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ARTICLE

Novel caffeine derivatives with antiproliferative activity

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Caffeine is probably the best known and most widely used psychoactive substance in the world. Beside its psychoactive effects, caffeine has been found to affect the cell cycle and DNA repair, as a consequence of the inhibition of ATM and ATR kinases. These two DNA damage response kinases, members of the phosphatidylinositol 3-kinase related protein kinase family, represent very attractive anticancer drug targets. Their inhibition can selectively sensitize cancer cells to DNA damaging agents and even kill various tumour cells in monotherapy. We developed a series of caffeine derivatives and evaluated their antiproliferative effects on 11 human tumour cell lines and compared them against caffeine and a standard ATR inhibitor VE-821. Although new caffeine derivatives did not achieve the overall potency of VE-821, several compounds exhibited enhanced antiproliferative activity compared to caffeine and in some cell lines showed at least comparable activity to VE-821.

Introduction

Caffeine is a naturally occurring CNS stimulant and one of the most frequently consumed psychoactive substances in the world. Apart from its well-known actions on the neural and cardiovascular systems, caffeine also exhibits notable effects on cell cycle function and regulation, apoptosis and DNA repair.^{1–3} Caffeine-prevented G2 arrest and imperfect DNA repair before S phase were observed more than 30 years ago, when it was found that caffeine increases the lethality of various cytotoxic agents such as nitrogen mustard.^{4,5} Since then caffeine has been the subject of many studies, which revealed the inhibition of ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) kinases as the major cause of these effects.^{6,7}

ATM and ATR are atypical serine/threonine protein kinases, which belong to the phosphatidylinositol 3-kinase (PI3K) related protein kinase (PIKK) family. They play a major role in the complex signal network of transduction pathways of DNA damage responses (DDR).^{8,9} ATM kinase is one of the main regulators in response to DNA double strand breaks (DSBs), hyperthermia, reactive oxygen species or replication stress. DSBs are the most dangerous type of DNA damage, and their repair is necessary for cell viability or correct cell division. They

are caused by ionizing radiation (IR), various cancer chemotherapeutics or by mechanical stress on chromosomes. The naming of ATM kinase originated from the rare multisystem disorder ataxia telangiectasia, which is caused by mutation in the *ATM* gene. As well as cerebellar ataxia and telangiectasia, patients suffer from neurodegeneration, cancer predisposition and extreme sensitivity to DNA damage due to genomic instability and cell cycle anomalies.¹⁰ ATR is, on the other hand, mainly a replication stress kinase and is responsible for proper DNA replication and reparation of single strand breaks (SSB). *ATR* gene mutation is embryonically lethal, possibly due to imperfect DNA replication in the absence of ATR.¹¹

In current cancer research, inhibition of ATM or ATR is desirable, because it provides an opportunity to enhance the conventional genotoxic cancer therapy (i.e., radiotherapy, chemotherapy). Not only can it increase the effectiveness of the treatment but it also enables specific targeting of tumour cells, which are often more sensitive to DDR intervention.^{12,13}

The earliest inhibitor of PI3K and PIKK to be discovered was a fungal metabolite wortmannin (Fig. 1). Wortmannin showed a very strong potency against almost all PI3K members. However, low selectivity, irreversible inhibition and high toxicity of this compound prevented its further use.^{14,15} The other naturally occurring inhibitor is the aforementioned caffeine. Methylxanthines in general are known to exert effects on the cell cycle and DNA repair, through the mechanism of inhibition of ATM and ATR kinases. The inhibition potency of caffeine is very weak (IC₅₀ value for ATM and ATR is 200 and 1 100 μM, respectively), and hence cannot itself be considered as a potential radiosensitizer, due to its numerous other effects on the human body. The concentration in blood needed for the desired effect would be associated with fatal tachyarrhythmias.^{12,16}

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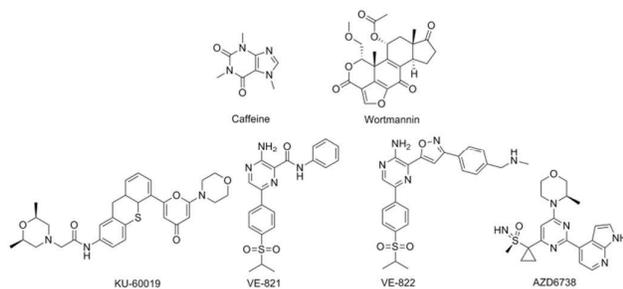


Figure 1. Structures of ATM and ATR inhibitors

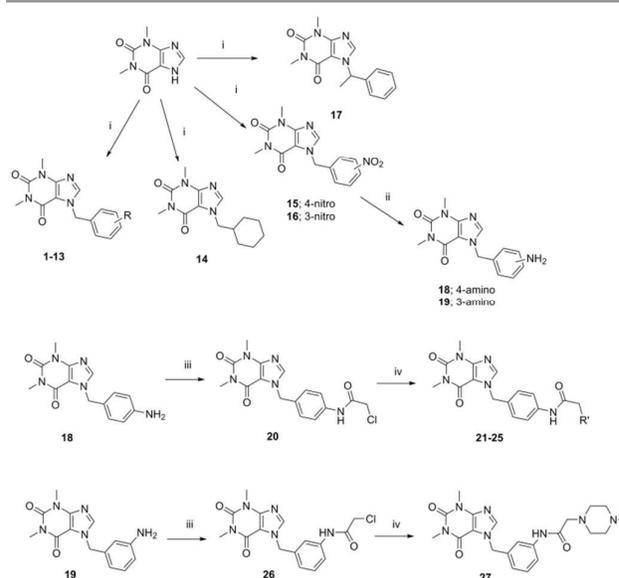
The attractiveness of ATR and ATM kinases is reflected in the efforts of several pharmaceutical companies and academic institutions for the development of new small molecule inhibitors for these kinases.^{16,17} Currently, the most advanced ATM inhibitor is KU-60019 (Fig. 1), which exhibited strong and safe radio- and chemosensitization of tumour cells and suppression of cell proliferation and migration.¹⁸ Among the ATR inhibitors, VE-822 (VX-970)^{19,20} and AZD6738^{21,22} are already in phase I of clinical trials for assessing safety and pharmacokinetics, both when administered alone and in combination with cytotoxic drugs. These compounds proved to be very effective in selective targeting of ATM or p53 deficient tumour cells, while they showed no severe toxicity on normal tissue.^{13,19}

Despite the extensive research devoted to these kinases, to date only a few promising inhibitors have been disclosed. The reason for the paucity of selective and potent inhibitors can be found in the absence of high-resolution information about the kinase domain structures of these kinases and in the high similarity between the members of PI3K and PIKK families, making the development of novel inhibitors an unmet goal.²³ In this work, we turned our efforts to caffeine and investigated the potency of several novel caffeine derivatives on the proliferation of 11 tumour cell lines, plausibly to find compounds with a highlighted antiproliferative effect caused by ATM or ATR inhibition. As a starting compound we used theophylline, to which were attached various non-polar aromatic or aliphatic substituents at position 7- via a methylene linker (i.e., benzyls, methylcyclohexyl). The selection of initial four substituents followed the Topliss scheme for mapping the structure-activity relationships.²⁴ The resulting series of 17 compounds were biologically evaluated; however, their poor aqueous solubility limited further biological testing. Hence, we tried to improve the physicochemical properties and possibly the activity by polar substituent attachments via an amide linker to positions 4- and 3- of the benzyl moiety, similarly as was applied in KU-60019.¹⁸ Unfortunately, this structural modification resulted in derivatives endowed with very low to no activity. Overall, a few of the prepared caffeine derivatives affected the proliferation of various tumour cell lines, and in some cell lines they exhibited at least comparable activity to that of the standard ATR inhibitor VE-821 (Fig. 1).

Results and discussion

Chemistry

The general synthetic pathway for the novel caffeine derivatives is displayed in Scheme 1 and described in Supplementary information. The initial series of 7-substituted caffeine derivatives (**1-17**) was obtained in good-to-excellent yields (62-92 %) by reaction of theophylline with differently substituted benzyl bromides, 4-methylcyclohexyl bromide or (1-bromoethyl)benzene in the presence of potassium carbonate in DMF.²⁵ The two nitrophenyl derivatives (**15, 16**) were then quantitatively reduced by zinc in acetic acid to the corresponding amino derivatives (**18, 19**).²⁶ These 4- and 3-amino derivatives (**18, 19**) were used as starting compounds for preparation of a second series of derivatives with enhanced solubility. The acylation of **18** and **19** with chloroacetyl chloride afforded intermediates **20** and **26**, which were subsequently treated with different secondary amines to afford the final compounds **21-25** and **27** with overall yields ranging from 25 to 37 %.



Scheme 1. Synthesis of caffeine derivatives (1-27). Reagents and conditions: (i) benzyl bromides, 4-methylcyclohexyl bromide or (1-bromoethyl)benzene, K_2CO_3 , DMF, 100 °C, 4 h, 62-92 %; (ii) Zn, AcOH, 12 h, 90-91 %; (iii) chloroacetyl chloride (Et_3N , THF, 12 h); (iv) various secondary amines, THF, 50 °C, 12 h, 25-37 %.

All synthesized compounds (**1-27**, except intermediates **20, 26**) were sufficiently characterized by 1H NMR, ^{13}C NMR and High-resolution Mass Spectrometry (HRMS) analyses. According to LC with UV detection ($\lambda = 254$ nm; see Supplementary information) the uncalibrated purity of the compounds was > 98%.

Cell proliferation after caffeine derivative treatment

Single dose testing of growth inhibition on the screening panel of 11 human tumour cell lines was performed with 25 different caffeine derivatives at a concentration of 20 μM . At the end of the incubation period (48 hours), cell proliferation was determined by WST-1 proliferation assay and related to the

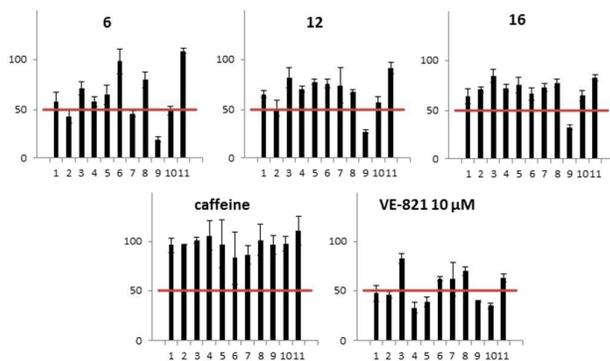


Figure 2. Decrease in cell proliferation after caffeine derivative treatment. Each graph represents proliferation of cells cultivated with the indicated caffeine derivative (**6**, **12** and **16**, with standards caffeine and VE-821) in percent relative to untreated control cells (100%). Numbers 1-11 define the appropriate cell line (1. Jurkat; 2. MOLT-4; 3. A549; 4. AGS; 5. COLO-201; 6. HT-29; 7. PANC-1; 8. SW-480; 9. A2780; 10. HeLa; 11. MCF-7). Red line delineates the 50% value. Error bars indicate \pm SD.

proliferation of untreated control cells. The one-dose data of all the screened compounds are in Supplementary Fig. S1, Tab. S2.

Amongst the caffeine derivatives tested, compound **6** showed the highest overall activity (Fig. 2, S1; Tab. 1, S2) as it caused a drop of proliferation to below 50% of the control in 4 cell lines (below 75% of control in 8 lines) from 11 lines tested, and its growth percentage (GP) value was 63 with the range from 19 to 108. Based on the GP values, derivatives **12** and **16** followed with GP values 67 (range 26-91) and 69 (range 32-84), respectively. As a positive control, we used a selective inhibitor of ATR, VE-821. Treatment with this inhibitor at a concentration of 10 μ M led to at least 50% proliferation decrease in 6 cell lines tested (10 lines below 75%) and its GP value was 52 (range 33-82). As expected, the parent compound of the series, caffeine at concentration 20 μ M exhibited almost no activity on cells' proliferation (GP 97, range 83-120).

Focussing on the cell types, A2780 (ovarian carcinoma) seemed to be the most sensitive cell line to the action of caffeine derivatives, as its proliferation dropped below 50% after treatment with 11 derivatives from a total of 25. GP value for A2780 was calculated as the mean of the percentage growth after each derivative treatment, and reached 61 with range from 26 to 103 (Fig. 3). On the other hand, the proliferation of MCF-7, A549, PANC-1 and SW-480 cell lines was almost unaffected by any of the prepared derivatives, including caffeine. Low activity (proliferation above 60%) was also observed after incubation with VE-821.

For two of the most sensitive cell lines, A2780 and MOLT-4, the cytotoxic dose dependency of compound **6** was evaluated and compared to VE-821. The dose response curves for compound **6** and VE-821 in A2780 ovarian cancer cell line and MOLT-4 leukemic cell line after 48 h are shown in Fig. 4. Half maximal inhibitory concentration (IC_{50}) was calculated from the measured results (Tab. S1). The caffeine derivative **6** affected the A2780 and MOLT-4 cells with IC_{50} values of 21 and 31 μ M, respectively; and VE-821 with 14 and 26 μ M, respectively.

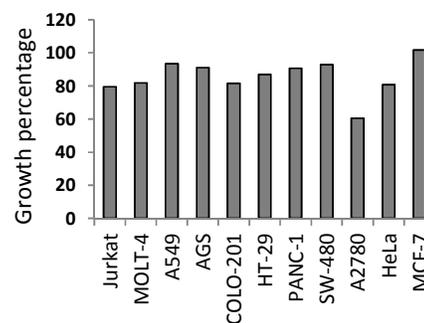


Figure 3. Overall sensitivity of tumour cell lines to caffeine derivatives. GP was calculated for each cell line as mean value of percentage expression of proliferation after caffeine derivative treatment (1-27). The order of cell lines in this Figure corresponds with numbers in Fig. 2.

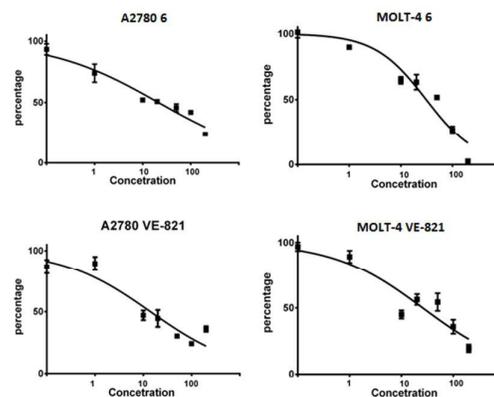


Figure 4. Cytotoxicity curves obtained by WST-1 assay showing the effect of tested compound **6** and VE-821 on the viability of A2780 (ovarian cancer) and MOLT-4 (acute T lymphoblastic leukaemia) cells after 48 h of exposure. X axis - concentration in μ mol/l; y axis - viability as percentage of control untreated cells.

To confirm that the derivative **6** acts as an inhibitor of ATR or ATM kinase we evaluated its effect on radiation-induced serine 345 phosphorylation of Chk1 (catalysed mainly by ATR) and threonine 68 phosphorylation of Chk2 (catalysed mainly by ATM).²⁷⁻²⁹ HL-60 cells were treated with the standard ATR inhibitor VE-821 or caffeine derivative **6** and irradiated by the dose of 6 Gy. The irradiation induces DNA damage, triggers DDR and results in significant increase in phosphorylated Chk1 and Chk2. The serine 345 phosphorylation of Chk1 was inhibited by standard ATR inhibitor VE-821 as well as by compound **6** in all tested concentrations (Fig. 5). As we expected, the ATR kinase inhibitor VE-821 did not prevent threonine 68 phosphorylation of Chk2. The changes in Chk2 phosphorylation in presence of derivatives **6** were not significant. From these data, we can assume that derivative **6** is a potent inhibitor of ATR.

Whilst caffeine was inefficient at a concentration of 20 μ M, several other derivatives bearing non-polar substitution at position 7- exhibited a moderate antiproliferative effect on various tumour cell lines. Introduction of a benzyl moiety itself (**1**) brought no activity improvement compared to caffeine; however, the activity was noticeably affected when the benzyl

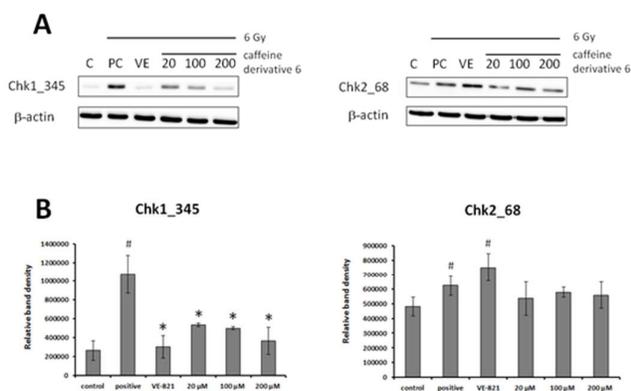


Figure 5. Changes in serine 345 phosphorylation of Chk1 (Chk1_345) and threonine 68 phosphorylation of Chk2 (Chk2_68) in HL-60 cells. Protein changes were detected 4 h after the irradiation by a dose of 6 Gy. The standard ATR inhibitor VE-821 (10 μ M) and caffeine derivative **6** (20, 100 and 200 μ M) were added 30 min before irradiation. To ensure equal protein loading, each membrane was reprobed and β -actin was detected. C – untreated control, PC – positive control (irradiation only), VE – VE-821. A) Representative blot of one of three independent experiments. B) Densitometric analysis of band intensity of proteins. Error bars indicate the standard deviation for $n = 3$ independent experiments. *significantly different to positive control (PC); # significantly different to untreated control (C); $P < 0.05$.

moiety contained small functional groups. From the GP values of the synthesized compounds we cannot deduce any direct dependence on different substituents on the aromatic ring. The most effective were compounds **6** and **12**, which both have a hydrophobic (isopropyl, methylthio) at position 4- of phenyl, whereas the derivatives with an electron-withdrawing substituent at this position (-Cl, -Br, -CN, -NO₂) were inactive. From this data we can assume that aliphatic substituents in this position enhance the antiproliferative activity. However, the third ranked most active compound **16** stands out somewhat as it bears the nitro group at position 3- of the benzyl. Experiments probing the effect of saturation of the non-polar part of the molecule in compound **14** seemed to indicate a negligible effect on activity compared to its aromatic counterpart **1**. Similarly, the introduction of a chiral centre to explore the effect of rotation of the aromatic ring relative to the core purine in **17** did not reveal any fluctuation in activity, possibly due to no topological preference in the binding site. In order to model partitioning of compounds **6** and **12**, we carried out a chromatographic analysis with Immobilized Artificial Membrane (IAM) column which mimic the lipid environment in cell membranes. Capacity factors proved that **6** ($k = 6.36$) interacts strongly with IAM column, whilst **12** ($k = 2.85$) exhibits significantly weaker retention. Comparing to **1** ($k = 0.99$), both compounds **6** and **12** are more hydrophobic, which may partially elucidate the observed enhancement of the biological activities by improved penetration to the cells. The second series was prepared with the aim of increasing the solubility as well as to investigate the effects of polar substitution on activity. Disappointingly, although the 4- and 3- substituted derivatives (**20-25**, **27**) had slightly better solubility, their antiproliferative activity was low to nil (GP

values of 88 – 103). Thus apparently only non-polar substituents are suitable for modification of the benzyl ring.

The results showed that beneficial modification of the benzyl moiety of the caffeine derivatives is very limited, and larger, polar and electron withdrawing groups all led to the ablation of activity. Therefore, investigation of modifications in the xanthine core will be the next step in seeking to uncover the plausible potential of novel caffeine hybrids.

Although the overall effects of the newly synthesized derivatives on cell proliferation are not very significant, certain amelioration compared to the standards is noteworthy. Compound **6** showed pronounced ATR inhibition and affected some cell lines (i.e. MOLT-4, A2780 or HeLa) in a manner similar to VE-821.

Conclusions

In this work, we prepared 25 novel caffeine derivatives through simple modification and examined their effect on the proliferation towards 11 tumour cell lines, which could result from ATR kinase inhibition. Caffeine itself was not active at the given concentration, but several new derivatives (**6**, **12** and **16**) exhibited similar, although not as strong, activities as the potent ATR inhibitor VE-821. Unfortunately, the active derivatives suffered from low aqueous solubility, which limits their biological evaluation. Efforts to increase the solubility and antiproliferative activity by modification of the benzyl moiety were not successful, leaving the xanthine core as the next target for modification.

Table 1. Antiproliferative effect of caffeine derivatives. Mean GP value was calculated for each caffeine derivative as the average of percentage expression of cell proliferation of 11 cell lines tested. The range of growth percentage as well as the three most sensitive cell lines with growth percentage values is indicated for each derivative (the complete Table is displayed in Supplementary Table S1).

Compound	R	Mean GP	Range of GP	The most sensitive cell lines
1	H	93	78-133	HeLa, A2780, PANC-1
2	4-MeO	84	51-106	A2780, Jurkat, HeLa
3	4-Me	83	41-103	A2780, Jurkat, HeLa
4	4-Cl	92	57-116	A2780, Jurkat, HeLa
5	3,5-diMe	78	26-105	A2780, Jurkat, HeLa
6	4- <i>i</i> Pr	63	19-108	A2780, MOLT-4, AGS
7	2-Me	81	42-98	A2780, Jurkat, COLO-201
8	4-CN	95	60-132	A2780, HeLa, COLO-201
9	4-Br	98	49-117	A2780, AGS, Jurkat
10	4-COOMe	81	43-106	A2780, HeLa, HT-29
11	4-CF ₃	78	46-96	A2780, MOLT-4, Jurkat
12	4-MeS	67	26-91	A2780, MOLT-4, HeLa
13	3-Br	73	34-112	A2780, MOLT-4, HeLa
14	^a	88	73-112	HeLa, SW-480, PANC-1
15	4-NO ₂	95	68-127	A2780, HeLa, Jurkat
16	3-NO ₂	69	32-84	A2780, Jurkat, HeLa
17	^a	96	76-130	COLO-201, HT-29, Jurkat
18	4-NH ₂	83	62-103	HT-29, HeLa, SW-480
19	3-NH ₂	86	65-104	A2780, COLO-201, Jurkat
21	<i>N</i> -methylpiperazine ^b	96	81-122	COLO-201, HT-29, PANC-1
22	Morpholine ^b	87	70-106	COLO-201, A2780, Jurkat
23	Piperidine ^b	88	75-98	A2780, MOLT-4, Jurkat
24	Diethylamine ^b	96	88-103	MCF-7, MOLT-4, A549
25	Diethanolamine ^b	103	93-124	A549, AGS, MCF-7
27	^a	101	73-120	A2780, Jurkat, MOLT-4
VE-821	–	52	33-82	COLO-201, HeLa, A2780
Caffeine	–	97	83-120	SW-480, AGS, Jurkat

^a Structure displayed in Scheme 1.

^b Substituents refer to R' in Scheme 1.

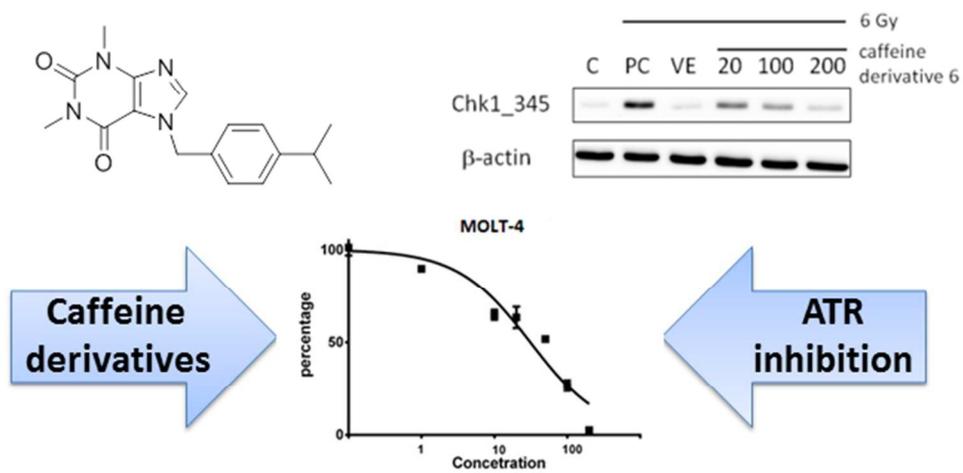
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