Aaptamine derivatives with CDK2 inhibitory activities from the South China Sea sponge *Aaptos suberitoides*

Qian-Qian He \(^a,b,\*\), Yu-Qing Man \(^a,b,\*\), Kun-Lai Sun \(^c\), Li-Juan Yang \(^a\), Yan Wu \(^a\), Jing Du \(^a\), Wei-Wei Chen \(^a\), Juan-Juan Dai \(^a\), Na Ni \(^a\), Shuang Miao \(^a\) and Kai-Kai Gong \(^a\)

\(^a\)Cancer Research Institute, Binzhou Medical University Hospital, Binzhou, China; \(^b\)Department of Pharmacy, Binzhou Medical University Hospital, Binzhou, China; \(^c\)Zhejiang Provincial Engineering Technology Research Center of Marine Biomedical Products, School of Food and Pharmacy, Zhejiang Ocean University, Zhoushan, China

**ABSTRACT**

Three new aaptamines (1–3) together with two known derivatives (4–5) were isolated from the South China Sea sponge *Aaptos suberitoides*. The structures of all compounds were unambiguously elucidated by spectroscopic analyses as well as the comparison with literature data. All the compounds were evaluated for their cytotoxic activities against five human cancer cell lines including H1299, H520, SCG7901, CNE-2 and SW680 cells. As a result, compounds 3–5 showed moderate cytotoxicities against H1299 and H520 cells with IC\(_{50}\) values ranging from 12.9 to 20.6 \(\mu\)g/mL. Besides, compounds 3–5 also showed potent inhibitory activities toward cyclin-dependent kinase-2 (CDK2) with IC\(_{50}\) values of 14.3, 3.0 and 6.0 \(\mu\)g/mL, respectively. In addition, compounds 3–5 significantly induced G1 arrests of H1299 cells at low concentrations. Drug affinity responsive target stability (DARTS) experiments were carried out and further demonstrated that compound 3 could effectively bind with CDK2 protein and protect it from the degradation by pronase.

**CONTACT**

Shuang Miao \(\text{keaimiaoshuang@126.com}\); Kai-Kai Gong \(\text{gongkaikai1005@163.com}\)

\*These authors contributed equally to this work.

Supplemental data for this article can be accessed online at [https://doi.org/10.1080/14786419.2021.2024533](https://doi.org/10.1080/14786419.2021.2024533).
1. Introduction

Though the cancers are diverse and heterogeneous, the commonality is the deregulation of cell cycle and the aberrant cell proliferation (Evan and Vousden 2001; Williams and Stoeber 2012). Cyclin-dependent kinases (CDKs), a key protein belonging to the serine/threonine kinase family, play a vital role in the regulation of the cell cycle and/or proliferation (Malumbres and Barbacid 2009). Among 9 subtypes, CDK2 is one of the most essential regulators for the cell cycle and it could facilitate the process of transition of G1 to S phase and DNA synthesis (Tadesse et al. 2020). Accumulating evidences confirmed that CDK2 is fundamentally linked to the proliferation of particular cancer types, such as ovarian cancer, glioblastoma, hepatocellular carcinoma and acute myeloid leukemia (Tadesse et al. 2019). It has been widely assumed that blocking CDK2 activity with selective inhibitors could provide a therapeutic benefit, while previous reported CDK2 inhibitors were nonspecific and limited by off-target effects (Lin et al. 2021). Thus, developing new CDK2 inhibitors represents a therapeutic opportunity for CDK2-dependent cancers.

Aaptamines are an intriguing class of marine alkaloids sharing a rare 1H-benzo[de]-1,6-naphthyridine skeleton and they have showed various biological activities, including antifouling (Diers et al. 2006), antiviral (Souza et al. 2007), antineoplastic (Gong, Miao et al. 2020; Trang et al. 2021), α-adrenoreceptor blocking (Ohizumi et al. 1984), and antioxidant activities (Utkina 2009). Until now, nearly one hundred of aaptamine derivatives were obtained from Demospongiae such as genera Aaptos, Suberites, Luffariella, Hymeniacidon, Suberea, and Xestospongia (Larghi et al. 2009). In particular, the genus Aaptos is the dominant species of producing 49 aaptamine derivatives (Yu, Yang, Sun, Li et al. 2014; He et al. 2020). In order to continue to find this kind of molecules with significant biological activities, we carried out chromatographic purification on the MeOH extracts of Aaptos suberitoides. Consequently, five aaptamine derivatives including three new ones (1–3) were isolated (Figure 1). Herein, we described the isolation, structure elucidation of these compounds, as well as the evaluation of their cytotoxicities and CDK2 inhibitory activities.
2. Results and discussion

2.1. Structure elucidation

Compound 1 was obtained as amorphous yellow solid with a molecular formula C_{13}H_{10}N_{2}O_{3} established by HR-ESI-MS (high resolution electrospray ionization mass spectrometry) ([M + H]^{+} m/z 243.0760; calcd. for 243.0764). The $^1$H NMR spectrum (Supplementary material, Table S1) displayed one set of coupled protons at $\delta_H$ 9.07 (d, $J = 4.3$ Hz) and 7.80 (d, $J = 4.3$ Hz), two isolated singlets at $\delta_H$ 8.83 and $\delta_H$ 7.16, and the characteristic signal for methoxy proton 8-OMe at $\delta_H$ 3.90 indicated that compound 1 was an aaptamine congener, with an 8-methoxybenzo[de][1,6]naphthyridine skeleton (Pham et al. 2013). Unlike the other aaptamine analogs which have been thus far reported, H-2 appeared as a singlet, thus indicating that C-3 was substituted. The $^1$H NMR spectra also exhibited a signal for another methoxyl group, observed at $\delta_C$ 4.23 (s), which could be assigned to C-3. Key HMBC correlations from 3-OMe ($\delta_C$ 154.0), from H-2 ($\delta_H$ 8.83, d) to C-3, C-3a ($\delta_C$ 139.8), from H-5 ($\delta_H$ 9.07, d) to C-6 ($\delta_C$ 122.4), C-6a ($\delta_C$ 139.1) and C-3a, from H-6 ($\delta_H$ 7.80, d) to C-5 ($\delta_C$ 154.8), C-7 ($\delta_C$ 108.6) and C-9b ($\delta_C$ 118.1), from H-7 ($\delta_H$ 7.16, s) to C-6, C-8 ($\delta_C$ 156.1), C-9 ($\delta_C$ 175.9), and C-9b, and from 8-OMe ($\delta_H$ 3.90) to C-8 ($\delta_C$ 156.1) confirmed this hypothesis (Supplementary material, Figure S6). Therefore, the structure of compound 1 was unambiguously assigned as 3-methoxy-demethyl(oxy)aaptamine.

Compound 2 was obtained as a purple-red solid with a molecular formula of C_{17}H_{17}N_{3}O_{4} established by HR-ESI-MS, which showed a pseudo-molecular-ion peak at m/z 328.1285 ([M + H]^{+} C_{17}H_{18}N_{3}O_{4}^{+}; cacld. for 328.1292). The NMR data of 2 (Supplementary material, Table S1) were closely related to compound 1, suggesting that they shared a common aaptamine skeleton with the same C-3-substituted benzo[de][1,6]naphthyridine moiety. Compared with compound 1, C-3 was upfield to $\delta_C$ 144.4 suggesting that C-3 was connected to a nitrogen atom (Yu, Yang, Sun, Ma et al. 2014). In addition, $^{13}$C NMR exhibited an additional carbonyl signal at $\delta_C$ 173.1 and three methylenes at $\delta_C$ 41.2, 30.6, 23.7 in the upfield. In the HSQC spectrum, the broad singlet peak at $\delta_H$ 8.46 was not directly attached to any carbon, and based on its highly deshielded nature, the proton was deduced to be an NH proton (Shaari et al. 2009). Interpretation of the COSY and HSQC spectra revealed the presence of continuous spin system NH-1’/H2-2’/H2-3’/H2-4’ attached to C-5’ on the basis of the HMBC correlation from H2-4’ ($\delta_H$ 2.44) to C-5’ ($\delta_C$ 173.1), from H2-3’ ($\delta_H$ 1.95) to C-5’. Additionally, the methoxyl ($\delta_C$ 51.3) must be attached to C-5’ on the basis of HMBC correlation of OCH$_3$ ($\delta_H$ 3.57)/C-5’ indicated the presence of a N-4-methylbutanolate fragment, which was unambiguously determined to be attached to C-3, based on the key HMBC correlations from H-1’ ($\delta_H$ 8.46) to C-2 ($\delta_C$ 129.7) and C-3a ($\delta_C$ 135.5) and from H2-2’ to C-3 ($\delta_C$ 144.4) (Supplementary material, Figure S13). Therefore, compound 2 was identified as 3-(N-4-methylbutanolate)aminodemethyl(oxy)aaptamine.

Compound 3 was also obtained as a purple-red amorphous solid, and its molecular formula was determined to be C$_{18}$H$_{19}$N$_{3}$O$_{4}$ by HRESIMS analysis which showed a pseudo molecular-ion peak at m/z 342.1439 ([M + H]^{+} C$_{18}$H$_{20}$N$_{3}$O$_{4}^{+}$; cacld. for 342.1448). Comparing the NMR data of 3 with those of 2 indicated that 3 was an analogue of compound 2, with the only difference at the C-3-substituted side chain.
The N-4-methylbutanoate in the structure of 2 was replaced by N-4-methylpentanoate in 3, which was confirmed by the COSY correlations of NH-1′/H2-2′/H2-3′/H2-4′/H2-5′, as well as the HMBC correlations from H-2′ ($\delta_H$ 3.55) to C-3 ($\delta_C$ 144.4) (Supplementary material, Figure S20). Thus, compound 3 was identified as 3-(N-4-methylpentanoate)aminodemethyl(oxy)aaptamine.

Due to the presence of ester bonds in compounds 2 and 3, some experiments were carried out to confirm whether 2 and 3 were natural products. Briefly, A. suberioides (100 g) was extracted and separated again by ethanol or CH$_3$CN in all process of experiments, and 40 fractions were obtained by ODS column eluting with a gradient of CH$_3$CN/H$_2$O (1:9 to 1:0). Next, all fractions were analyzed by HPLC-MS (YMC, C18, acetonitrile from 10% to 100%, 1 mL/min). As expected, 2 (in fraction 7) and 3 (in fraction 21) were successfully detected (Supplementary material, Figure S28), which demonstrated that 2 and 3 were not artificial products formed by reacting with MeOH. In fact, methyl ester is a very common group for all the classes of natural products (Venditti 2020), however, it is still necessary to eliminate the suspicion of artifacts by re-conducting the isolation work using artifact-free extraction solvent or other useful methods.

In addition to these three compounds, a spectroscopic data comparison with the literature allowed the known compounds to be identified as 3-(phenethylamino)demethyl(oxy)aaptamine (4) and 3-(isopentylamino)demethyl(oxy)aaptamine (5) (Shaari et al. 2009).

### 2.2. Biological evaluations

#### 2.2.1. Cytotoxicities of all the isolated compounds

All the compounds were tested for their in vitro cytotoxicities against the H1299, H520, SCG7901, CNE-2 and SW680 cell lines with cisplatin (DDP) as a positive control. As shown in Table S2 (Supplementary material), compounds 3–5 showed different level of cytotoxic activities against these five cancer cells with the IC$_{50}$ values ranging from 12.9 to 42.1 $\mu$g/mL (Gong, Qianqian et al. 2020), whereas 1 and 2 were inactive to all the selected cell lines at the concentration of 50 $\mu$g/mL. A preliminary structure-activity relationship implied that different C-3 substitutions probably contributed to the various degrees of cytotoxicities of demethyl(oxy)aaptamines which is consistent with previous reports (Shaari et al. 2009). Specifically, comparing the cytotoxicity data of 2 and 3 revealed that extending the length of alkyl chain between N atom and carbonyl group might be positive for the cytotoxic activity. In addition, the comparison between the cytotoxic activity data of 4 and 5 showed that electron withdrawing groups did not result in a significant increase of general cytotoxicity. However, the in-depth structure-activity relationship about aaptamines would be further analyzed based on the enough numbers of compounds.

#### 2.2.2. CDK2 inhibitory activities of all the isolated compounds

CDK2 is an important target in developing anticancer drugs. On the basis of the obtained cytotoxicities data, we further investigated the CDK2 inhibitory activities of compounds 3–5. Results showed that 4 and 5 strongly inhibited CDK2 activity with
IC$_{50}$ values of 3.0 and 6.0 μg/mL whereas 3 displayed weaker inhibitory effect with IC$_{50}$ value of 14.3 μg/mL. This is the first report of aapamine family for the CDK2 inhibitory activity (Gong, He et al. 2020).

2.2.3. **Cell cycle assay of compounds 3–5**

CDK2 and Cyclin E could modulate the cell cycle of G1 to S period. Therefore, inhibiting CDK2 activity can block the G1 period of cell cycle, subsequently induced cell death. Cell cycle assay was performed to further testify the inhibitory activity against CDK2 of compounds 3–5. H1299 cells were respectively treated with three compounds (5.0 μg/mL of compound 3 and 5, 3.0 μg/mL of compound 4) for 48 h. Cell cycle analysis revealed that the G1 population of control group was 46.67 ± 1.37%, and that of 3–5 was 54.64 ± 1.01%, 59.38 ± 1.34%, 64.15 ± 1.25%, indicating a remarkable increment of G1 population of H1299 cells after treatment with different concentrations of compounds (Supplementary material, Figure S26), which is consistent with the results of CDK2 inhibitory activity.

2.2.4. **Direct interaction of compound 3 with CDK2**

Drug affinity responsive target stability (DARTS) is a robust method for detection of small molecule protein targets (Dal Piaz et al. 2016; Lomenick et al. 2009). This method is based on the principle that the target protein becomes more stable toward proteases when binding to a small molecule (Lomenick et al. 2011). Thus, it can be used to validate potential protein-ligand interactions. DARTS and western blot assay was performed to monitor the degradation of p-CDK2 protein by pronase. In this experiment, compound 3 was selected to validate the direct interaction with p-CDK2, and the CDK2 inhibitor PHA-793887 was used as the positive control. Results showed that after treating with 0.2 mg/mL of compound 3, the p-CDK2 protein was completely digested by pronase at the concentrations of 1:1600 and 1:400. In addition, the comparison suggested that compound 3 could protect p-CDK2 from degradation by pronase at the higher concentration of 1 mg/mL (Supplementary material, Figure S27). Given all this, it could be deduced that 3 could bind with phospho-CDK2 protein and protect it from degradation by pronase, which further validated it directly interacted with CDK2 and inhibited its kinase activity.

3. **Experimental**

3.1. **General experimental procedures**

UV spectra were taken on a Beckman DU640 spectrophotometer (Beckman Coulter Inc., Brea, CA, USA). $^1$H-, $^{13}$C-NMR, DEPT, and 2D NMR spectra were recorded on NMR spectra were measured on a JEOL JNM-ECP600 spectrometer (JEOL Ltd., Tokyo, Japan), with δ in ppm with solvent residual signals as internal standards (DMSO-$d_6$: δH 2.50 ppm, δC 39.5 ppm), and J in Hz. HRMS data were obtained on a Thermo Scientific LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, USA). Cell counting kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Kyushu Island, Japan). ADP-Glo$^\text{TM}$ kinase assay kit was purchased from Promega (Madison, WI 53711 USA). Phospho-CDK2 antibody was purchased from Bioss (Catalog: bs-3483R, Bioss, Beijing).
Recombinant human CDK2/Cyclin A2 was obtained from Abcam (Catalog: ab196060, Abcam, Abcam (Shanghai) Trading Co., Ltd., China). Pronase was purchased from Sangon (Sangon Biotech (Shanghai) Co., Ltd). PHA-793887 was purchased from Beyotime (Beyotime Biotechnology, Shanghai). HPLC-MS was performed on an Thermo Scientific Ultimate 3000 chromatograph with a YMC C18 column (250 × 4.6 mm, 5 μm particle size), interfaced with an LTQ XL mass spectrometer system (Thermo Scientific).

3.2. Animal material

The marine sponge *A. suberitoides* was collected from the South Sea (Xisha Islands area) at a depth of 12 m, and was frozen immediately after collection. The specimen was identified by Dr. Nicole J. de Voogd (National Museum of Natural History, Leiden, the Netherlands). The voucher specimen (NO. XS-2012-30) was deposited at State Key Laboratory of Marine Drugs, Ocean University of China, Qingdao, Shandong, China.

3.3. Extraction and isolation

The frozen sample of *A. suberitoides* (5.75 kg, wet weight) was homogenized and then extracted with MeOH three times (5 L × 3, each, three days) at room temperature, and the solution was evaporated in vacuum to yield a crude extract (346 g). After desalting with anhydrous methanol, the left residue (246 g) was subjected to vacuum liquid chromatography (VLC) on a silica gel column (100–200 mesh, 500 g) and eluted with a gradient of petroleum ether/ethyl acetate (v/v 100:1, 50:1, 25:1, 10:1, 5:1, 2:1, 1:1, 1:2, 1:4) and CH₂Cl₂/MeOH (v/v 20:1, 10:1, 5:1, 0:1) yield seven fractions (Fr.1–Fr.7). Each fraction was detected by HPLC and was preliminarily bioassayed for cytotoxicities, and Fr. 5 and 6 were found to be the most active fractions containing the main aaptamines of this species. Thus, Fr. 5 (4.03 g) was separated by reversed-phase silica gel CC eluted with a gradient of MeOH/H₂O (v/v 20:80, 30:70, 35:65, 40:60, 45:55, 60:40) to afford six sub-fractions (Fr.5.1–Fr.5.6). Fr.5.5 (203 mg) was further separated by semi-preparative RP-HPLC (C18, acetonitrile/water, v/v 40:60, 1.5 mL/min) to obtain compounds 2 (3.70 mg, t<sub>R</sub> 17.7 min) and 3 (4.36 mg, t<sub>R</sub> 23.7 min). Fr.5.6 (325 mg) was further separated by semi-preparative RP-HPLC (C18, acetonitrile/water, v/v 45:55, 1.5 mL/min) to obtain compounds 4 (6.63 mg, t<sub>R</sub> 38.1 min) and 5 (3.77 mg, t<sub>R</sub> 41.9 min). Fr.6 (1.247 g) was separated by silica gel CC eluted with a gradient of CH₂Cl₂/MeOH (v/v 40:1, 20:1, 10:1, 5:1, 1:1, 0:1) to afford seven sub-fractions (Fr.6.1–Fr.6.7). Fr.6.1 (568 mg) was separated by reversed-phase silica gel CC eluted with a gradient of MeOH/H₂O (v/v 40:60, 45:55, 60:40, 80:20, 100:0) to afford five sub-fractions (Fr.6.1.1–Fr.6.1.5). Fr.6.1.2 (105 mg) was further separated by semi-preparative RP-HPLC (C18, acetonitrile/water, v/v 18:82, 1.5 mL/min) to obtain compound 1 (3.57 mg, t<sub>R</sub> 12.5 min).

3.4. Spectral data

3-methoxy-demethyl(oxy)aaptamine (1): amorphous yellow solid; UV (MeOH) λ<sub>max</sub> (log ε) = 205 (2.88), 235 (2.90), 265 (2.86), 360 (2.70) nm; for ¹H-NMR (DMSO-<i>d</i>₆, 600 MHz)
and $^{13}$C-NMR (DMSO-$d_6$, 150 MHz) spectroscopic data, see Table S1 (Supplementary material); HRESIMS $m/z$ 243.0760 [M + H]$^+$ (calcd for C$_{13}$H$_{11}$N$_2$O$_3$, 243.0764).

3-(N-4-methylbutanoate)aminodemethyl(oxy)aaptamine (2): purple-red amorphous solid; UV (CH$_3$OH): $\lambda_{\text{max}}$ (log $\varepsilon$) = 205 (3.06), 235 (3.09), 265 (3.04), 360 (2.87) nm; for $^1$H-NMR (DMSO-$d_6$, 600 MHz) and $^{13}$C-NMR (DMSO-$d_6$, 150 MHz) spectroscopic data, see Table S1 (Supplementary material); HRESIMS $m/z$ 328.1285 [M + H]$^+$ (calcd for C$_{17}$H$_{18}$N$_3$O$_4$, 328.1292).

3-(N-4-methylpentanoate)aminodemethyl(oxy)aaptamine (3): purple-red amorphous solid; UV (CH$_3$OH): $\lambda_{\text{max}}$ (log $\varepsilon$) = 205 (2.75), 235 (2.77), 265 (2.73), 360 (2.60) nm; for $^1$H-NMR (DMSO-$d_6$, 600 MHz) and $^{13}$C-NMR (DMSO-$d_6$, 150 MHz) spectroscopic data, see Table S1 (Supplementary material); HRESIMS $m/z$ 342.1439 [M + H]$^+$ (calcd for C$_{18}$H$_{20}$N$_3$O$_4$, 342.1448).

4. Conclusions

In summary, three new aaptamines together with two known ones were obtained from the sponge *A. suberitoides*. All compounds possessed the same 8-methoxybenzo[de][1,6]naphthyridine skeleton with diverse side chains which resulted in different levels of cytotoxicities toward selected cancer cell lines. ADP-Glo™ Kinase assay demonstrated that compounds 3–5 could potently inhibit the activity of CDK2 kinase which was further validated by cell cycle assay and DARTS. This study discovered a new scaffold of CDK2 inhibitor and found a new target of aaptamine family.

Acknowledgements

Special thanks are given to G.-Q Li (School of Medicine and Pharmacy, Ocean University of China, Qingdao, China) for providing the sponge sample *Aaptos suberitoides*.

Disclosure statement

There are no conflicts to declare.

Funding

This work was supported by the National Natural Science Foundation of China (81903537, 81803423 and 81903102), Natural Science Foundation of Shandong Province (ZR2018BB024), Traditional Chinese Medicine Technology Development Foundation of Shandong (2019-0521), and the Science and Technology Development Foundation of Binzhou (2015ZC0304), Research Foundation of Binzhou Medical University (BY2015KYQD31 and BY2017KJ17).

ORCID

Kai-Kai Gong http://orcid.org/0000-0003-2575-5533
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