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Cycloartanes with Anticancer Activity Demonstrate Promising inhibition of the Mrckα and Mrckβ Kinases

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Authors' contributions

This work was carried out in collaboration between all authors. Conceived experiments and designed the experiments by authors HICL, NJT and JB. Performed experiments by authors NJT, HICL, JB and CTW. Analyzed the data by authors NJT, HICL and CTW. Wrote the paper by authors HICL, NJT and JB. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: The role of Kinases in cancer onset and progression has made kinases a target for the control of some cancers. Recent discoveries that kinases are most effectively inhibited by small molecules have also resulted in an increased search for small molecule kinase inhibitors. Cycloartanes are small molecules found in many medicinal plants including the Jamaican Ball Moss (*Tillandsia recurvata*). Recent studies on *T. recurvata* have demonstrated that it possesses anticancer activity. Cycloartane-3,24,25-triol, an analog of a cycloartane identified in Ball moss was also shown to have inhibitory activity against MRCKα kinase. This study was as such set up to determine the MRCKα/β kinase inhibition activity of other cycloartanes in Ball Moss and their analogs. MRCKα/β kinases has been identified as an important kinase implicated in cancer onset and progression and as such a potential drug target.
Methodology: Kinase inhibition activity of 6 cycloartanes was investigated using the

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ligand-kinase binding assay. The WST-1 reagent assay was also used to determine the antiproliferation activity of the cycloartanes against some prostate and breast cancer cell lines.

Results: Cycloart-23-ene-3,25-diol (1), Cycloartane-3,24,25-triol (2), Cycloart-25-ene-3,24-diol (3), 3,23-Dioxo-9,19-cyclolanost-24-en-26-oic acid (4), 24,25-Dihydroxycycloartan-3-one (5) inhibited the MRCKα kinase with K_d of 0.21 μM, 0.25μM, 0.36 μM, 3.0 μM and 2.1 μM respectively. Hydroxycycloart-23-en-3-one,25, (6) showed no inhibition against the MRCKα kinase. Compounds 1, 3, 4, 5 inhibited the MRCKβ kinase with K_d of 4.7 μM, 1.10 μM, 3.2 μM and 9.8 μM, respectively. Three of the six cycloartanes exhibited antiproliferation activity against two prostate and breast cancer cell lines each.

Conclusion: Cycloart-23-ene-3,25-diol (1) showed the most promising activity against the MRCKα/β kinase out of the 6 cycloartanes screened demonstrating an interesting structure activity relationship profile when compared with the other molecules. Cycloart-23-ene-3,25-diol (1) deserves further studies to determine its *in vivo* activity as well.

Keywords: *Cycloart-23-ene-3; 25-diol; cycloartane; MRCK kinase; cancer; ball moss; Tillandsia recurvata.*

1. INTRODUCTION

The human kinome comprise over 500 protein kinases which play import roles in many cell signalling pathways [1]. Protein kinases are as such considered major druggable targets as their over-expression and/or dysregulation result in many diseases including various cancers [2]. The myotonic dystrophy related Cdc42-binding kinases (MRCKα/β) are amongst some of the kinases that have been identified and are implicated in cell growth and differentiation [3,4]. The Cdc42-binding kinases (MRCKα/β) belong to the Rho GTPase family of kinases which are considered to act like molecular switches in cells [5]. Specifically, MRCKα/β kinases are responsible for the regulation of the actin cytoskeleton of cells [6]. The actin cytoskeleton in all eukaryotic cells is important as it provides the structural framework required for cell motility and division. Based on the ability of the MRCKα/β to aid cell motility by activating or working in concert with other kinases, their over-expression in cancer cells may as such lead to metastasis which usually lead to poor prognosis [7]. For example, it has been shown that squamous cell carcinoma cells require Cdc42 and its effector kinases MRCKα/β to invade surrounding fibroblasts [8]. In another example, studies on the effect of MRCK kinases in cancer progression seem to suggest that MRCKα kinase in particular mediates the activation of LIMK1 kinase which has been shown to be over-expressed in breast and prostate cancer cells [9,10]. Functional studies have confirmed the fact that increasing LIMK1 expression in breast cancer cell lines results in increased cellular migration and invasion in vivo and ex-vivo while inhibition of LIMK1 over-expression limited the rapid spread of the cells [11,12]. Cdc42 mutations have recently been found in melanoma cells and given that Cdc42 has been shown to be involved in two alternative types of individual invasion movements, Cdc42 effector kinases are thus seen as attractive druggable targets [13,14].

Few reports exist on MRCKα/β kinase inhibitors. Lowe et al. [15] recently reported the inhibition of MRCKα kinase by a cycloartane type compound while Choi et al. [16] demonstrated the binding affinity of phorbol esters to the c-domain of the MRCKα/β kinase. Heikkila et al. have also reported on the inhibition of MRCK kinases using co-crystal structure analysis of 3 kinase inhibitors [3]. While the search for more potent and safe small kinase inhibitors remains a major priority within the drug discovery community, our research has focused on identifying more cycloartanes with MRCKα/β kinase inhibition properties.

Cycloartanes belong to the triterpene class of natural compounds and are known to possess diverse biological properties [17-20]. Cycloartanes are present in many plants and are known to be major constituents of the Jamaican Ball Moss (*Tillandsia recurvata*) and other *Tillandsia* species [21-23]. The Jamaican Ball Moss has been reported to show anticancer activity against different histogenic cancer cell lines through the induction of apoptosis, kinase inhibition and antiangiogenic activity amongst other possible mechanisms [15,24,25]. Based on the preliminary antiproliferation activity of cycloartanes isolated from the Jamaican Ball Moss and some analogs, there is therefore reasonable link the anticancer properties of the plant [24] to the presence of the cycloartanes in addition to other yet to be identified molecules in the plants. Our recent findings that Cycloartane-3,24,25-triol potently inhibits MRCKα kinase has motivated us to search for other cycloartanes with MRCKα/β inhibitory activity in an attempt to identify the most active cycloartane kinase inhibitors as well as carryout any structure activity relationship (SAR) analysis that might exist between active members of these class of small molecule.

2. MATERIALS AND METHODS

2.1 Compound Isolation and Procurement

Cycloart-23-ene-3,25-diol, Cycloart-25-ene-3,24-diol were identified and isolated from samples of Tillandsia recurvata as previously reported [15,22]. For this study, samples of these two cycloartanes were sourced commercially from ChemFaces Biochemical Co Ltd, Wuhan, China alongside Cycloartane-3,24,25-triol, 3,23-Dioxo-9,19-cyclolanost-24-en-26-oic acid, Hydroxycycloart-23-en-3-one,25 and 24,25-Dihydroxycycloartan-3-one which are close analogs of the cycloartanes identified in *T. recurvata*. The structures of the 6 cycloartanes are presented in Fig. 1.

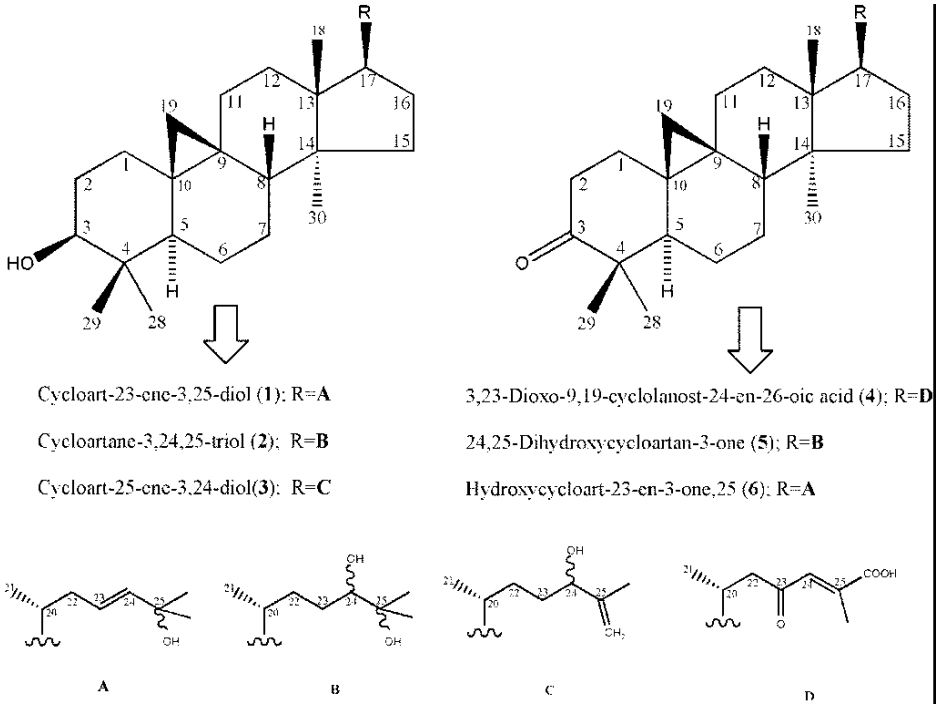

Fig. 1. Structures of cycloartanes used in this study

2.2 Compound Handling

Compound handling was carried out as previously described [15]. Briefly, An 11-point 3-fold serial dilution of each test compound was prepared in 100% DMSO at 100x final test concentration and subsequently diluted to 1x in the assay (final DMSO concentration = 1%). Primary screening was done at a single concentration of 10 µM. K_{ds} were determined using a compound top concentration of 30,000 nM. If the initial K_d determined was < 0.5 nM (the lowest concentration tested), the measurement was repeated with a serial dilution starting at a lower top concentration.

2.3 Kinase Inhibition Assay

Competition binding assays were run as previously described [26,27]. In summary, kinases were fused to T7 phage strains and for the other assays, kinases were produced in HEK-293 cells after which they were tagged with DNA for quantitative PCR detection (data not shown). The binding assays use streptavidin-coated magnetic beads treated with biotinylated small molecule ligands for 30 minutes at room temperature which generated affinity resins for the kinase assays. The liganded beads were blocked with excess biotin and washed with blocking buffer (Sea Block (Pierce), 1 % BSA, 0.05 % Tween 20, 1 mM DTT) to remove unbound ligand and to reduce non-specific phage binding. Binding reactions were assembled by combining kinases, liganded affinity beads and test compounds in 1x binding buffer (20% SeaBlock, 0.17x PBS, 0.05% Tween 20, 6 mM DTT). Compounds were prepared as 40x stock solution in 100% DMSO and diluted directly into the assay with a final DMSO concentration of 2.5%. The assay plates were incubated at room temperature on a shaker for 1 hour and the affinity beads were washed with wash buffer (1x PBS, 0.05% Tween 20). The beads were then re-suspended in elution buffer (1x PBS, 0.05 % Tween 20, 0.5µM non-biotinylated affinity ligand) and incubated for 30 minutes at room temperature with shaking. The kinase concentration in the eluates was measured by quantitative PCR. K_ds were determined using a standard dose response curve run in duplicate using the hill equation. Curves were determined using a non-linear least square fit with the Levenberg-Marquardt algorithm.

2.4 Biological Assay

2.4.1 Cell lines and culture medium

Four human tumor cell lines (Prostate-PC-3; prostate-DU-145; Breast-MDA-MB-231 and breast-MCF-7) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). The four tumor cell lines were maintained in minimum essential media supplemented with 10% fetal calf serum (Thermo Scientific, USA), 1% L-glutamine, 2% penicillin–streptomycin and 0.2% gentamicin all from Corning Cellgro Mediatech, Inc. (Manassas, USA).

2.4.2 Cell proliferation assay

For the assessment of the antiproliferation activity of the cycloartanes, the WST-1 (4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1, 3-benzene disulfonate) (Roche) colorimetric assay was used [28]. Briefly, cells were trypsinized and plated into 96 well plates in 50µl of media and incubated overnight. Approximately 18 hours after plating, 50µl of media containing the required drug concentration was added to each well. Cells were plated at a density so that 72 hours post drug addition, the cells will be in log phase (500-2000 cells/well). The compounds and extracts were solubilized in DMSO. The cells are allowed to proliferate for 72 h 37°C in humidified atmosphere of 5% CO_2. The experiment was

terminated using WST-1 (Roche) 10µl per well and absorbance read at 450 nm/690 nm. Antiproliferation activity was assessed as a percentage of proliferation of untreated cells and IC_{50} values determined using Graphpad Prism software. All experiments were carried out at least in duplicate and the means and standard deviations determined.

3. RESULTS AND DISCUSSION

3.1 Kinase Inhibition Screen

Cycloartane-3,24,25-triol (2) inhibited eight kinases (1.77%) by ≥50% out of a total of 451 kinases screened in a primary screen kinase inhibition assay (Table 1) using a single concentration of 10 µM (Fig. 2). The remaining kinases that were inhibited by <50% are listed in Supplement Table 1. The results of inhibition binding constant (k_d) determinations carried out for all 6 cycloartanes (Fig. 1) used in this study are presented in Table 2. Fig. 3 presents the phylogeny of the DMPK family kinases where the MRCK kinases belong.

Table 1. Results of primary screen for kinase inhibitory activity by Cycloartane-3,24,25-triol at a single concentration of 10 µM

Gene Symbol	% Inhibition	Disease link	Reference
DMPK2	54	Neurodegeneration	[33]
ERK3	52	Cancer, cardiovascular	[34]
HUNK	59	Cancer	[35]
MRCKA	76	Cancer	[4]
MRCKB	61	Cancer	[4]
PLK4	51	Cancer	[36]
RIOK3	54	Cancer	[37]
RIPK1	52	Neurodegeneration, cancer	[38,39]

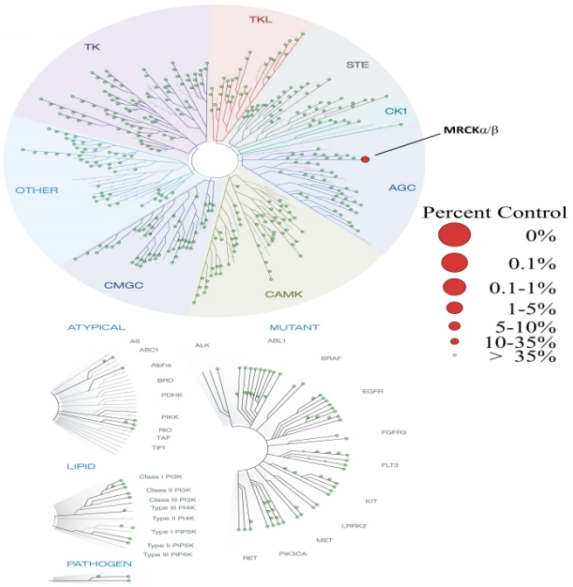

Fig. 2. Primary screen Treespot diagram depicting the selectivity of compound 2 ag MRCK kinases. Green dots indicate inhibition <65 at 10µM

Table 2. $K_d$determination of the kinase inhibitory activity of cycloartanes

Compound	K_d - µM	
	MRCKα	MRCKβ
Cycloart-23-ene-3,25-diol (**1**)	0.21	4.7
Cycloartane-3,24,25-triol (**2**)	0.25	NA
Cycloart-25-ene-3,24-diol (**3**)	0.36	1.10
3,23-Dioxo-9,19-cyclolanost-24-en-26-oic acid (**4**)	3.0	3.2
24,25-Dihydroxycycloartan-3-one (**5**)	2.1	9.8
Hydroxycycloart-23-en-3-one,25, (**6**)	NA	NA

NT=Not tested; NA=Not active

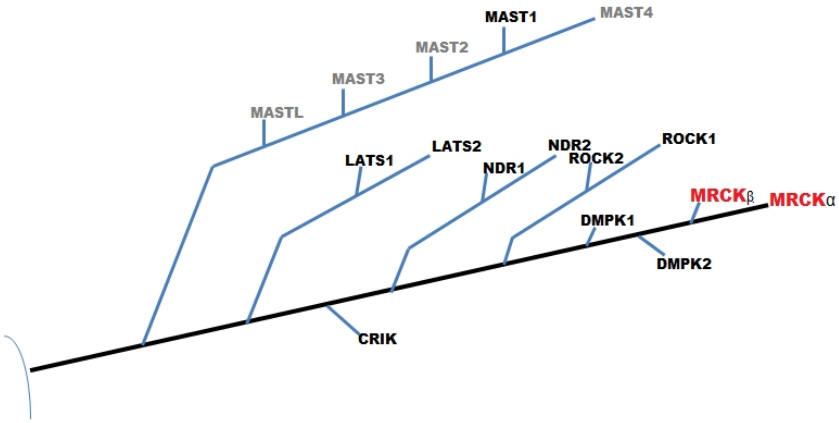

Fig. 3. Phylogenic relationship of DMPK family kinases. Compounds 1 & 2 showed selective inhibition of MRCKα/β

3.2 Antiproliferation Activity of Cycloartanes Against Prostate and Breast Cancer Cells

The results of the antiproliferation activity of 6 cycloartanes against prostate and breast cancer cell lines are presented in Table 3. The compounds inhibited proliferation of the four cell lines tested at concentrations ranging from 1.38 µM – 7.69 µM.

Table 3. Antiproliferation activity of cycloartanes against prostate and breast cancer cell lines

Cycloartanes	Prostate cancer		Breast cancer	
	PC-3	DU-145	MDA-MB-231	MCF-7
Cycloart-23-ene-3,25-diol (1)	2.20±0.18	1.92±0.06	3.46±0.97	>100
Cycloartane-3,24,25-triol (2)	2.09±0.20	1.59±0.30	1.38±0.31	7.17±1.10
Cycloart-25-ene-3,24-diol (3)	>100	>100	>100	>100
3,23-Dioxo-9,19-cyclolanost-24-en-26-oic acid (4)	>100	>100	>100	>100
24,25-Dihydroxycycloartan-3-one (5)	>100	>100	>100	>100
Hydroxycycloart-23-en-3-one,25, (6)	5.05±0.33	7.69±0.42	>100	>100

4. DISCUSSION

Cdc42-MRCK and Rho-ROCK kinase signaling have been shown to cooperate in cell invasion and in the event of deleterious mutations, may result in rapid progression and metastasis of cancer cells [7]. The Rho family GTPases play important intracellular roles as signal transducers necessary for cell growth, morphogenesis, motility, cytoskeleton formation, endocytosis and exocytosis [29].These functions are required for normal development, wound healing as well as the acquired transformation and metastasis of cancer cells [30]. The Cdc42-effector kinases (MRCKα/β) are now known to contribute to the cell migration and invasion process alone or in concert with other kinases such as the ROCK and LIMK1 [7,31]. Given recent advances in kinase research and the discovery of the role of MRCKα/β in cancer cell migration and invasion, these kinases are now considered possible targets for cancer therapeutics [8,13,14].

Kinases have been shown to be best inhibited by small molecules thus the recent surge in interest in small molecule kinase inhibitors [26,32]. Our research has focused on Cycloartanes as inhibitors of the MRCKα/β kinases because they fit the profile of small molecules given their <500 molecular weights and the activity previously demonstrated by compound 2 against the MRCKα kinase [15]. The low K_d activity demonstrated by some of the cycloartanes is comparable to the activity of several kinase inhibitors against this group of kinases. For example, a recent study screened 159 kinase inhibitors out of which 11 inhibited the MRCKβ by over 80% at 3 μM [3]. Despite the discovery that MRCKα/β are known to contribute to cell migration and invasion process alone or in concert with other kinases such as the ROCK and LIMK1, Compound 2 showed inhibitory activity against MRCKα/β but not against ROCK and LIMK1 kinases in this study(Supplement Table 1). In terms of the comparison of the inhibition of MRCKα versus MRCKβ, All of the 5 active compounds demonstrated higher activity against the MRCKα (K_d=0.21μM–3.0 μM) than against MRCKβ (K_d=1.101μM–9.8μM). The best activity against MRCKβ was exhibited by compound 3 while compound 2 was inactive.

The 6 cycloartanes used in this study have demonstrated interesting SAR profiles. Structurally, compounds 1-3 have a 3β-hydroxyl group and differ from each other only in the side chain attached to carbon 17 (Fig1). Compounds 4-6 differ from compounds 1-3 in that they carry 3-oxo group instead of the 3β-hydroxyl group. In terms of cross similarity of the side chain attached to carbon 17, compounds 1 and 6 have the same side chain (A) while 2 and 5 also have a similar side chain (B) attached to carbon 17 (Fig 1). Compounds 3 and 4 have distinct side chains attached to carbon 17. In terms of the kinase inhibition activity of the 6 compounds, the theoretical octanol/water partition coefficients for the compounds were analysed and no correlation between Log P and kinase inhibition was established [40]. In compounds 1–3, the presence of the tertiary hydroxyl at C-25 lead to higher activity compared to the presence of the hydroxyl group at either C-24 alone or both at C-24 and C-25. The presence of the 3-oxo group certainly hinders kinase inhibition compared to the presence of the 3β-hydroxy group. For example, compound 2 with a 3β-hydroxy group has an 8 fold higher binding affinity to MRCKα kinase compared to compound 5 which has a 3-oxo group and the same side chain on C-17 as compound 2 while compound 6 totally has no inhibitory properties compared to compound 1 which differs from it only in the C-3 position.

The results of the antiproliferation activity seem to also parallel the results of the kinase inhibition as only compounds 1 and 2 exhibited antiproliferation activity against both prostate and breast cancer cell lines. Compound 6 showed antiproliferation activity only against the prostate cancer cell line. Only compound 2 exhibited activity against all four cell lines tested. The MCF-7 breast cancer cell line showed the least sensitivity to the cycloartanes as it was

inhibited by only compound 2 and at IC_{50} values 3-5 times weaker than for the other cell lines.

5. CONCLUSION

In conclusion, this study has shown that cycloartane type compounds are promising inhibitors of the Cdc42-binding kinases (MRCKα/β). Further studies are required to determine the binding domains as well as examine structural modification options that could be carried out to optimize the binding affinity of cycloartanes to select kinases. The antiproliferative activity of cycloartanes against prostate and breast cancer cell lines combined with their kinase inhibitory properties makes these compounds good candidate for development into anticancer drugs. Further studies especially *in vivo* will be carried out to further evaluate the efficacy and safety of compounds 1 and 2 alone or in combination with existing anticancer drugs.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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