Bioorganic & Medicinal Chemistry Letters xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Benzimidazole derivatives protect against cytokine-induced apoptosis in pancreatic β-Cells

Nik Khairunissa Nik Abdullah Zawawi [a,b,†], Sajid Ali Rajput [c,†], Muhammad Taha [a,b,*], Norizan Ahmat [a,b,*], Nor Hadiani Ismail [a,b], Noraishah Abdullah [b], Khalid Mohammed Khan [d], M. Iqbal Choudhary [a,c,d,*]

[a] Atta-ur-Rahman Institute for Natural Product Discovery, Universiti Teknologi MARA (UiTM), Puncak Alam Campus, 42300 Bandar Puncak Alam, Selangor D.E., Malaysia
[b] Faculty of Applied Science, UiTM Shah Alam, 40450 Shah Alam, Selangor D.E., Malaysia
[c] Dr. Panjwani Center for Molecular Medicine and Drug Research, International Centre for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan
[d] H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan

ARTICLE INFO

Article history:
Received 29 May 2015
Revised 6 August 2015
Accepted 8 August 2015
Available online xxxx

Keywords:
Pancreatic β-cells
Benzimidazole
Cytokines
Apoptosis
Glucose-stimulated insulin secretion

ABSTRACT

Apoptotic cell death is the cause of the loss of insulin-producing β-cells in all forms of diabetes mellitus. The identification of small molecules capable of protecting cytokine-induced apoptosis could form the basis of useful therapeutic interventions. Here in, we present the discovery and synthesis of new benzimidazole derivatives, capable of rescuing pancreatic β-cells from cytokine-induced apoptosis. Three hydrazone derivatives of benzimidazole significantly increased the cellular ATP levels, reduced caspase-3 activity, reduced nitrite production and increased glucose-stimulated insulin secretion in the presence of proinflammatory cytokines. These findings suggest that these compounds may protect β-cells from the harmful effects of cytokines and may serve as candidates for therapeutic intervention for diabetes.

Diabetes mellitus is a chronic metabolic disorder, characterized by the presence of persistent hyperglycaemia resulting from defects in insulin secretion, insulin action or both.[1,2] The decline in β-cell mass due to apoptosis and insulin producing function underlie much of the pathology of both type 1 and type 2 diabetes mellitus. Apoptotic death of pancreatic β-cells is a hallmark of all forms of diabetes. The β-cells are highly sensitive to apoptotic damages induced by multiple stress factors, such as inflammatory and oxidative assault.[3–5] The inflammatory cytokines, such as interleukin-1β (IL-1β), interferon-γ (INF-γ) and tumour necrosis factor-α (TNF-α) are important mediators in impaired function, apoptosis and progressive loss of β-cells. These cytokines activate intracellular signalling pathways that drive β-cell apoptosis.[6–9] IL-1β, TNF-α and INF-γ induce the expression of transcription factor NF-κB and STAT1. Whereas, the downstream signalling was reported to occur through nitric oxide (NO), which disrupts mitochondrial physiology by inhibiting the electron-transport chain, resulting in a decrease in glucose oxidation rates, ATP production, increase ROS production and decrease in insulin biosynthesis and secretion. Caspase-3 also mediates β-cell apoptosis and cause a loss of glucose-stimulated insulin secretion (GSIS).[10,11,5,12]

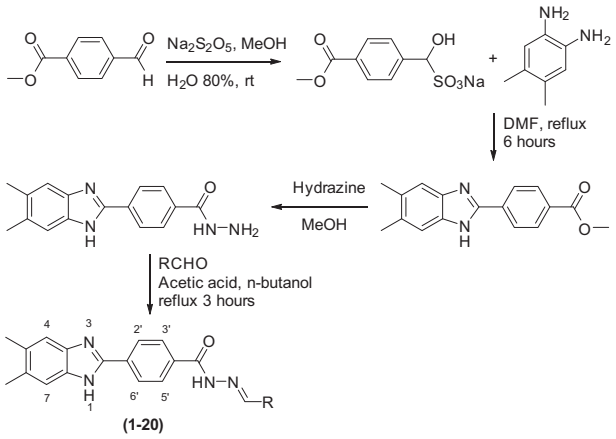
Current treatments for diabetes fail to halt the decline in functional β-cell mass; therefore, strategies to prevent β-cell dysfunction and apoptosis are urgently needed. Strategies to discover small molecules, capable of inhibiting β cell apoptosis, may have a great therapeutic potential, and can be used in combination with traditional diabetic therapies. Benzimidazole nucleus is an important pharmacophore with unique chemical and biological properties.[13–17] Benzimidazoles have been found to possess anti-inflammatory, antispasmodic, antihistaminic, analgesic, antimicrobial, antiproliferative, antitumor, anti-HIV-RT, antiulcer, anticancer, anti-tubercular, and cycloxygenase inhibitor activities.[18–22] In our continuous efforts to discover bioactive compounds.[23–29] We screened broad range of compounds and found benzimidazoles as prominent class of compounds on the bases of that we synthesized a series of benzimidazole derivatives and investigated their ability to protect the rat insulinoma cell line INS-1E from cytokine-induced apoptosis.[30] However, the mechanism of protection by which the compound protected β-cells against cytokine-induced cell death is not reported in this communication.

* Corresponding authors. Tel.: +60 182901765 (M.T.); tel.: +60 03 55435590; fax: +60 03 55444562 (N.A.); tel.: +92 (21) 4824924/4824925; fax: +92 (21) 4819018/4819019 (M.I.C.).

E-mail addresses: taha_hej@yahoo.com, muhamm9000@puncakalam.uitm.edu.my (M. Taha), noriz118@salam.uitm.edu.my (N. Ahmat), hej@cyber.net.pk, iqbal.choudhary@iccs.edu (M.I. Choudhary).

† Authors equally contributed.

http://dx.doi.org/10.1016/j.bmcl.2015.08.022
0960-894X/



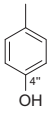
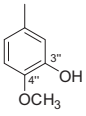
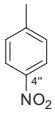
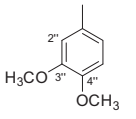
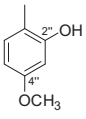
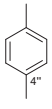
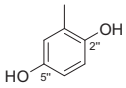
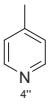
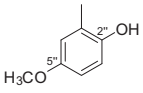
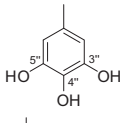
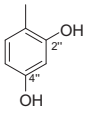
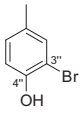
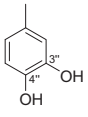
Scheme 1. Synthesis of benzimidazole benzohydrazide (**1**–**20**).

The synthesis of the target compounds began with the synthesis of sodium metasulfite adduct according to literature protocol.[31] The resulting sulfite adduct was refluxed with 4,5-dimethyl-*O*-phenylenediamine in DMF for 6 h to give the arylester substituted benzimidazole. The benzohydrazide of benzimidazole was formed by refluxing arylester of benzimidazole with methanolic hydrazine hydrate (Scheme 1). The synthesis of benzimidazole benzohydrazide Schiff bases (**1**–**20**) was accomplished by reacting different aldehydes (Table 1) with benzimidazole benzohydrazide in *n*-butanol in the presence of catalytic amount of acetic acid as shown in Scheme 1.

The ability of these compounds in protecting the rat insulinoma cell line INS-1E from cytokine-induced apoptosis were tested using cellular ATP levels as a surrogate for cell viability.[32] After 48 h incubation with a cytokine cocktail of IL-1β, INF-γ and TNF-α resulted in more than 2-fold decreased in ATP levels (Fig. 1A). However, only seven of the compounds tested protect against cytokine effects in β-cells (Fig. 1A). In dose-dependency study, compound **19** completely restored the β-cell ATP levels at 2 μM concentration (Fig. 2A) with an EC_{50} value of 0.968 μM (Table 2). Similarly, compounds **4** and **5** also increased β-cell ATP levels by more than 90% of untreated controls (Fig. 2B), whereas compounds **6** and **7** were slightly less potent in increasing the cellular ATP levels with their EC_{50} values of 2.57, 3.17, 7.28 and 16.71 μM, respectively (Table 2).

Structure–activity relationship (SAR) of active benzimidazole analogues was determined from the primary screening. It was observed that increases in the cellular ATP levels in β-cells mainly depend upon three parameters: the functional groups on substituted phenyl moiety, and the number and the position of the functional groups on the phenyl ring. As shown in Table 2, hydroxyl (–OH) and methoxy ($-OCH_3$) groups and their respective positions on the phenyl ring were the preferred substitution required for the activity. The benzimidazole derivatives with hydroxyl group on the phenyl ring were found to have an activity pattern, such that the more hydroxyl group, the greater is the activity. Thus, the tri-hydroxyl containing compounds were found to be more potent than di- and mono-hydroxyl group containing analogues. Compounds **4**, **5**, **6**, and **7**, all having dihydroxyl group on the phenyl moiety showed excellent to moderate activity in increasing the cellular ATP levels, depending upon the position of the hydroxyl groups (Table 2). These compounds had one hydroxyl group at *meta* position, while the other hydroxyl group was moved to carbon at 3rd, 4th, and 5th positions on the phenyl moiety. Compound **19**, which has the tri-hydroxyl group on phenyl ring, was found to be the most potent suppressor of β-cell apoptosis among all

Table 1
Benzimidazole benzohydrazide derivatives **1**–**20**

Compd no.	R	Yield (%)	Compd no.	R	Yield (%)	Compd no.	R	Yield (%)
1		91	**8**		89	**15**		90
2		90	**9**		90	**16**		92
3		92	**10**		91	**17**		92
4		91	**11**		90	**18**		89
5		92	**12**		90	**19**		93
6		92	**13**		93	**20**		90
7		89	**14**		93			

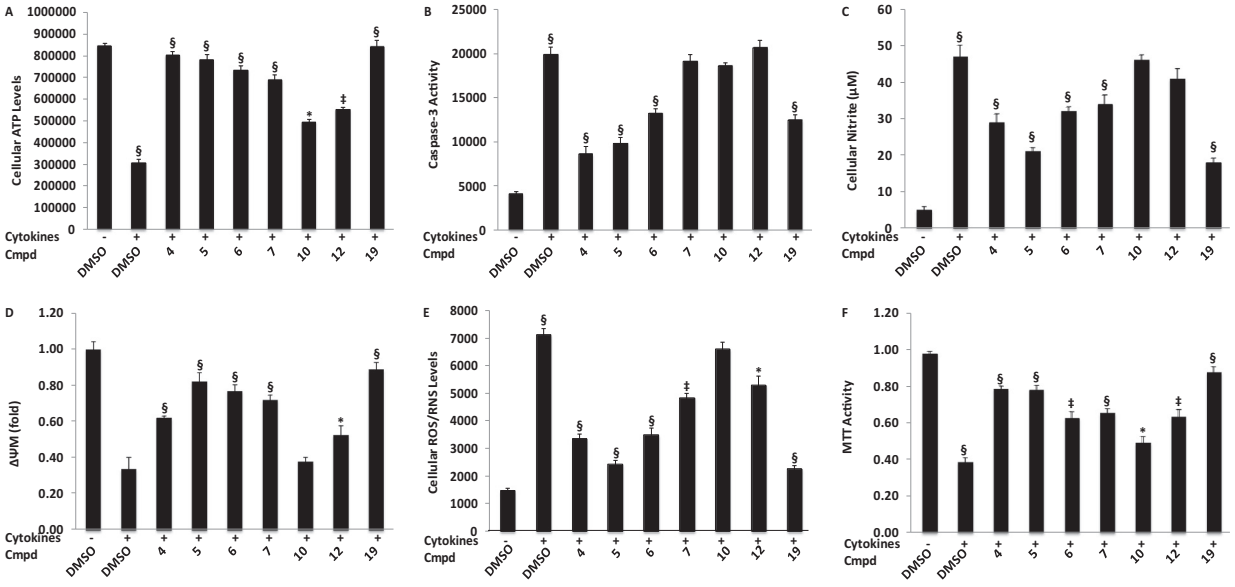


Figure 1. Effects of benzimidazole derivatives on cellular ATP levels, caspase-3 activity, cellular nitrite production, and mitochondrial physiological parameters in β-cells and after exposure to a cytokine cocktail. (A–F) INS-1E cells were treated for 48 h with a cocktail of proinflammatory cytokines (IL-1β 20 ng/mL, INF-γ 50 ng/mL, and TNF-α 50 ng/mL) in the presence or absence of benzimidazole derivatives, and were assessed for cellular ATP levels (A), caspase-3 activity (B), and nitrite production (C), mitochondrial membrane potential (ΔΨm) (D), reactive oxygen species (ROS) production (E), and MTT activity (F). Concentrations used were based on molecular weight and as follows: 5 μM for compounds **4**, **5**, and **19**; 10 μM for compounds **6** and **7**; and 20 μM for compounds **10** and **12**. Data represent the mean ± standard deviation of 24 independent wells. ∗ indicates $p<0.05$, ‡ <0.01 and § <0.001, respectively, as compared to the cytokine treatment alone.

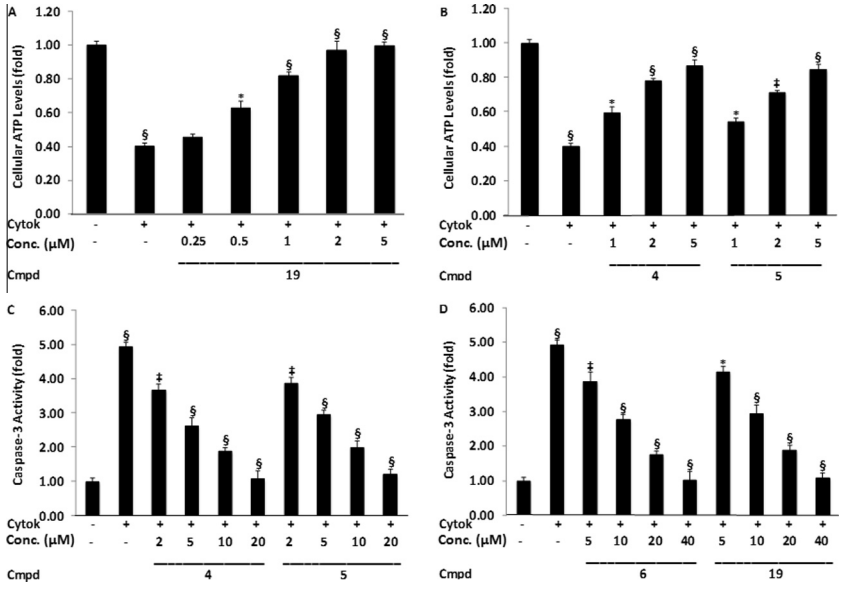


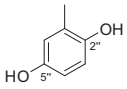
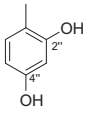
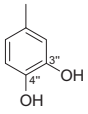
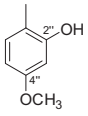
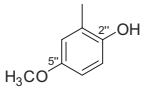
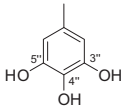
Figure 2. Dose-dependency study for cellular ATP levels and caspase-3 activity. INS-1E cells were treated with cytokine cocktail (see Supporting data) in the presence of increasing concentrations of compounds **4**, **5**, **6** and **19** (A–D). Cellular ATP levels (A–B) and caspase-3 activities (C–D) were measured and normalized to untreated controls. Data are presented as the mean ± standard deviation of 12 independent wells. ∗ indicates $p<0.05$, ‡ <0.01 and § <0.001, respectively, relative to cytokine-treated cells.

benzimidazole derivatives (Table 2). Whereas, compounds **10** and **12**, having a single hydroxyl group on phenyl ring, only showed partial activity (Table 2).

In the follow-up study with the initial hit compounds, caspase-3 activity was measured as a direct readout of apoptosis. Caspase-3 activity was found to have increased by more than 5-fold after 48 h exposure of INS-1E cells to cytokine cocktail (Fig. 1B). This increase in caspase-3 activity was largely suppressed by compounds **4**, **5**, **6**, and **19**, which significantly decreased the caspase-3 activity (Table 2). However, compounds **7** and **10** were found to be not effective, whereas compound **12** even caused increased caspase-3 activity (Fig. 1B). In dose-dependency study, we demonstrated that compounds **4** and **5** reduced the expression of cleaved caspase-3 activity (Fig. 2C) with IC_{50} values of 8.81 and 9.48 μM, respectively (Table 2). Whereas compounds **6** and **19** were only partially effective in reducing caspase-3 activity (Fig. 1D) with IC_{50} values of 16.38 and 18.47 μM, respectively (Table 2). These results indicated that compounds **4**, **5**, **6** and **19** were able to halt the apoptotic process in the presence of inflammatory cytokines.

Pro-inflammatory cytokines were reported to induce the production of nitric oxide, which drives β-cell death by both apoptosis and necrosis.[33] Cellular production of nitrite was used as a surrogate for NO levels.[24] In the cellular nitrite production assay, cellular nitrite levels were found to be increased by more than

Table 2
Effect of benzimidazole derivatives on induced cytokines in INS-1E cells

Compd no.	R	INS-1E cells viability target potency (μM)							
		ATP		Caspase-3		Nitrite		GSIS	
		Max. act.	EC_{50} (μM)	Max. act.	IC_{50} (μM)	Max. act.	IC_{50} (μM)	Max. act.	EC_{50} (μM)
4		93	2.57	84	8.81	71	17.28	71	13.28
5		89	3.17	82	9.48	79	11.42	84	8.32
6		86	7.28	75	16.38	72	17.48	64	ND
7		73	16.71	22	>30	68	17.59	53	ND
10		54	29.28	15	>30	18	>30	IA	ND
12		61	25.28	12	>30	20	>30	IA	ND
19		98	0.968	68	18.47	85	9.47	91	5.41

Max. act. = maximum activity, IA = inactive, ND = not determine.

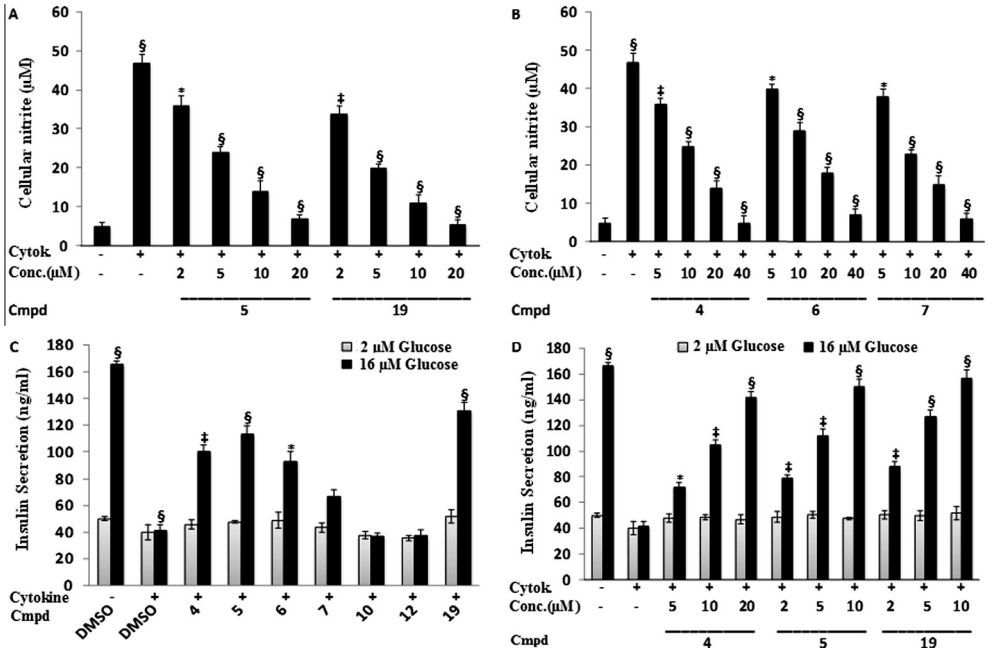


Figure 3. Correlation between inhibition of cellular nitrite production and restoration of glucose-stimulated insulin secretion (GSIS). Cellular nitrite production was measured after treatment with cytokine cocktail (see Supporting data) with increasing concentrations of (A) compounds **5** and **19**, (B) compounds **4**, **6**, and **7**. Data represent the mean ± standard deviation of 12 independent wells. (C) Glucose-stimulated insulin secretion was measured in the presence of 2 μM glucose and 16 μM glucose in the absence or presence of cytokines and benzimidazole derivatives. (D) Compounds **4**, **5**, and **19** were also assessed for their dose-dependent increase in insulin secretory function of β-cells. Data represent the mean ± standard deviation of 2 independent experiments. ∗ indicates $p<0.05$, ‡ <0.01 and **§** <0.001, respectively, relative to cytokine-treated cells.

6-fold following 48 h cytokine exposure, which was significantly decreased by many of the test compounds in varying levels (Fig. 1C). Compounds **19** and **5** induced a dose-dependent decrease in the production of nitrite (Fig. 3A) and were an excellent suppressors of the series with IC_{50} values of 9.47 and 11.42 μM, respectively (Table 2). Compounds **4**, **6** and **7** moderately inhibited the cellular nitrite production (Fig. 3B) with IC_{50} values of 17.28, 17.48 and 17.59 μM, respectively (Table 2). In contrast to caspase-3 activity, compound **7** was found to be active in nitrite assay. These results suggest that only certain position of hydroxyl group on benzimidazole derivatives are considered important in assessing this activity and as expected, compounds **10** and **12** exhibited less effect on cellular nitrite production (Fig. 1C).

Finally, we evaluated the effects of these compounds on GSIS in INS-1E cells. Glucose-stimulated insulin secretion (GSIS) is the gold standard for β-cell specific functions. Exposure of β-cells to cytokine treatment reduced GSIS to 4-folds in comparison to no cytokine treatment (Fig. 3C). This loss of insulin secretion by β-cells was significantly improved by the addition of 5 μM of compound **19** to the cytokine cocktail; with insulin secretion was elevated to more than 3-fold in comparison to untreated controls (Fig. 3C). Compound **19** was the most potent compound which restored GSIS in a dose-dependent manner (Fig. 3D) with an EC_{50} value of 5.41 μM and 91% of activity (Table 2). Compounds **4** and **5** were also significantly enhanced insulin secretion in a dose dependent manner (Fig. 3D) with EC_{50} values of 13.28 and 8.32 μM, respectively (Table 2). Compounds **6** and **7** were found to be partially effective in restoring GSIS in the presence of cytokines (Fig. 3C). These results recommend that cellular nitrite levels are correlated with GSIS in INS-1E cells. We can observe that compounds that are capable of reducing nitrite production in the present of cytokine treatment also restore GSIS.

In the presence of pro-inflammatory cytokines, we have summarized that compounds **4**, **5**, and **19** were the most potent among all the analogues, in which they increased the cellular ATP levels, inhibited caspase-3 activity, decreased nitrite production and restored GSIS in a dose-dependent manner. These results show that benzimidazole derivatives may protect pancreatic β-cells against cytokine-induced apoptosis. However, the protective role of each compound in β-cells and their relevant mechanisms need further investigation in order to establish these findings.

Acknowledgements

INS-1E cells were generously provided by Paolo Meda, Department of Morphology, University of Geneva Medical School, Switzerland. Authors would like to acknowledge the Ministry of Education Malaysia and Universiti Teknologi MARA for the financial support under RAGS grant 600-RMI/RAGS/5/3/ (2/2012).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.08.022.

References and notes

1. Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, *Diabetes Care* **1997**, *20*, 1183.
2. Diagnosis and Classification of Diabetes Mellitus. American Diabetes Association, *Diabetes Care* **2013**, *36*, S67.
3. Bluestone, J. A.; Herold, K.; Eisenbarth, G. *Nature* **2010**, *464*, 1293.
4. Cnop, M.; Welsh, N.; Jonas, J. C.; Jörns, A.; Lenzen, S.; Eizirik, D. L. *Diabetes* **2005**, *54*, S97.
5. Soldevila, G.; Buscema, M.; Doshi, M.; James, R. F.; Bottazzo, G. F.; Pujol-Borrell, R. *J. Autoimmun.* **1991**, *4*, 291.
6. Robertson, P. P.; Harmon, J.; Tran, P. O. T.; Poitout, V. *Diabetes* **2004**, *53*, S119.
7. Lenzen, S.; Drinkgern, J.; Tiedge, M. *Free Radic. Biol. Med.* **1996**, *20*, 463.
8. Donath, M. Y.; Shoelson, S. E. *Nat. Rev. Immunol.* **2011**, *11*, 98.
9. Donath, M. Y.; Størling, J.; Berchtold, L. A.; Billestrup, N.; Mandrup-Poulsen, T. *Endocr. Rev.* **2008**, *29*, 334.
10. Eizirik, D. L.; Colli, M. L.; Ortis, F. *Nat. Rev. Endocrinol.* **2009**, *5*, 219.
11. Beg, A. A.; Baltimore, D. *Science* **1996**, *274*, 782.
12. Darville, M. I.; Eizirik, D. L. *Diabetologia* **1998**, *41*, 1101.
13. Taha, M.; Ismail, N. H.; Jamil, W.; Rashwan, H.; Kashif, S. M.; Sain, A. A.; Adenan, M. I.; Anouar, E. H.; Ali, M.; Rahim, F.; Khan, K. M. *Eur. J. Med. Chem.* **2014**, *84*, 731.
14. Taha, M.; Ismail, N. H.; Imran, S.; Selvaraj, M.; Rashwan, H.; Farhanah, F. U.; Rahim, F.; Selvarajan, K. K.; Ali, M. *Bioorg. Chem.* **2015**, *61*, 36.
15. Abdullah, N. K. N. Z.; Taha, M.; Ahmat, N.; Wadood, A.; Ismail, N. H.; Rahim, F.; Ali, M.; Abdullah, N.; Khan, K. M. *Bioorg. Med. Chem.* **2015**, *23*, 3119.
16. Saify, Z. S.; Kamil, A.; Akhtar, S.; Taha, M.; Khan, A.; Khan, K. M.; Jahan, S.; Rahim, F.; Perveen, S.; Choudhary, M. I. *Med. Chem. Res.* **2014**, *23*, 4447.
17. Khan, K. M.; Khan, M.; Saleem, M.; Taha, M.; Perveen, S.; Choudhary, M. I. *J. Pak. Chem Soc.* **2013**, *35*, 901.
18. Sondhi, S. M.; Bhattacharjee, G.; Jameel, R. K.; Shukla, R.; Raghubir, R.; Lozach, O.; Meijer, L. *Cent. Eur. J. Chem.* **2004**, *2*, 1.
19. Garuti, L.; Roberti, M.; Pizzirani, D.; Pession, A.; Leoncini, E.; Cenci, V.; Hrelia, S. *Il Farmaco* **2004**, *59*, 663.
20. Kumar, D.; Jacob, M. R.; Reynolds, M. B.; Kerwin, S. M. *Bioorg. Med. Chem.* **2002**, *10*, 3997.
21. Paramashivappa, R.; Phani Kumar, P.; Subba Rao, P. V.; Srinivasa, R. A. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 657.
22. Ozkay, Y.; Tanah, Y.; Karaka, H.; Iskdag, I. *Eur. J. Med. Chem.* **2010**, *45*, 3293.
23. Taha, M.; Ismail, N. H.; Imran, S.; Rokei, M. Q. B.; Saad, S. M.; Khan, K. M. *Bioorg. Med Chem.* **2015**, *23*, 4155.
24. Taha, M.; Ismail, N. H.; Khan, A.; Shah, S. A. A.; Anwar, A.; Halim, S. A.; Fatmi, M. Q.; Imran, S.; Rahim, F.; Khan, K. M. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 3285.
25. Taha, M.; Ismail, N. H.; Baharudin, M. S.; Lalani, S.; Mehboob, S.; Khan, K. M.; yousuf, S.; Siddiqui, S.; Rahim, F.; Choudhary, M. I. *Med. Chem. Res.* **2015**, *24*, 1310.
26. Taha, M.; Ismail, N. H.; Jamil, W.; Khan, K. M.; Salar, U.; Kashif, S. M.; Rahim, F.; Latif, Y. *Med. Chem. Res.* **2015**, *24*, 3166.
27. Rahim, Fazal; Ullah, Khadim; Ullah, Hayat; Wadood, Abdul; Taha, Muhammad; Rehman, Ashfaq Ur; uddin, Imad; Ashraf, Muhammad; Shaukat, Ayesha; Rehman, Wajid; Hussain, Shafqat; Khan, Khalid Mohammed *Bioorg. Chem.* **2015**, *58*, 81.
28. Rahim, F.; Ullah, H.; Javid, M. T.; Wadood, A.; Taha, M.; Ashraf, M.; Shaukat, A.; Junaid, M.; Hussain, S.; Rehman, W.; Mehmood, R.; Sajid, M.; Khan, M. N.; Khan, K. M. *Bioorg. Chem.* **2015**, *62*, 15.
29. Mesaik, M. A.; Khan, K. M.; Rahim, F.; Taha, M.; Haider, S. M.; Perveen, S.; Khalid, A. S.; Abdalla, O. M.; Soomro, S.; Voelter, W. *Bioorg. Chem.* **2015**, *60*, 118.
30. Chou, D. H. C.; Bodycombe, N. C.; Carrinski, H. A.; Lewis, T. A.; Clemons, P. A.; Schrelber, S. L.; Wagner, B. K. *ACS Chem. Biol.* **2010**, *8*, 729.
31. Taha, M.; Ismail, N. H.; Lalani, S.; Fatmi, M. Q.; Atia-tul-Wahab; Siddiqui, S.; Khan, K. M.; Imran, S.; Choudhary, M. I. *Eur. J. Med. Chem.* **2015**, *92*, 387.
32. Chou, D. H. C.; Holson, E. B.; Wagner, F. F.; Tang, A. J.; Maglathin, R. L.; Lewis, T. A.; Schreiber, S. L.; Wagner, B. K. *Chem. Biol.* **2013**, *19*, 669.
33. Vieira, H.; Kroemer, G. *IUBMB Life* **2003**, *55*, 613.