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Bioorganic & Medicinal Chemistry 16 (2008) 3626–3631
Bioorganic & Medicinal Chemistry

Synthesis and anti-cancer activity of benzothiazole containing phthalimide on human carcinoma cell lines

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Received 5 December 2007; revised 3 February 2008; accepted 5 February 2008
Available online 8 February 2008

Abstract—Phthalic anhydride is a highly toxic substance, facing, however, the problem of hydrolysis. In fact, it is rapidly hydrolyzed in aqueous medium, generating phthalic acid as the final product, which is almost harmless to viable cells. Here we describe the 'one pot' condensation reaction for the synthesis of phthalic imide derivative (benzothiazole containing phthalimide), exhibiting in vitro cytotoxic potential on human cancer cell lines. We further demonstrated that both caspase-dependent and -independent pathways are involved in our novel benzothiazole containing phthalimide induced apoptosis on cancer cells.

1. Introduction

Cancer chemotherapy targeting tumour progression represents one of the most relevant challenges of chemists and oncologist. In order to gain new insights into the complexity of the disease, robust screening methods for evaluating different natural or synthetic drugs have been carried out from the science community.

In this respect, the benzothiazole constitutes an important scaffold of drugs, possessing several pharmacological functions, rendering this molecule and its derivatives powerful antitumour agents,[1–6] neurotransmission blocker,[7–9] calmodulin (CaM) antagonists,[10] neuroprotective agent[11,12] and agents exhibiting other interesting biological activities.[13–19] Recently, structurally novel benzothiazole derivatives have been shown to have improved biological activity. Some of the 2-amino derivatives have been reported to possess cytotoxicity on tumour cells which are comparable to that of cisplatin.[1] Cantharidin-containing benzothiazole represents our recently explored series possessing antitumour properties in hepatocellular carcinoma, breast cancer, acute myelogenous leukaemia and non-small cell lung carcinoma. At the same time, the cytotoxicity towards non-malignant haematological disorder bone marrow cells is significantly low. Our results also demonstrated that cantharimide analogues bearing 2-amino-benzothiazole moiety were effective to minimize the original cytotoxicity, whilst maintaining potency towards cancer cell lines in vitro. As part of our on-going studies of cantharimide analogues, further modifications were elucidated and evaluated in vitro, in order to expand structure–activity

Keywords: Antiproliferation; Benzothiazole; Neoplasm; Phthalimide.
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doi:10.1016/j.bmc.2008.02.005

relationship studies for possibly finding novel active templates. Replacement of the bicyclic counterpart is however the most straightforward structural modification (Fig. 1). The phthalimide substituted benzothiazole was then synthesized and its biological activity screened on cancer cell lines. Our results showed that it mediates a significant cytotoxic response to cancer cell lines according to striking differences in electronic property and potency.

2. Results and discussion

2.1. Identification of our novel synthesized benzothiazole containing phthalimide

To confirm the structure of our novel benzothiazole containing phthalimide, we have performed both 1H spectrum and 13C spectrum NMR analysis.

2.2. Cytotoxic activity of benzothiazole containing phthalimide on human cancer cell lines

The human hepatoma cell line SKHep1, the Burkitt's lymphoma cell line (B cell type) CA46 and the chronic myelogenous leukaemia (CML) K562 were firstly selected for the purpose of preliminary anti-cancer screening of novel synthesized compounds. As shown in Figure 2, our novel synthesized benzothiazole containing phthalimide showed significant cytotoxicity towards all the three human cancer cell lines tested. The mean concentration of this benzothiazole containing phthalimide compound reducing by 50% the cellular adenosine triphosphate (ATP) content in all the cancer cell lines was found to be around 69 μM (25 μg/mL). Phthalic anhydride, on the other hand, did not show significant cytotoxicity in all the three cell lines tested. The results are shown in Figure 3. Figure 4 demonstrates that incubating the SKHep1 hepatoma cells with 110 μM (40 μg/mL) of benzothiazole containing phthalimide after 48 h showed morphological changes including cell rounding and detachment from the substratum.

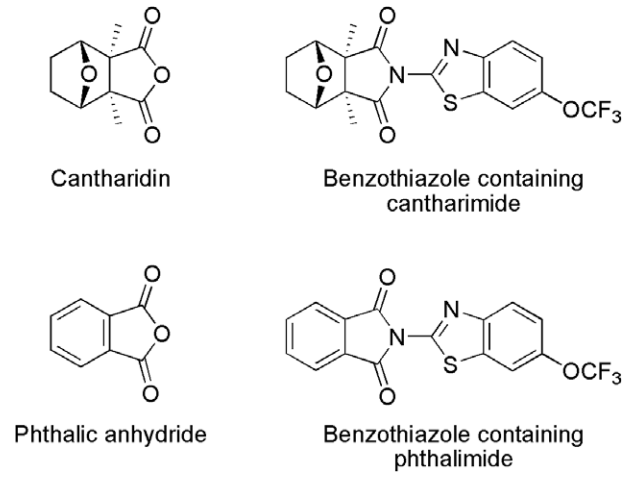


Figure 1. Chemical structures of cantharidin, phthalic anhydride, benzothiazole containing cantharimide and benzothiazole containing phthalimide.

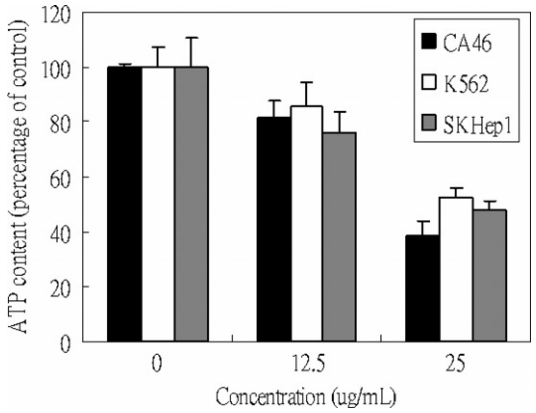


Figure 2. Growth inhibitory activity of benzothiazole containing phthalimide on CA46, K562 and SKHep1 human cancer cells after 48 h as investigated by quantitating the cellular ATP levels. Reported results represent the mean + standard deviation from triplicate tests. The figure shows a representative experiment taken from three independent experiments giving similar results.

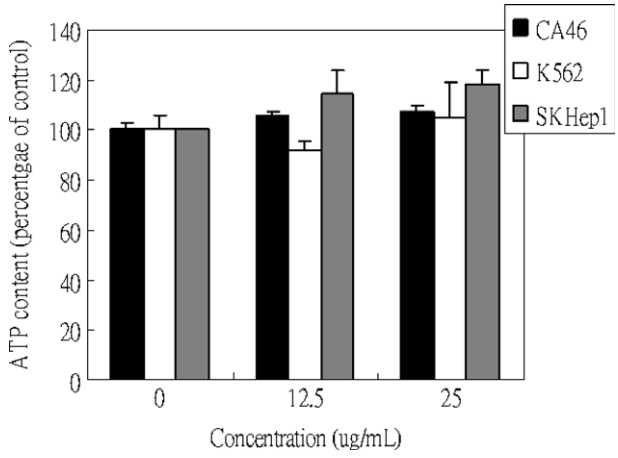


Figure 3. Effects of phthalic anhydride (16.9 μM = 25 μg/mL) on cell proliferation of CA46, K-562 and SKHep1 human cancer cells after 48 h as investigated by quantitating the cellular ATP levels. Reported results represent the mean + standard deviation from triplicate tests. The figure shows a representative experiment taken from three independent experiments giving similar results.

2.3. Caspase-dependent and -independent pathways are involved in benzothiazole containing phthalimide induced apoptosis on human cancer cells

Failure to activate apoptotic pathways in response to drug treatment may lead to resistance of tumours cells to anti-cancer therapies. Therefore, factors affecting caspase activation and apoptosis might be important determinants for the drug sensitivity. In addition to caspase-dependent apoptosis, caspase-independent forms of cell death may also play a crucial role for treatment response. Among the different caspases, caspase 3 is considered to be the central machinery of apoptosis. In conclusion, insights into the mechanisms regulating apoptosis as well as other forms of cell death pathways provide information greatly valuable for the development of novel strategies targeting resistance of tumour

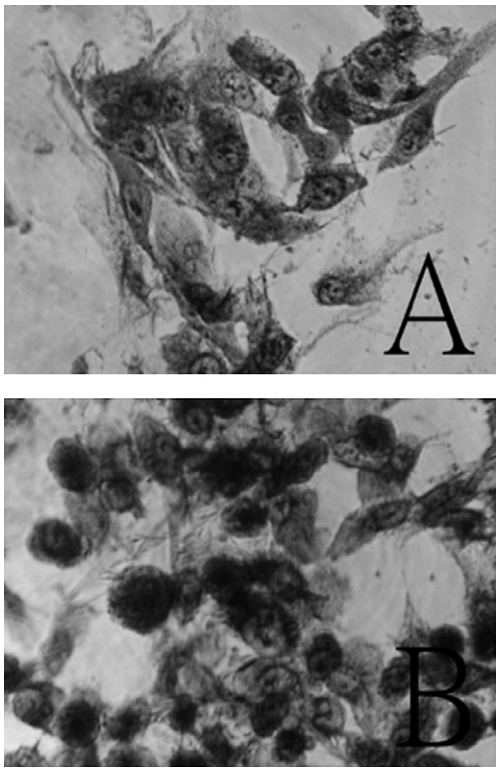


Figure 4. Morphological analysis for the effects of benzothiazole containing phthalimide on SKHep1 hepatoma cells after 48 h of incubation and methylene blue staining. (A) Untreated control. (B) Benzothiazole containing phthalimide at 110 μM (40 μg/mL).

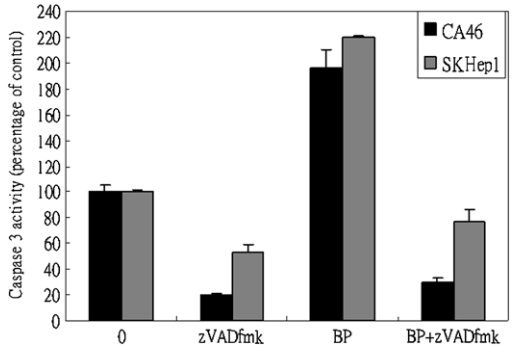


Figure 5. Caspase 3 activity assay to study the possible apoptotic potential of benzothiazole containing phthalimide (BP) at a concentration of 110 μM (40 μg/mL) on CA46 and SKHep1 human cancer cells after 48 h incubation. The zVADfmk (20 μM) is a caspase inhibitor used to inhibit the activation of caspase 3. Reported results represent the mean + standard deviation from triplicate tests. The figure shows a representative experiment taken from three independent experiments giving similar results.

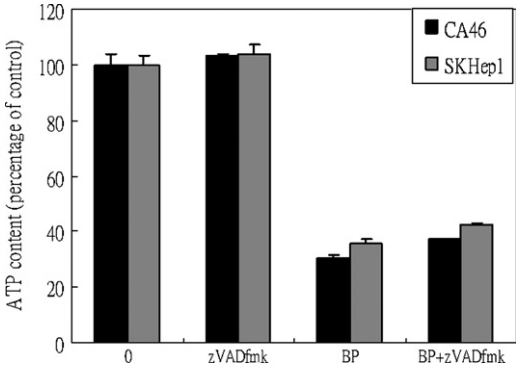


Figure 6. Analysis of the possible reversal effects of caspase inhibitor zVADfmk (20 μM) on the cytotoxicity of benzothiazole containing phthalimide (BP) at a concentration of 110 μM (40 μg/mL) on CA46 and SKHep1 human cancer cells after 48 h of incubation. Reported results represent the mean + standard deviation from triplicate tests. The figure shows a representative experiment taken from three independent experiments giving similar results.

cells. Since both CA46 and SKHep1 cancer cells were found to be more susceptible to the action of our benzothiazole containing phthalimide (Fig. 2), we further selected them to study the possible involvement of caspase 3 for the biological activity of benzothiazole containing phthalimide. As anticipated, we found that the benzothiazole containing phthalimide at a concentration of 110 μM (40 μg/mL) for 48 h could significantly activate caspase 3 as determined by the luminescence kit purchased from Promega (Fig. 5) while pre-incubating the cancer cells with 20 μM of the pan-caspase inhibitor (zVADfmk, purchased from Promega) for 2 h before the addition of the tested compound could significantly inhibit the proteolytic activity of caspase 3. However, pre-incubating the cancer cells with the caspase inhibitor could only partially reverse the benzothiazole containing phthalimide mediated cytotoxic activity [increased from a mean of 30.2–37.1% for CA46 and 37.8–42.6% for SKHep1 for their cellular ATP content when compared with untreated control (Fig. 6)]. Thus we speculated that both caspase-dependent and -independent apoptotic pathways are important for the actions our benzothiazole containing phthalimide on these two cancer cell lines.

As mentioned before, the selectivity between normal and malignant cells is one of the critical issues for the research and development of chemotherapeutic reagents. Accordingly, we have also tested the tolerance of non-malignant haematological disorder bone marrow cells. We found that the trend of inhibition of our benzothiazole containing phthalimide on bone marrow cells showed similar toxicity as those on cancer cells [with 50% of cellular ATP content reduction around 69 μM (25 μg/mL)].

In order to identify its physicochemical properties in relation to its biological activity or binding, Log *P* and π values were estimated using ChemDraw Ultra 11 (Fig. 7). In the presence of OCF_3 substituting group, the overall hydrophobicity (i.e., Log *P*) increases significantly for both phthalimide and cantharidimide in relation to the corresponding H derivatives. The extra gain of hydrophobicity changing from cantharidimide to phthalimide (entries 2 and 4) suggests that aromatic structure of phthalimide could show dominant effect on hydrophobicity. However, the overall substituent hydrophobicity constant (i.e., π_{OCF3}) remains unchanged. For the case of using hepatoma cell line SKHep1, the structural modification on imide, however, did not appear

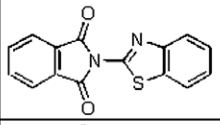
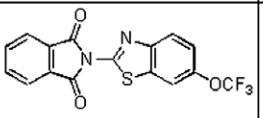
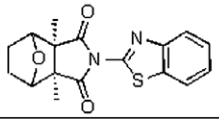
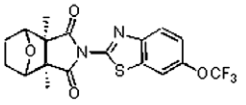
Entry	Imide type	6-Subsituting group	Structure	Log P*	π_{OCF3}	IC50 (μM)
1	Phathalimide	H		3.6	-	-
2	Phathalimide	OCF_3		5.13	1.53	69
3	Cantharidimide	H		3.15	-	-
4	Cantharidimide	OCF_3		4.18	1.53	15.2 to 30.3[18]

Figure 7. Physicochemical properties in relation to each compound's biological activity or binding. Log *P* and π values of our benzothiazole containing phthalimide and related compounds. *Log *P* values are predicted using ChemDraw Ultra 11.

to significantly alter the hydrophobicity; on the contrary, it induced a significant drop of 50% inhibitory concentration (more than a double), suggesting a further investigation on structure–activity relationship.

3. Conclusions

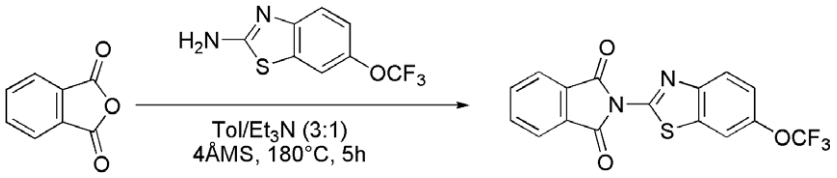
In conclusion, we have here described a facile synthesis of an anti-cancer compound, which showed significant cytotoxic activity towards three human cancer cell lines tested. Further experimental investigation for other potential partners with benzothiazole is on-going to select potential combinations not only with improved ability to discriminate between non-malignant cells and carcinoma cells but may also possess anti-microbial activity including the inhibition of β-lactamase, which could be versatile in textile industry.

4. Experimental

4.1. Preparation of phthalic imide

For the synthesis of phthalic imide derivative, a 'one pot' condensation reaction was employed as a key step with moderate yield (Scheme 1). Based upon the similarity in structure, one would predict that benzothiazole containing cantharimide and phthalimide would be metabolized and degraded in a similar manner, retaining potential applications as active anti-cancer drugs.

Unless otherwise indicated, all reactions were carried out under nitrogen atmosphere. NMR spectra were recorded on a Varian 500 MHz Fourier transform spectrometer. ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra were recorded relative to residual protiated solvent; a positive value of the chemical shift denotes a resonance downfield from TMS. Mass analyses were performed on a Finnigan model Mat 95 ST mass spectrometer. Phthalic anhydride and 2-amino-6-trifluoromethoxy-benzothiazole were purchased from Sigma–Aldrich. All other chemicals were purchased from commercial suppliers and used without further purification. Toluene and triethylamine were freshly distilled from sodium under nitrogen. All reactions were monitored by analytical thin-layer chromatography (TLC) on Merck aluminium-precoated plates of silica gel $60F_{254}$ with detection by spraying with 5% (w/v) dodecamolybdophosphoric acid in ethanol or 5% (w/v) ninhydrin in ethanol and subsequent heating. E. Merck silica gel 60 (230–400 mesh) was used for flash chromatography. Chemical



Scheme 1. Condensation of phthalic anhydride with benzothiazole.

structure was confirmed by the nuclear magnetic resonance including 1H and 13C spectra, respectively.

A mixture of phthalic anhydride (50 mg, 0.34 mmol), 2-amino-6-trifluoromethoxy-benzothiazole (410 mg, 1.7 mmol), dried toluene and dried triethylamine (6 mL; 2:1, v/v) in sealed tube was heated for 5 h at 180 °C. Concentration followed by flash chromatography gave the product (80.5 mg) in 65% yield and $R_f = 0.91$ (DCM/MeOH, 10:1). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.39 (d, $J = 8.5$ Hz, 1H), 7.77 (s, 1H), 7.88–7.90 (m, 2H), 8.06–8.08 (m, 2H), 8.14 (dd, $J = 9$ Hz and 1.5 Hz, 1H); ^{13}C NMR (dimethylsulfoxide, DMSO-d_6): δ 115.7, 120.9, 124.3, 124.9, 131.7, 134.4, 136.3, 145.9, 146.0, 148.6, 154.4, 165.1; MS (ESI) *m*/*z* (relative intensity) 387.3 [(M+Na)$^+$, 100); HRMS (ESI) [M+Na]$^+$ Calcd for $C_{16}H_7F_3N_2NaO_3S$ 387.0027, found 387.0024.

4.2. Bioassay of phthalic imide

Human Burkitt's lymphoma cell line (B cell type) CA46, chronic myelogenous leukaemia (CML) K562 and hepatoma cell line SKHep1 were used for preliminary anti-cancer screening of the novel synthesized compound. Cancer cells seeded in the 96 wells microtitre plates for 24 h were prepared for the benzothiazole containing pthalimide screening. The benzothiazole containing pthalimide was prepared as a stock concentration of 69 mM (25 mg/mL) in DMSO. Phthalic anhydride purchased from Sigma chemical was also prepared as a stock concentration of 16.9 mM (25 mg/mL) in DMSO. Both compounds were added at a starting concentration of 25 μg/mL and followed by a serial of twofold dilutions and incubated for a further 48 h. Untreated control received either total complete medium or 0.1% of DMSO. Afterwards, the evaluation of possible antiproliferative and cytotoxicity of our novel synthesized compound was assayed by the One Step ATP lite assay purchased from PerkinElmer (Netherlands) according to the technical manual provided.[19]

4.3. Caspase 3 activity assay

CA46 and SK-Hep1 cancer cells were incubated with 110 μM (40 μg/mL) of benzothiazole containing phthalimide for 48 h. Twenty micrograms of total cellular protein was analysed for the caspase 3 activity using the PerkinElmer Trupoint caspase 3 assay system according to the manufacturer's instruction.[19]

Acknowledgements

We acknowledge a donation fund from Mr. K.C. Lo to Dr. J.C.O. Tang for the establishment of Lo Ka Chung Centre for Natural Anti-cancer Drug Development (Laboratory B) and a Niche area grant offered by the Hong Kong Polytechnic University to Dr. F. Cheung (BB8R). Drs. C.H. Chui and F.Y. Lau are the honourary tutors kindly offered by Professor J. Sung and Professor G.Y.M. Cheng from Department of Medicine and Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong. Dr. C.H. Chui is supported by the post of 'Senior Research Fellow' kindly offered by Dr. J.C.O. Tang (Niche Area Grant: BB8P to Dr. J.C.O. Tang). Special thank is given to Professor R. Gambari and Dr. C.H. Chui who gave us valuable advice on the anti-cancer drug screening experiments and their constructive criticism on this paper. Professor R. Gambari is sponsored by AIRC (Italian Association for Cancer Research). Lastly, we would like to thank the assistance from Miss Monica Kwok, Dr. R.S.M. Wong and Professor G.Y.M. Cheng for providing the cancer cell lines and non-malignant haematological disorder bone marrow samples for our study.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.02.005.

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