prior to end of incubation time. Finally, the fluorescence of resorufin (excitation: 535 nm, emission: 590 nm) was measured using a microplate spectrophotometer. Viability of control cells (0.1% DMSO) was set to 100%.

Immunoblotting: Immunoblotting was performed according to standard protocol as described earlier.[33] Signals were detected using IRDye800- or IRDye680- conjugated secondary antibodies and an infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Fluorimetric caspase-3 activity assay: Activation of caspase-3 was detected as described previously.[33] Briefly, cells plated in 96-well microtiter plates were lysed in caspase assay buffer (20 mM HEPES, 84 mM KCl, 10 mM $MgCl_2$, 200 µM EDTA, 200 µM EGTA, 0.5% NP-40, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 5 µg/mL aprotinin) after the indicated incubation time. Subsequently, reaction buffer containing the pro-fluorescent caspase-3 substrate Ac-DEVD-AMC was added. In the final step, increase in AMC fluorescence, indicating caspase-3 activity, was measured over a period of at least 60 min.

Sphingosine kinase inhibition assays: The inhibition of the sphingosine kinase was measured as reported previously[18a] with

minor changes. Briefly, the compounds of interest (10 µM) were incubated with sphingosine kinase (SK) 1 or 2 (0.5 U/mL or 0.1 U/mL final concentration, respectively) for 15 min. Then 5 µM sphingosine and ATP (final concentration, assay volume of 10 µL) were added and incubated for 30 or 60 min (SK1 and 2, respectively) at room temperature. Afterwards the detection reagent (BellBrook Labs) was added and incubated for 60 min in the dark. The buffer contained 50 mM HEPES (pH 7.5), 4 mM $MgCl_2$, 100 µM sodium orthovanadate, 1 mM dithiothreitol, 0.01 % Brij 35 and 2 mM egtazic acid. Assays were performed in triplicates in two independent experiments (**3a** and **3c** at SK1 with n=1). Relative fluorescence intensity of the compounds was calculated relative to specific fluorescence intensity (total fluorescence intensity minus blank was set to 100%). Inhibition values are derived by calculating 100 minus relative fluorescence intensity. Values are reported as global means of all replicates with standard error of mean.

Acknowledgements

The authors cordially thank the GRK 2158 (Deutsche Forschungsgemeinschaft) for financial support.

Supporting Information:

Supporting Information to this article can be found online at https://doi.org/j.bmc.

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