Cancer stem cells CD133 inhibition and cytotoxicity of certain 3-phenylthiazolo[3,2-α]benzimidazoles: design, direct synthesis, crystal study and in vitro biological evaluation

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ABSTRACT

Cancer stem cells (CSCs) have been objects of intensive study since their identification in 1994. Adopting a structural rigidification approach, a novel series of 3-phenylthiazolo[3,2-α]benzimidazoles 4a-d was designed and synthesised, in an attempt to develop potent anticancer agent that can target the bulk of tumour cells and CSCs. The anti-proliferative activity of the synthesised compounds was evaluated against two cell lines, namely; colon cancer HT-29 and triple negative breast cancer MDA-MB-468 cell lines. Also, their inhibitory activity against the cell surface expression of CD133 was examined. In particular, compound 4b emerged as a promising hit molecule as it manifested good antineoplastic potency against both tested cell lines (IC₅₀ = 9 and 12 μM, respectively), beside its ability to inhibit the cell surface expression of CD133 by 50% suggesting a promising potential of effectively controlling the tumour by eradicating the tumour bulk and inhibiting the proliferation of the CSCs. Moreover, compounds 4a and 4c showed moderate activity against HT-29 (IC₅₀ = 21 and 29 μM, respectively) and MDA-MB-468 (IC₅₀ = 23 and 24 μM, respectively) cell lines, while they inhibited the CD133 expression by 14% and 48%, respectively. Finally, a single crystal X-ray diffraction was recorded for compound 4d.

Introduction

Cancer stem cells (CSCs) paradigm spurted over the past few decades as an answering solution for the ambiguity of haematological malignancies as well as solid tumours regarding intra-tumoural heterogeneity and tumour dormancy. Moreover, the observation that tumour has the proclivity to resist chemotherapy and even radiotherapy, besides metastatic relapse that can occur more than a decade post initial treatment and clinical cure, suggests a more circuitous aetiology for the malignancies. This surveillance devoted the scientists to abandon the postulation that tumour is a mass of homogeneous cancer cell population, but rather to devoted the scientists to abandon the postulation that tumour is a more circuitous aetiology for the malignancies. This surveillance suggests slow rate of proliferation rendering them resistant to conventional chemotherapy, and relapse. In this insight, targeting CSCs that are the culprit that fuels tumour development, progression, metastasis, and relapse. In this view, CSCs are beheld as a distinct subpopulation of the differentiated tumour cells that ultimately acquire CSC-like features.

In this view, CSCs are beheld as a distinct subpopulation of tumour cells that exhibit exclusive characteristics. Indeed, three main key properties of CSCs render them highly distinguishable; (1) differentiation, as they are capable to give rise to a hierarchy of progenitor and aberrantly differentiated cells, (2) self-renewal capacity, which conserves an intact stem cell pool, (3) homeostatic control that guarantees a balance between differentiation and self-renewal in response to environmental stimuli. Moreover, cunningly, the CSCs mimic their normal counterparts as they possess slow rate of proliferation rendering them resistant to conventional treatment. Accordingly, CSCs are regarded as the main culprit that fuels tumour development, progression, metastasis, and relapse. In this insight, targeting CSCs that are the “beating heart” of the tumour is a judicious goal for establishing a platform of effective cancer therapy.

Pertaining to their close similarity to normal cells, it is problematic to segregate CSCs from non-CSCs within a tumour. But for the
presence of surface cell antigens, the identification and separation of
tumour initiating cells from more differentiated tumour cells
would not have been possible. Five surface antigens whose
expression is thought to indicate stem cell like properties namely,
CD133, CD44, CD24, CDCP1, and CXCR4 proved to be useful for
the identification and characterisation of CSCs within a tumour. CD133
(Prominin-1 or AC133) is a transmembrane pentaspan pro-
tein antigen found on stem-like cells of various tissues and
malignancies, like brain, colon, breast, liver, pancreas, kidney,
lung, endometrium, ovary, and bone. The supporting evi-
dence that CD133 is a useful CSC marker further proved that CD133
is a useful CSC marker. Accordingly, it is thought to be a predictive indicator
for neoplasm identification. Targeting CD133 might be a successful strategy
for combating cancer.

In our previous work, we synthesised a series of 2-((benzimidazol-2-yl)thio)-1-arylethan-
ones (Series 1, Figure 1) that proved to possess good anti-proliferative activity toward
HT-29 colon cancer cell line besides its capability to inhibit cell surface expression
of CD133 in HT-29 cancer cells. Inspired by these findings and as a part of our ongoing efforts
towards developing potent anticancer agents, we designed a new series of
3-phenylthiazolo[3,2-a]benzimidazoles (Series 2, Figure 1) based on a benzimidazole
scaffold that proved to be affirmative for the anti-proliferative activity
beside the CD133 inhibitory potential.

Our judicious design aimed at improving the potency of the
disclosed compounds by increasing the selectivity of the synthe-
sised compounds. This was achieved through limiting the free
rotation around the single bonds in the thiocarbon linker by incorporating the linker in a cyclised thiazole ring. This interven-
tion afforded compounds that are frozen into a rigid structure
thus exhibiting less isomers which augments their selectivity at the target proteins. Moreover, our design was based on previous SAR findings that highlighted the substitution of the pendant pheno-
nyl ring by an electron-donating group to be profitable for the
inhibition of both the bulk tumour cells and the CSCs.

**Materials and methods**

**Chemistry**

Melting points were determined using a Gallenkamp melting point
apparatus and are uncorrected. Infrared (IR) Spectra were recorded
as KBr disks using the Perkin Elmer FT-IR (Fourier transform infra-
red) Spectrum BX apparatus. Mass spectra were measured on an
Agilent Triple Quadrupole 6410 QQQ LC/MS (Liquid chromatog-
raphy/Mass spectroscopy) equipped with an ESI (electrospray ion-
isation). NMR spectra were recorded on a Bruker NMR
spectrometer. 1H spectrum was run at 500 MHz and 13C spectrum
was run at 125 MHz in deuterated dimethyl sulfoxide (DMSO-d6).
Chemical shifts are expressed in δ values (ppm) using the solvent
peak as internal standard. All coupling constant (J) values are
given in hertz. The abbreviations used are as follows: s, singlet; d,
doublet; m, multiplet. Elemental analyses were carried out at the
Regional Center for Mycology and Biotechnology, Al-Azhar
University, Cairo, Egypt. Analytical thin layer chromatography (TLC)
on silica gel plates containing UV indicator was employed rou-
tinely to follow the course of reactions and to check the purity of
products. All reagents and solvents were purified and dried by
standard techniques.

**General procedure for synthesis of sulphate salts 3a–d**

To a solution of the appropriate acetophenone 1a–d (5 mmol) in
acetic acid (10 ml), 2-mercaptobenzimidazole 2 (0.75 g, 5 mmol)
and conc. sulphuric acid (50 mmol) were added. The reaction
mixture was heated under reflux for 2 h. The solid product
obtained upon cooling was filtered off, washed with cold water
then with petroleum ether, and recrystallised from ethanol to
afford the corresponding sulphate salts 3a–d, respectively.

**General procedure for preparation of 3-(3-arylimidazo[2,1-b]thiazole 4a–d**

To a suspension of the appropriate sulphate salts 3a–d (2 mmol)
in water (10 ml), an aqueous solution of sodium bicarbonate was
added. The reaction mixture was stirred at room temperature for
2 h. The solid formed was collected by filtration, washed with
water, dried, and crystallised from ethanol to afford compounds
4a–d, respectively.

**3-(3-Methoxyphenyl)benzo[4,5]imidazo[2,1-b]thiazole (4a).** White crystals
(yield 80%), m.p. 173–175 ºC; 1H NMR (DMSO-d6) δ ppm: 3.84 (s, 3H, OCH3), 7.13
(d, 1H, Ar-H, J = 7.5 Hz), 7.20–7.32 (m, 6H, Ar-H), 7.54 (t, 1H, Ar-H, J = 8.0 Hz),
7.71 (d, 1H, Ar-H, J = 8.0 Hz); 13C NMR (DMSO-d6) δ ppm: 55.86 (OCH3), 109.23,
112.01, 114.68, 116.44, 119.23, 120.86, 121.47, 123.61, 130.61, 130.69, 133.66,
148.64, 157.03, 159.92; Anal. Calcd. for C16H12N2O2S: C, 68.55; H, 4.31; N, 9.99;
Found C, 68.73; H, 4.28; N, 10.12.

**4-(Benzimidazol-2-yl)benzo[2,1-b]thiazole (4b).** White crystals (yield 78%), m.p. 190–192 ºC; 1H NMR (DMSO-d6) δ ppm: 3.82 (s, 3H, OCH3), 6.99 (d, 1H, Ar-H, J = 8.0 Hz), 7.09 (s, 1H, Ar-H),
7.12 (t, 2H, Ar-H, J = 7.5 Hz), 7.27–7.31 (m, 3H, Ar-H), 7.69
(d, 1H, Ar-H, J = 8.0 Hz), 9.67 (s, 1H, OH, D2O exchangeable); 13C NMR (DMSO-d6) δ ppm: 56.23 (OCH3), 107.63, 112.07, 113.40,
116.14, 119.11, 120.06, 120.72, 123.86, 123.48, 130.22, 134.22, 148.17, 148.67, 148.77, 156.94; Anal. Calcd. for C19H14N2O2S: C, 64.85; H, 4.08; N, 9.45; Found C, 65.14; H, 4.02; N, 9.34.
3-(3,4,5-Trimethoxyphenyl)benzo[4,5]imidazo[2,1-b]thiazole (4c). White crystals (yield 83%), m.p. 177–179°C; 1H NMR (DMSO-d6) δ ppm: 3.79 (s, 6H, OCH3), 3.83 (s, 3H, OCH3), 7.07 (s, 2H, Ar-H), 7.22–7.38 (m, 4H, Ar-H), 7.71 (d, 1H, Ar-H, J = 8.0 Hz); 13C NMR (DMSO-d6) δ ppm: 56.62 (OCH3), 60.71 (OCH3), 106.95, 108.79, 112.23, 119.19, 120.89, 123.58, 124.64, 130.21, 133.83, 139.20, 148.68, 153.59, 156.93; Anal. Calcd. for C18H16N2O3S: C, 63.51; H, 4.14; N, 7.59; O, 7.22 ppm: 5.28 (s, 2H, NH2), 6.73 (d, 2H, Ar-H, J = 8.0 Hz), 7.22–7.38 (m, 4H, Ar-H), 7.59 (d, 2H, Ar-H, J = 8.0 Hz), 7.68 (d, 1H, Ar-H, J = 8.0 Hz); Anal. Calcd. for C12H16N2O5S: C, 63.69; H, 4.69; N, 8.23; Found C, 63.69; H, 4.69; N, 8.11.

4-(Benzol[4,5]imidazo[2,1-b]thiazol-3-yl)aniline (4d). White crystals (yield 75%), m.p. 177–179°C; 1H NMR (DMSO-d6) δ ppm: 7.38 (m, 4H, Ar-H), 7.68 (d, 1H, Ar-H, J = 8.0 Hz); 13C NMR (DMSO-d6) δ ppm: 56.62 (OCH3), 60.71 (OCH3), 106.95, 108.79, 112.23, 119.19, 120.89, 123.58, 124.64, 130.21, 133.83, 139.20, 148.68, 153.59, 156.93; Anal. Calcd. for C18H16N2O3S: C, 63.51; H, 4.14; N, 7.59; O, 7.22 ppm: 5.28 (s, 2H, NH2), 6.73 (d, 2H, Ar-H, J = 8.0 Hz), 7.22–7.38 (m, 4H, Ar-H), 7.59 (d, 2H, Ar-H, J = 8.0 Hz), 7.68 (d, 1H, Ar-H, J = 8.0 Hz); Anal. Calcd. for C12H16N2O5S: C, 63.69; H, 4.69; N, 8.23; Found C, 63.69; H, 4.69; N, 8.11.

X-ray crystallographic analysis

The measurements of the crystal of compound 4d were performed on a Bruker SMART APEX II D8 Venture diffractometer with graphite-monochromated Mo Kα radiation (λ = 0.71073 Å) at 100 K. The structure was solved by direct method and refined with SHELXTL. E-maps provided the positions of all the non-H-atoms. The full-matrix least-squares refinement was carried out on F2 using anisotropic temperature factors for all non-H-atoms. Crystallographic data for the structure reported in this paper have been deposited at the Cambridge Crystallographic Data Centre and allocated with the deposition number: CCDC 1429525.

Biological evaluations

In vitro anti-proliferative activity

Anti-proliferative activity of the synthesised 3-phenylthiazolo[3,2-a]benzimidazoles 4a–d was evaluated at Stem Cell Therapy and Tissue Reengineering Program, King Faisal Specialized Hospital and Research Center, Riyadh, Saudi Arabia. Anti-proliferative activity was measured by the cell growth inhibition assay. This method and refined by SHELXTL27. Crystallographic data of compound 4d was conducted by a single crystal X-ray study for such compound unambiguously defines its exact structure. Crystal packing of 4d showed the intermolecular hydrogen bonds N3A—H1NA...N2A and N3B—H1NB...N2B (Supplementary data). The crystallographic data and hydrogen-bond geometry of 4d are presented in Tables 1 and 2, 4d was prepared according to Scheme 1. In a one-pot two-components heterocyclisation process, sulphate salts 3a–d were obtained via the reaction of acetophenone 1a–d with 2-mercaptobenzimidazole 2 in refluxing acetic acid in the presence of five equivalents of sulphuric acid. Next, neutralisation of such sulphate salts 3a–d was carried out through stirring with aqueous solution of sodium bicarbonate to furnish the 3-phenylthiazolo[3,2-a]benzimidazoles 4a–d, with 75–83% yield (Scheme 1). All spectral and elemental analyses were consistent with the proposed structures of the prepared compounds.

CD133 expression measure by flow cytometry

HT-29 and MDA-MB-468 cells harvested, washed, and then the cells were stained with conjugated monoclonal antibodies CD133-APC (Miltenyi Biotec, Bergisch Gladbach, Germany). The analyses were performed on a BD LSR II™ (BD Biosciences, San Jose, CA). Debris and cell clusters were excluded during side-scatter and forward-scatter analyses.

Results and discussion

Chemistry

In the present work, target compounds 4a–d were prepared according to Scheme 1. Characterisation of compound 4d was conducted by a single crystal X-ray structural analysis. The structure was solved with direct method and refined by SHELXTL27. Crystallographic data of compound 4d is deposited with the Cambridge Crystallographic Data Centre with deposition number CCDC 1429525. The crystallographic structure of 4d is represented in Figure 2. The single crystal X-ray study for such compound unambiguously defines its exact structure. Crystal packing of 4d showed the intermolecular hydrogen bonds N3A—H1NA...N2A and N3B—H1NB...N2B (Supplementary data). The crystallographic data and hydrogen-bond geometry of 4d are presented in Tables 1 and 2.
Biological evaluation

Anti-proliferative activity of the prepared 3-phenylthiazolo[3,2-d]benzimidazoles 4a–d was evaluated against human colon cancer cell line HT-29 and triple negative breast cancer cell line MDA-MB-468 using the WST-1 assay as described by Ngamwongsatit et al\textsuperscript{26}. 5-Fluorouracil was used as a positive control for its well-known clinical utility for managing malignant carcinomas. The anti-proliferative activities were expressed as growth inhibitory concentration (IC\textsubscript{50}) values. Among the tested compounds, compound 4b bearing a terminal phenyl ring substituted with two electron-donating groups proved to be the most potent against both cell lines; HT-29 and MDA-MB-468 with IC\textsubscript{50} values of 9 and 12 µM, respectively. Compared to the positive control 5-FU (IC\textsubscript{50} values of 15 and 41 µM, respectively), these results proclaimed compound 4b to be superior to the reference drug. Moreover, compounds 4a and 4c showed moderate activity against HT-29 cell line with IC\textsubscript{50} values of 21 and 29 µM, respectively, which are comparable to that of 5-FU (IC\textsubscript{50} = 15 µM). Luckily, both compounds evidenced good antineoplastic potency against MDA-MB-468 cell line relative to 5-FU (IC\textsubscript{50} values of 23, 24, and 41 µM, respectively). Unfortunately, compound 4d bearing a p-amino phenyl group did not manifest any significant anti-proliferative activity against the colon cancer cell line HT-29 while it demonstrated moderate activity against triple negative breast cancer cell line MDA-MB-468 (53 µM) (Table 3).

Moreover, the inhibitory effect of the prepared 3-phenylthiazolo[3,2-d]benzimidazoles 4a–d on cell surface expression of CD133 was evaluated at 10 µM via flow cytometry. The analysis was performed on a BD LSR II\textsuperscript{TM} (BD Biosciences). The results expressed as a side population inhibition (%). Scrutinizing the obtained results disclosed that compound 4a bearing a m-methoxy phenyl group only limitedly inhibited the CD133 by 13%, whereas compound 4b that possesses an extra electron donating group; an additional p-hydroxy group significantly inhibited the CD133 expression by 50%. Coherent to these findings, compound 4c that has three methoxy groups grafted on its terminal phenyl ring.

Table 2. Hydrogen-bond geometry (Å, °) of 4d.

| D–H···A       | D–H   | H···A   | D–A   | D–H···A   |
|--------------|-------|--------|-------|-----------|
| N3A–H1NA···N2A | 0.91  | 3.19   | 3.095 | 168       |
| N3B–H1NB···N2B | 0.93  | 2.18   | 3.046 | 155       |

Symmetry codes: (i) x, y, z – 1.

Table 3. In vitro anti-proliferative activity of compounds 4a–d against colon HT-29 and breast MDA-MB-468 cancer cell lines.

| Comp. | R<sub>1</sub> | R<sub>2</sub> | R<sub>3</sub> | HT-29 | MDA-MB-468 |
|-------|--------------|--------------|--------------|-------|-------------|
| 4a    | OMe          | H            | H            | 21 ± 1.5 | 24 ± 1.7     |
| 4b    | OMe          | OH           | H            | 10 ± 0.7 | 13 ± 0.9     |
| 4c    | OMe          | OMe          | OMe          | 29 ± 2.3 | 24 ± 2.2     |
| 4d    | H            | NH<sub>3</sub> | H            | NA<sup>b</sup> | 53 ± 4.7     |
| 5-Fluorouracil | | | | 16 ± 1.6 | 41 ± 3.8 |

<sup>a</sup>IC\textsubscript{50} values are the mean ± SD of three separate experiments.<br><sup>b</sup>NA: compounds having IC\textsubscript{50} value >100 µM.

Figure 2. An ORTEP diagram of final X-ray structure of compound 4d.
produced a comparable inhibition of CD133 (48%). These findings are in accordance with the previous conclusion that outlined the importance of incorporation of a lipophilic electron-donating group represented by a terminal phenyl ring substituted with a methoxy or a hydroxyl group. Regrettably, compound 4d containing a p-amino phenyl ring did not display any marked activity against the cell surface expression of CD133 (Table 4).

It is worth noting that the cytotoxic activities were decreased in the order of 4b > 4a > 4c > 4d, while the order was 4b > 4c > 4a > 4d for the inhibitory activity towards CD133 surface expression, which suggesting absence of a correlation between the two activities that may be attributable to the different phenotypic characteristics and different proliferative potentials.

Conclusions

In conclusion, we designed and synthesised a novel series of 3-arylthiazolo[3,2-a]benzimidazoles 4a–d (Series 2) based on structural rigidification of a series of 2-((benzimidazol-2-yl)thio)-1-arylethan-1-ones (Series 1) that proved to have antineoplastic activity and inhibitory activity of cell surface expression of CD133. The anti-proliferative activity of the synthesised compounds was evaluated against two cell lines: colon cancer cell line HT-29 and triple negative breast cancer cell line MDA-MB-468. Moreover, their inhibitory activity against the cell surface expression of CD133 was determined in an attempt to explore their potential to eradicate CSCs as well as the tumour bulk cells. Compound 4b emerged as a promising hit molecule as it manifested excellent antineoplastic potency against both tested cell lines (IC_{50} values of 9 and 12 μM, respectively) beside its ability to inhibit the cell surface expression of CD133 by 50% suggesting a promising potential of effectively controlling the tumour by eradicating the tumour bulk and inhibiting the proliferation of the CSCs. Moreover, compounds 4a and 4d exhibited good anti-proliferative activity against both cell lines and also significant inhibition potential of cell surface expression of CD133. On the other hand, structure of compound 4d was further substantiated via X-ray single crystal analysis.

Acknowledgements

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project no. RG-1436–038.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project no. RG-1436–038.

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| Table 4. Inhibition (%) of cell surface expression of CD133 on HT-29 cancer cells at 10 μM. |
| Comp. | Inhibition (%) ± SD |
|-------|---------------------|
| Untreated | – |
| 4a    | 14 ± 1.0 |
| 4b    | 50 ± 4.3 |
| 4c    | 48 ± 3.8 |
| 4d    | NA |

*Values are the mean ± SD of three separate experiments.

Compounds having inhibition % < 10.
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