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Original Article

SYNTHESIS AND *IN-VITRO* ANTIPROLIFERATIVE ACTIVITY OF 2, 3-ARYL SUBSTITUTED 1, 3-BENZOXAZIN-4-ONE DERIVATIVES

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ABSTRACT

Objective: The aim of the present work was to design and synthesize 2, 3-aryl substituted 1, 3-benzoxazin-4-one derivatives and evaluate them for *in-vitro* antiproliferative activity against human breast adeno-carcinoma cells.

Methods: The compounds were synthesized and screened for *in-vitro* antiproliferative activity against MCF-7 cell lines using 96 well plate method.

Results: 3 out of 9 synthesized compounds showed good *in-vitro* inhibition of MCF-7 cell lines. Compound 2 showed least IC_{50} (highest active) i.e. 0.89 µg followed by compound 4 (IC_{50}= 1.02 µg) and compound 3 (IC_{50}= 1.19 µg). 4 compounds showed more than 90 % inhibition at 100 µg after 48 h incubation.

Conclusion: This class of compounds showed some initial promising activity, which can be further expanded by synthesizing and testing more analogs of this kind against MCF-7 cell lines which may give good leads to proceed.

Keywords: 1, 3-benzoxazin-4-one, Antiproliferative activity, MCF-7 cell lines, Molecular docking.

INTRODUCTION

4H-1, 3-benzoxazin-4-one derivatives have become an important class of compounds due to their interesting biological properties. Benzoxazinone derivatives have been used as antiphlogistics, antifungal and antibacterial agent [1]. Various benzoxazinones such as 1, 3-benzoxazinones, 1, 4 and 2, 4-benzoxazinones have been synthesized and tested for several biological activities.

These include anti-inflammatory, anticancer and anticoagulant activities. Certain benzoxazinones have shown good potential as 5-HT (1A) receptor antagonists with promising potent 5-HT reuptake inhibitor activity [2]. Apart from this, many benzoxazinones have shown antiviral activity. A few of these were active against herpes simplex virus (HSV) too [3].

Some others were found to be active against human cytomegalo virus (CMV) by inhibiting CMV protease [4]. Certain benzoxazinones have been accredited with inhibitory activity against HIV reverse transcriptase enzyme which has given some prominence to this class of compounds [5].

Cancer is a significant worldwide health problem generally due to the lack of widespread and comprehensive early detection methods [6]. Indeed, the struggle to combat cancer is one of the greatest challenges of mankind. Over-expression of EGFR (a receptor tyrosine kinase) has been associated with aggressive disease and poor patient prognosis in a range of human tumor types (e.g. breast, lung, ovarian, prostate, and squamous carcinoma of head and neck) [7]. Disruption of signal transduction of EGFR has been shown to have an antiproliferative and therapeutic effect [8]. Recently, some substituted benzoxazinones (bioisosters of marketed drugs like Gefitinib and Lapatinib) have shown epidermal growth factor receptor (EGFR) inhibitory effect [9], which is involved in cell growth, survival and differentiation of cancer.

In the present work, 9 derivatives of 2, 3-substituted benzoxazin-4-one were synthesized and *in-vitro* antiproliferative assay was performed for all compounds using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method on MCF-7 cell lines (EGFR positive cell lines). Instead of substitutions on 4 and 6 positions, we attached ring substitutions at 2 and 3 positions of benzoxazine ring to study their effect on *in-vitro* activity.

MATERIALS AND METHODS

All Solvents, reagents and chemicals used in this work were purchased from Aldrich, Spectrochem Pvt. Ltd., S. D. Fine Chem. Pvt. Ltd. and used as such. The reactions were monitored by thin layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (silica gel 60 F^{254}). Purification of synthesized compounds was performed by column chromatographic technique using silica gel 100-200 mesh (E. Merck). Melting points were determined by using laboratory melting point apparatus (Toshniwal Pvt. Ltd.) and are uncorrected. Chemical tests were performed to confirm the presence of functional groups and required elements. IR spectra of the synthesized compounds were recorded on FT-IR Affinity-1 (Shimadzu) IR Spectrometer. Mass spectra were recorded on LC-MS (Shimadzu). NMR spectra were recorded on Bruker 400 MHz spectrometer using DMSO-*d*6 as a solvent.

Synthesis

Step-I: Synthesis of phenyl salicylate

Phenyl salicylate was prepared by esterification of salicylic acid with phenol in presence of phosphorous oxychloride [10]. Equimolar quantities of salicylic acid and phenol were refluxed for 6 h in presence of small quantity of phosphorous oxychloride to give phenyl salicylate. The product was washed with water and dried. The reaction was monitored by thin layer chromatography (TLC) with hexane:ethyl acetate (3:1) as a solvent system and was purified by column chromatography.

Step-II: Synthesis of N-phenyl salicylamide

Solvent free microwave irradiation method was followed to prepare N-phenyl salicylamide [11]. Equimolar quantities of phenyl salicylate and aniline were mixed and subjected to microwave irradiation for 5 min. The reaction was stopped when the temperature reached 180 ºC. The reaction mixture was cooled to room temperature and the mixture was treated with dichloromethane. The insoluble impurities were filtered off and the solvent was evaporated to get N-phenyl salicylamide. The product was then washed with 2 M HCl to remove access of aniline and then purified by column chromatography using hexane-ethyl acetate (3:1) as a solvent system.

Step-III: Synthesis of derivatives

The acid catalyzed condensation was carried out to synthesize the final derivatives of 1, 3-benzoxazin-4-one [12]. (0.85 g (0.004 M) of N-phenyl salicylamide and equimolar quantity of aldehydes were added to 20 ml of chloroform and 10 ml of glacial acetic acid. The reaction mixture was refluxed for 2 h at 50 ºC with continuous passage of HCl gas through the reaction vessel. The reaction was monitored by TLC using different solvent systems as specified in table 1. The reaction vessel was cooled and the chloroform was evaporated on rotary evaporator. 200 ml of cold water was added to the reaction mixture. The precipitate was triturated with 5 % NaOH solution and again washed with water. The product was re-crystallized from ethanol and purified with column chromatography.

Synthesis of derivatives

Nine 2, 3-aryl substituted 1, 3-benzoxazin-4-ones derivatives were synthesized (Fig.1) (Table 1). All compounds were tested for their *in-vitro* antiproliferative activity against MCF7 cell lines.

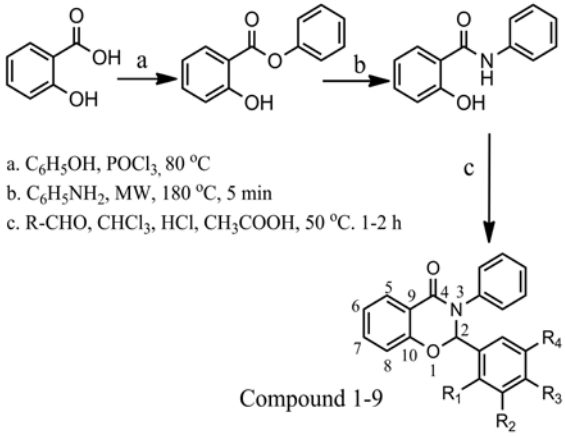

Fig. 1: Synthetic scheme of all derivatives

Table 1: 1, 3-benzoxazin-4-one derivatives

Compound Code	R_1	R_2	R_3	R_4	Molecular weight	% yield	Melting Point (ºC)	R_f
1	H	H	$N(CH_3)$	H	344.41	84.86	238-240	0.36*
2	H	H	OH	H	317.34	78.05	254-256	0.67*
3	OH	CH_3	H	H	361.39	78.82	241-243	0.82*
4	OH	H	H	CH_3	347.36	67.74	235-237	0.20#
5	OH	H	H	OCH_3	331.36	82.22	232-234	0.39#
6	H	H	OCH_3	OCH_3	315.17	81.73	220-222	0.24*
7	H	H	OH	OCH_3	331.36	73.25	247-249	0.40*
8	H	H	CH_3	H	317.34	70.25	276-278	0.80*
9	OH	H	H	H	347.36	74.87	250-252	0.30**

*Hexane: Ethyl acetate (1:1), #Benzene:Chloroform(1:1), **Benzene: Methanol(3:1)

Synthesis of 2-[4-(dimethylamino) phenyl]-3-phenyl-2, 3-dihydro-4H-1, 3-benzoxazin-4-one (1)

It was synthesized by using 0.60 g (0.004 M) of 4-dimethyaminobenzaldehyde in the above procedure mentioned under the synthesis of derivatives. It yielded 1.18 g (85 %) of reddish brown solid product. Mp. 238-240 ºC. FTIR (KBr, cm^{-1}): 3294 (Aromatic -C-H str.), 3026 (-C-H str.), 1722 (-C=O str.), 1620 (-C=O str.), 1197 (-C-N str.), 896 (-C-H bending); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.50 (s, 3H), 1.39 (s, 2H), 6.93 (t, *J*=8 Hz, 2H), 7.02 (d, *J*=1.6 Hz, 2H), 7.33-7.66 (m, 9H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 35.41, 127.59, 138.11, 142.82, 144.37, 147.51, 156.13, 168.23. LCMS (ESI, *m/z*) 345.2 $(M+H)^+$.

Synthesis of 2-(4-hydroxyphenyl)-3-phenyl-2, 3-dihydro-4H-1, 3-benzoxazin-4-one (2)

It synthesized by using 0.49 g (0.004 M) of 4-hydroxybenzaldehyde in the above procedure mentioned under the synthesis of derivatives. It yielded 1.0 g (78 %) of greenish white solid product. Mp. 254-256 ºC. FTIR (KBr, cm^{-1}): 3319 (Aromatic –O-H str.), 3022 (Aromatic -C-H str.), 1593 (-C=O str.), 1161 (-C-N str.), 825 (-C-H bending); ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.90- 7.22 (m, 6H), 7.52-7.72 (m, 7H), 9.53 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 114.27, 127.59, 138.11, 142.82, 169.33. LCMS (ESI, *m/z*) 318.4 $(M+H)^+$.

Synthesis of 2-(2-hydroxy-3-methylphenyl)-3-phenyl-2, 3-dihydro-4H-1, 3-benzoxazin-4-one (3)

It was synthesized by using 0.55 g (0.004 M) of 2-hydroxy-3-methylbenzaldehyde in the above procedure mentioned under the synthesis of derivatives. It yielded 1.10 g (82 %) of yellowish white solid product. Mp. 241-243 ºC. FTIR (KBr, cm^{-1}): 3377 (Aromatic –O-H str.), 2999 (Aromatic -C-H str.), 2929 (-C-H str.), 1610 (-C=O str.), 1174 (-C-N str.), 866 (-C-H bending); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.88 (s, 3H), 4.23 (s, 1H), 6.93 (d, *J*= 8 Hz, 1H), 7.32 (d, *J*= 8.6 Hz, 1H), 7.33-7.66 (m, 9H), 7.65 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 47.41, 127.55, 135.11, 142.82, 142.99, 143.37, 147.51, 156.13, 171.03. LCMS (ESI, *m/z*) 331.2 $(M)^+$.

Synthesis of 2-(2-hydroxy-5-methylphenyl)-3-phenyl-2, 3-dihydro-4H-1, 3-benzoxazin-4-one (4)

It was synthesized by using 0.55 g (0.004 M) of 2-hydroxy-5-methylbenzaldehyde in the above procedure mentioned under the synthesis of derivatives. It yielded 0.98 g (73 %) of yellowish white solid product. Mp. 235-237 ºC. FTIR (KBr, cm^{-1}): 3377 (Aromatic –O-H str.), 2999 (Aromatic -C-H str.), 2929 (-C-H str.), 1610 (-C=O str.), 1174 (-C-N str.), 866 (-C-H bending); ^{1}H NMR (400 MHz, DMSO-d6): δ 1.88 (s, 3H), 4.23 (s, 1H), 6.93 (d, J= 8 Hz, 1H), 7.32 (d, J=8.6 Hz, 1H), 7.33-7.66 (m, 9H), 7.65 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d6): δ 47.41, 127.55, 135.11, 142.82, 142.99, 143.37, 147.51, 156.13, 171.03. LCMS (ESI, *m/z*) 331.2 $(M)^+$.

Synthesis of 2-(2-hydroxy-5-methoxyphenyl)-3-phenyl-2, 3-dihydro-4H-1, 3-benzoxazin-4-one (5)

It was synthesized by using 0.61 g (0.004 M) of 2-hydroxy-5-methoxybenzaldehyde in the above procedure mentioned under the synthesis of derivatives. It yielded 1.05 g (75 %) of white solid product. Mp. 232-234 ºC. FTIR (KBr, cm^{-1}): 3315 (Aromatic –O-H str.), 3026 (Aromatic -C-H str.), 2966 (-C-H str.), 2852 (-O-C str.), 1618 (-C=O str.), 1238 (-C-O-C str.), 1155 (-C-N str.), 896 (-C-H bending); ^{1}H NMR (400 MHz, DMSO-d6): δ 3.73 (s, 3H), 4.00 (s, 1H), 7.09 (d, J=8 Hz, 1H), 7.23 (d, J=8.6 Hz, 1H), 7.55-7.66 (m, 9H), 7.99 (s, 1H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 40.31, 127.55, 135.11, 140.82, 142.99, 144.37, 168.23. LCMS (ESI, *m/z*) 347.76 $(M)^+$.

Synthesis of 2-(3, 4-dimethoxyphenyl)-3-phenyl-2, 3-dihydro-4H-1, 3-benzoxazin-4-one (6)

It was synthesized by using 0.67 g (0.004 M) of 3, 4-dimethoxybenzaldehyde in the above procedure mentioned under the synthesis of derivatives. It yielded 1.15 g (79 %) of yellowish solid product. Mp. 220-222 ºC. FTIR (KBr, cm^{-1}): 3296 (Aromatic -C-

H str.), 3026 (Aromatic -C-H str.), 2922 (-C-H str.), 2852 (-O-C str.), 1620 (-C=O str.), 1236 (-C-O-C str.), 1153 (-C-N str.), 896 (-C-H bending); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 3.60 (s, 3H), 4.00 (s, 3H), 6.90- 7.09 (m, 5H), 7.23 (m, 4H), 7.55 (d, J= 8 Hz, 1H), 7.62 (d, J= 8 Hz, 1H), 7.82 (s, 1H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 40.21, 135.11, 143.00, 144.37, 157.68, 169.22. LCMS (ESI, *m/z*) 361.06 $(M)^+$.

Synthesis of 2-(4-hydroxy-3-methoxyphenyl)-3-phenyl-2, 3-dihydro-4H-1, 3-benzoxazin-4-one (7)

It was synthesized by using 0.61 g (0.004 M) of 4-hydroxy-3-methoxybenzaldehyde in the above procedure mentioned under the synthesis of derivatives. It yielded 0.95 g (68 %) of greenish white solid product. Mp. 247-249 ºC. FTIR (KBr, cm^{-1}): 3315 (Aromatic –O-H str.), 3026 (Aromatic -C-H str.), 2966 (-C-H str.), 2852 (-O-C str.), 1618 (-C=O str.), 1238 (-C-O-C str.), 1155 (-C-N str.), 896 (-C-H bending); ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.73 (s, 3H), 4.00 (s, 1H), 7.09 (d, *J*=8 Hz, 1H), 7.23 (d, *J*=8.6 Hz, 1H), 7.55-7.66 (m, 9H), 7.99 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 40.31, 127.55, 135.11, 140.82, 142.99, 144.37, 168.23. LCMS (ESI, *m/z*) 347.76 $(M)^+$.

Synthesis of 2-(4-methylphenyl)-3-phenyl-2, 3-dihydro-4H-1, 3-benzoxazin-4-one (8)

It was synthesized by using 0.48 g (0.004 M) of 4-methylbenzaldehyde in the above procedure mentioned under the synthesis of derivatives. It yielded 1.04 g (82 %) of brown colored solid product. Mp. 276-278 ºC. FTIR (KBr, cm^{-1}): 3250 (Aromatic –C-H str.), 3022 (Aromatic C-H str.), 1693 (-C=O str.), 1111 (-C-N str.), 876 (-C-H bending); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.34 (s, 3H), 7.10- 7.22 (m, 6H), 7.55-7.62 (m, 7H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 114.27, 126.77, 129.10, 127.59, 138.11, 142.82, 169.33. LCMS (ESI, *m/z*) 315.37 $(M)^+$.

Synthesis of 2-(2-hydroxyphenyl)-3-phenyl-2, 3-dihydro-4H-1, 3-benzoxazin-4-one (9)

It was synthesized by using 0.49 g (0.004 M) of 2-hydroxybenzaldehyde in the above procedure mentioned under the synthesis of derivatives. It yielded 0.90 g (70 %) of greenish white solid product. Mp. 250-252 ºC. FTIR (KBr, cm^{-1}): 3399 (Aromatic –O-H str.), 3024 (Aromatic C-H str.), 1593 (-C=O str.), 1122 (-C-N str.), 885 (-C-H bending); ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.22 (s, 1H), 6.90- 7.22 (m, 6H), 7.63-7.73 (m, 7H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 124.27, 127.59, 136.11, 142.82, 171.31. LCMS (ESI, *m/z*) 317.17 $(M)^+$.

***In-vitro* Antiproliferative activity**

Maintenance of cell lines

MCF-7 cells were procured from national centre for cell sciences (NCCS) Pune, India and were grown in 75 cm^2 tissue culture flasks containing Eagle's minimum essential medium (EMEM) supplemented with 10 % fetal bovine serum (FBS), 1 % L- glutamine and 50 µg/ml Gentamycin sulphate at 37 ºC in a CO_2 incubator in an atmosphere of humidified 5 % CO_2 and 95 % air. The cells were maintained by routine sub culturing in 75 cm^2 tissue culture flasks. Preservation of tumor cells from the first and second passage of transplantation were stored in liquid nitrogen in cryovials containing Iscove's Modified Dulbecco's Media (IMDM) supplemented with 10 % FBS and 10 % dimethyl sulphoxide (DMSO) as a preservative at concentration of 10^6 cells/ml. This constituted the tumor bank. After every 10 passages, tumor cell line was discarded and new passage was started using the original tumor cells from the tumor bank.

MTT assay method

The assay was performed as per the reported protocols [13, 14]. Exponentially growing MCF-7 cells were harvested from 75 cm^2 tissue culture flask and a stock cell suspension ($1x10^5$ cell/ml) was prepared. A 96-well flat bottom tissue culture plate was seeded with 2 x 10^3 cells in 0.1 ml of EMEM medium supplemented with 10% FBS and allowed to attach for 24 h. Test compounds were prepared just prior to the experiment in DMSO to get 10000 µg/ml**.** After 24 h of incubation, cells were treated with test compounds to get a final dose of 12.5, 25, 50 and 100 µg in respective wells and incubated for 48 h. The cells in the control group received only the medium containing 0.2 % DMSO. Each treatment was performed in duplication. After the treatment, drug containing media was removed and washed with 200 µl of FBS. To each well of the 96 well plate, 100 µl of MTT reagent (Stock: 1 mg/ml in serum free medium) was added and incubated for 4 h at 37 ºC. After 4 h of incubation, the plate was inverted on tissue paper to remove the MTT reagent. To solubilize formazan crystals in the wells, 100 µl of 100 % DMSO was added to each well. The optical density was measured by micro-titer plate reader at 590 nm**.** Percentage cytotoxicity of each compound was calculated by using the formula (Control - Blank) - (Test - Blank) X 100 / (Control-Blank).

RESULTS AND DISCUSSION

In vitro antiproliferative activity (MTT assay)

The percentage inhibition of test compounds was measured after 24 h and 48h. After 24 h incubation, 4 out of 9 compounds (2, 7, 8 and 9) showed more than 75 % inhibition at 100 µg (Fig. 2). After incubation for 48 h, 8 out of 9 compounds showed more than 75 % inhibition at 100 µg and 4 compounds showed more than 90 % inhibition when tested with the same maximum dose (Fig. 3). All compounds inhibited MCF-7 cells in a dose dependent manner after 24 as well as 48 h (Fig. 2 and 3). Compound 2 showed the maximum activity with an IC_{50} of 0.89 µg followed by compound 4 and 3 with an IC_{50} of 1.02 and 1.19 µg respectively. Compound 2 also showed 77.26 % inhibition of cell lines at 12.5 µg and 98.18 % inhibition at highest dose (100 µg) after 48 h incubation. Compound 9 also showed 98.38 % inhibition of cell lines at 100 µg but its IC_{50} was found to be more compared to compound 2, 3 and 4. All other compounds showed less activity among which, compound 6 was least active with an IC_{50} of 51.55 µg.

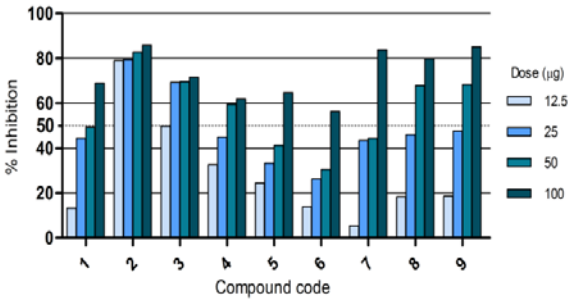

Fig. 2: Inhibition of MCF-7 cells after 24 h inhibition

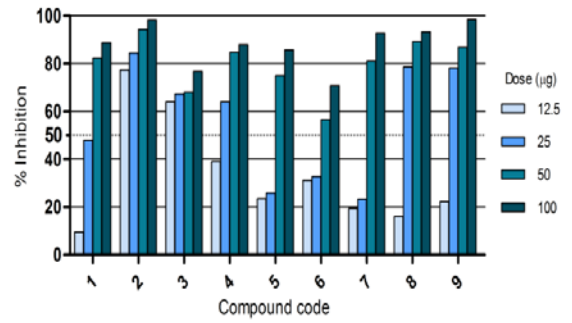

Fig. 3: Inhibition of MCF-7 cells after 48 h inhibition

CONCLUSION

All synthesized compounds showed activity against MCF-7 cell lines. Few compounds (2,3 and 4) showed promising activity by inhibiting the MCF-7 cell lines by more than 90 %. From this study, we could conclude that benzoxazinone derivatives with substitutions at 2 and 3 positions can be a good class of compounds against MCF-7 cell

lines. This class of compounds may further be explored by synthesizing more compounds and testing them against MCF-7 cell lines.

CONFLICT OF INTERESTS

Declared None

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