Bioorganic & Medicinal Chemistry Letters 23 (2013) 3101–3104

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Synthesis, characterization and anti-tumor activity of novel thymoquinone analogs against pancreatic cancer

Mujahid Yusufi [a,†], Sanjeev Banerjee [b,†], Momin Mohammad [c], Sandhya Khatal [d], K. Venkateswara Swamy [d], Ejazuddin M. Khan [a], Amro Aboukameel [b], Fazlul H. Sarkar [b,c,*], Subhash Padhye [a,b,*]

[a] ISTRA, Department of Chemistry, Abeda Inamdar College, University of Pune, Azam Campus, Pune 411001, India
[b] Department of Pathology, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI 48201, USA
[c] Department of Oncology, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI 48201, USA
[d] Department of Bioinformatics and Computer Science, Dr. D. Y. Patil Biotechnology and Bioinformatics Institute, Dr. D. Y. Patil Vidyapeeth, Pune 411044, India

ARTICLE INFO

Article history:
Received 9 January 2013
Revised 25 February 2013
Accepted 1 March 2013
Available online 14 March 2013

Keywords:
Thymoquinone analogs
Molecular Docking
Pancreatic cancer
Thymoquinone
Chemosensitisation
Gemcitabine

ABSTRACT

Thymoquinone (**TQ**), isolated from the seeds of *Nigella sativa*, show moderate efficacy against pancreatic cancer. In the present work we report synthesis and characterization of novel **TQ** analogs appended with gallate and fluorogallate pharmacophores and evaluation of their effects against pancreatic cancer cell lines for cell viability and induction of apoptosis. The efficacy of the analogs alone or in combination with Gemcitabine was assessed in vitro. LC–MS spectra of **ATQTHB** and **ATQTFB** showed major peaks corresponding to expected M+1 fragment at 316.34 and 322.34 respectively. Molecular docking studies revealed good fit for these analogs in the COX-2 protein cavity with better binding energies compared to parent **TQ** compound. Present **TQ** analogs exhibit superior anti-proliferative activity, excellent chemo-sensitizing activity against pancreatic cancer in vitro and in combination with Gemcitabine.

Pancreatic cancer is the fourth most common cancer with a five year survival rate of less than 3% amongst afflicted patients. In United States, approximately 43,920 new cases of pancreatic cancer are expected to be diagnosed in 2012, with 37,390 deaths anticipated.[1] The high rate of mortality associated with this disease is in part due to indolent nature of tumor growth presenting metastasis at time of diagnosis as well as, intrinsic and drug induced resistance to commonly used chemotherapies. As a consequence, disease-free survival time even after complete resection of the tumor and adjuvant treatments is less favourable. Since, currently available cytotoxic chemotherapeutic agents and other modalities of treatment have been found to be inadequate there is a dire need to develop novel translational drugs from clinical perspective for the treatment of pancreatic cancer.

Thymoquinone (**TQ**, 2-methyl, 5-isopropyl 1,4-benzoquinone) isolated from the seeds of *Nigella sativa* has been shown to exhibit anti tumor activity against breast, lung, prostate, liver, colon and pancreatic cancer.[2–4] We recently summarized the biological basis and therapeutic activities of **TQ** and reviewed existing analogs reported in literature for use in cancer therapy.[3,5,6] However, despite limited attempts originating from various laboratories, none of the analogs have proven efficacious as monotherapy or as therapeutic adjunct against pancreatic cancer.

In our previous endeavor, we have synthesized di-substituted benzoquinones bearing structural similarity with **TQ** which have shown promising results against pancreatic cancer cell lines.[7] In the same study we also reported that **TQ** exhibits growth inhibition and chemo-sensitization to standard chemo drugs like Gemcitabine and Oxaliplatin against chemo-resistant pancreatic cancer cell line-MiaPaCa-2 (7). Recently, Effenberger et al. have reported terpene conjugates of **TQ** which have been found to be more potent than **TQ** against human HL-60 leukemia, multidrug-resistant KB-V1/Vbl cervix, 518A2 melanoma and MCF-7/Topo breast cancer cells.[8] The 4-acylhydrazones and 6-alkyl analogs of **TQ** have also been found to be inhibitory against MCF-7/Topo breast cancer cells and HL-60 leukemia cells.[9]

In order to broaden the scope and propensities of these activities it is necessary to prepare different analogs of **TQ** through modification of its carbonyl functionalities or through nucleophilic additions of desirable groups and linkages. However, such modifications have not provided significant therapeutic advantage. Taking clue from our previous efforts where we showed that the

* Corresponding authors. Tel.: +91 8390025533 (S.P.).
E-mail address: sbpadhye@hotmail.com (S. Padhye).
† These authors contributed equally.

0960-894X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2013.03.003

gallate pharmacophore and its fluorinated analog when appended to chalcones and curcumin enhance the anticancer effect of the parent compounds by several folds, we were motivated to combine these pharmacophores with **TQ**.[10,11] Conceptually, these may behave as novel and potent agents against pancreatic cancer because of the retention and accompanying pleiotropic effects exhibited by **TQ**. In the present work we have employed a different strategy by synthesizing 3-aminothymoquinone (**ATQ**) as the initial building block and then synthesizing its Schiff base derivatives **ATQTHB** and **ATQTFB** with 2,3,4-trihydroxybenzaldehyde and 2,3,4-trifluorobenzaldehyde respectively.

The starting building compound, viz. 3-amino Thymoquinone (**ATQ**) was synthesized with the procedure described by Moore and co-workers with slight modifications.[12] A mixture of **TQ** (1 mmol, 0.164 g) and sodium azide (1.3 mmol, 0.084 g) in ethanol was refluxed for 3 h in the presence of 3 ml of glacial acetic acid (Fig 1). The compounds **ATQTHB** and **ATQTFB** were synthesized by condensation of equimolar quantities of **ATQ** (0.179 mg, 1 mmol) and 2,3,4-tri-hydroxy and 2,3,4-trifluoro benzaldehyde respectively in absolute alcohol in presence of 0.5 ml of concentrated hydrochloric acid as catalyst with continuous stirring at 75 °C for 4 h (Fig. 1). The reaction mixture was poured on crushed ice and the resulting precipitate was filtered, washed with water and purified by Column chromatography. The details of synthesis and spectroscopic characterization are provided in Supplementary material. The LC-MS spectra of **ATQTHB** and **ATQTFB** (Fig. 2A–D) showed major peaks corresponding to expected M+1 fragment at 316.34 (with retention time of 2.08 min) and 322.34 (with retention time of 2.77 min) respectively. The IR spectra of **ATQ** showed an intense carbonyl band at 1647 cm^{-1} and two medium intensity peaks at 3254 and 3310 cm^{-1} due to N–H stretches of the amino group. **ATQTHB** exhibited quinone carbonyl stretch in the range 1637–1639 cm^{-1} while the azomethine band was observed in the region 1595–1599 cm^{-1}. The broad band at 3375–3495 cm^{-1} was assigned to hydroxyl groups on the side chain. **ATQTFB** compound showed the carbonyl band at 1637 cm^{-1} while the azomethine band was observed in the range 1556–1564 cm^{-1}. A distinct band observed at 1058 cm^{-1} was due to C–F stretching vibration.[16] The ^{1}H NMR spectrum of parent **ATQ** compound showed proton of the amino group at 6.42 ppm which was found to be absent in the condensed Schiff base products (**ATQTHB** and **ATQTFB**). Additionally appearance of the azomethine hydrogen in the region 9.0–9.18 ppm confirmed the Schiff Base formation. The aromatic hydrogen atoms were located in the range of 6.9–8.0 ppm. In case of **ATQTFB** downfield values of aromatic hydrogen atoms were due to the presence of fluorine and aromatic signals appearing as multiplets were due to *ortho*- and *meta*-coupling of neighbouring hydrogen or fluorine atoms with coupling constants in the range of 7, 2 and 14, 11 Hz respectively. On the other hand the protons of hydroxyl groups in **ATQTHB** appeared as three singlet in the range of 10.20–11.42 ppm. The ^{13}C NMR of **ATQ** exhibited signals from the aliphatic carbons in the range of 8.59–25.84 ppm, olefinic carbon atoms at 106.6–148.9 ppm and quinone carbonyl carbons at 183.6 and 184.7 ppm respectively. In both these compounds the aliphatic carbon signals were found in the range of 9.9–10.1 ppm, while the olefinic and aromatic carbons atoms appeared in the range of 102–149 ppm. The azomethine and carbonyl carbons were observed in the range of 150–162.7 ppm.[17]

The COX pathway is known to elicit growth related signals and regulate cell proliferation contributing to development of pancreatic cancer.[18–23] The majority of human pancreatic carcinomas show over-expression of COX-2, while their benign counterpart lack this expression[24–26] suggesting that these tumors can be targeted selectively through COX pathway. The COX-2 inhibitor Celecoxib has been shown to reduce pancreatic cancer growth in animal models and humans.[27–30] Arafat and co-workers[31] have shown that TQ induced apoptosis and inhibited proliferation in pancreatic ductal adenocarcinoma cells as well as synthesis of MCP-1, TNF-α, interleukin (IL)-1beta and COX-2 in dose- and time-dependent manner. TQ has also been shown to exert an

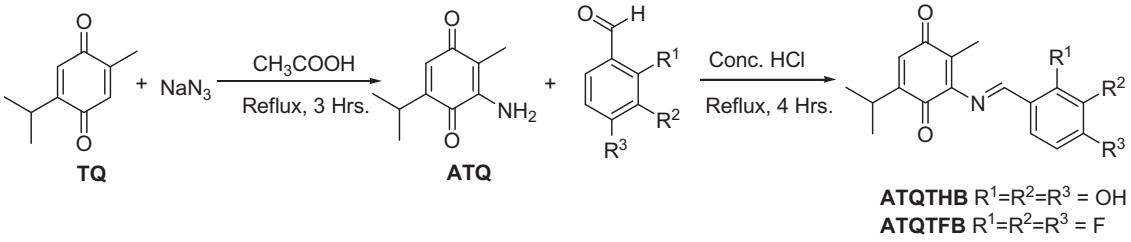

Figure 1. Schematic representation of synthesis of 3-amino-TQ and its Schiff Base analogs.

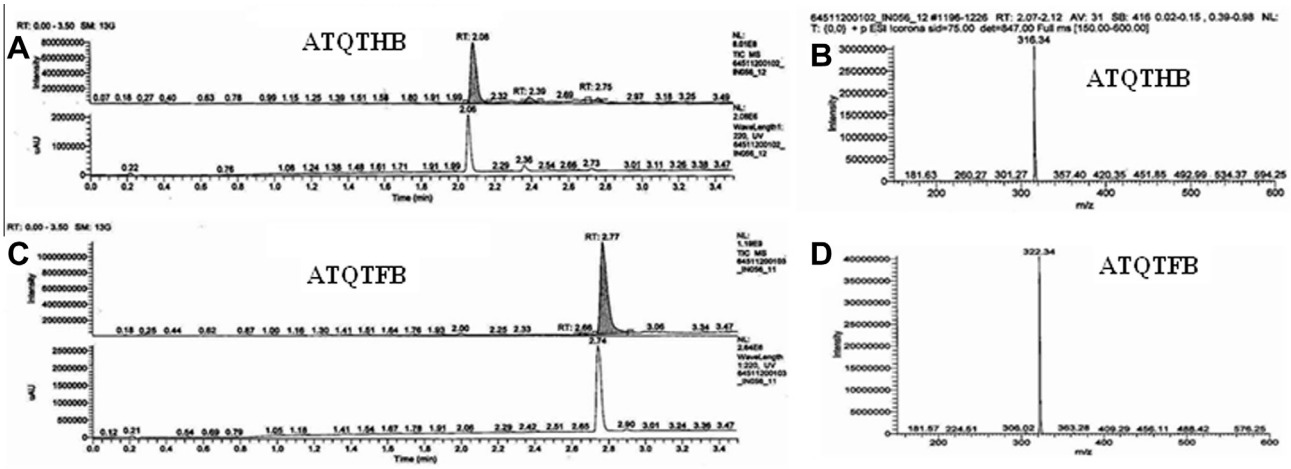

Figure 2. (A and B) LC–MS spectra of **ATQTHB**. (C and D) LC–MS spectra of **ATQTFB**.

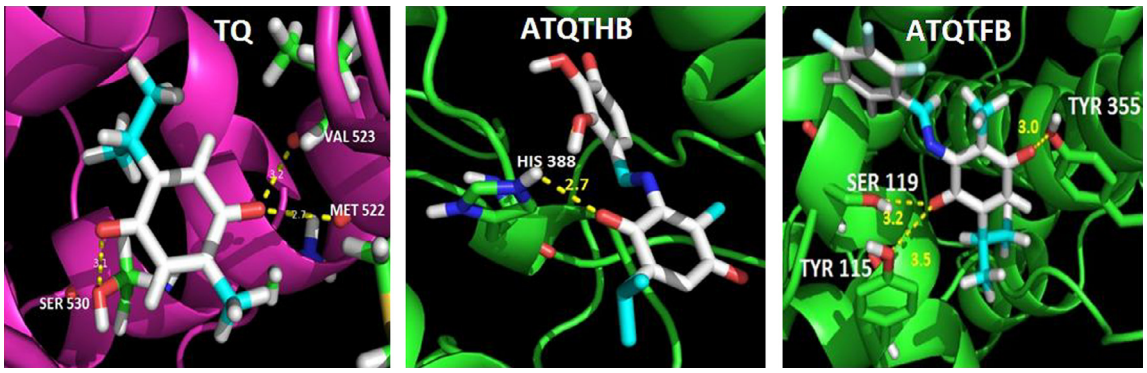

Figure 3. Binding of **TQ**, **ATQTHB** and **ATQTFB** into active site of COX-2 as assessed by molecular docking studies.

Table 1
Docking results and consensus scores of synthesized **TQ**, **ATQTHB** and **ATQTFB** analogs

Compounds	Binding energy (Kcal/mol)	No. of H bonds	Residues	Bond distance (Å)
TQ	−6.8	3	SER 530	3.1
			MET 522	2.7
			VAL 523	3.2
ATQ-THB	−8.1	1	HIS 388	2.7
ATQ-TFB	−7.9	3	TYR 115	3.5
			SER 119	3.2
			TYR 355	3.0

anti-inflammatory effect in a mouse model of ovalbumin-induced allergic airway inflammation by inhibiting COX-2 protein expression.[32] Hence, we have conducted a molecular docking study of TQ and its derivatives in the COX-2 protein cavity by using AutodockVina. [13–15]

TQ, **ATQTFB** and **ATQTHB** compounds when docked into the active site of COX-2 were found to give a good fit confirming that modification of the parent compound with additional pharmacophore does not introduce any major steric change in the **TQ** moiety except allowing additional hydrogen bonding interactions (Fig. 3, Table 1). The best binding energy was exhibited by **ATQTHB** (−8.1 kcal/mol) followed by **ATQTFB** (−7.9 kcal/mol) compared with **TQ** (−6.50 kcal/mol) respectively. **TQ** undergoes hydrogen bonding interactions with three amino acid residues, viz. SER 530 (3.1 Å), MET 522 (2.7 Å) and VAL 523 (3.2 Å) respectively in the COX cavity via its quinone carbonyl group. Similar H-bonding interactions are seen for **ATQTHB** (HIS 388, 2.7 Å) and **ATQTFB** (TYR 115, 3.5 Å, SER 119, 3.2 Å, TYR 355, 3.0 Å) respectively. These interactions lead to stabilization of the compounds in the protein cavity. Based on these considerations, compound **ATQTHB** has better stability in the COX-2 protein cavity than parent **TQ** molecule and anticipated to show more enhanced anticancer activity.

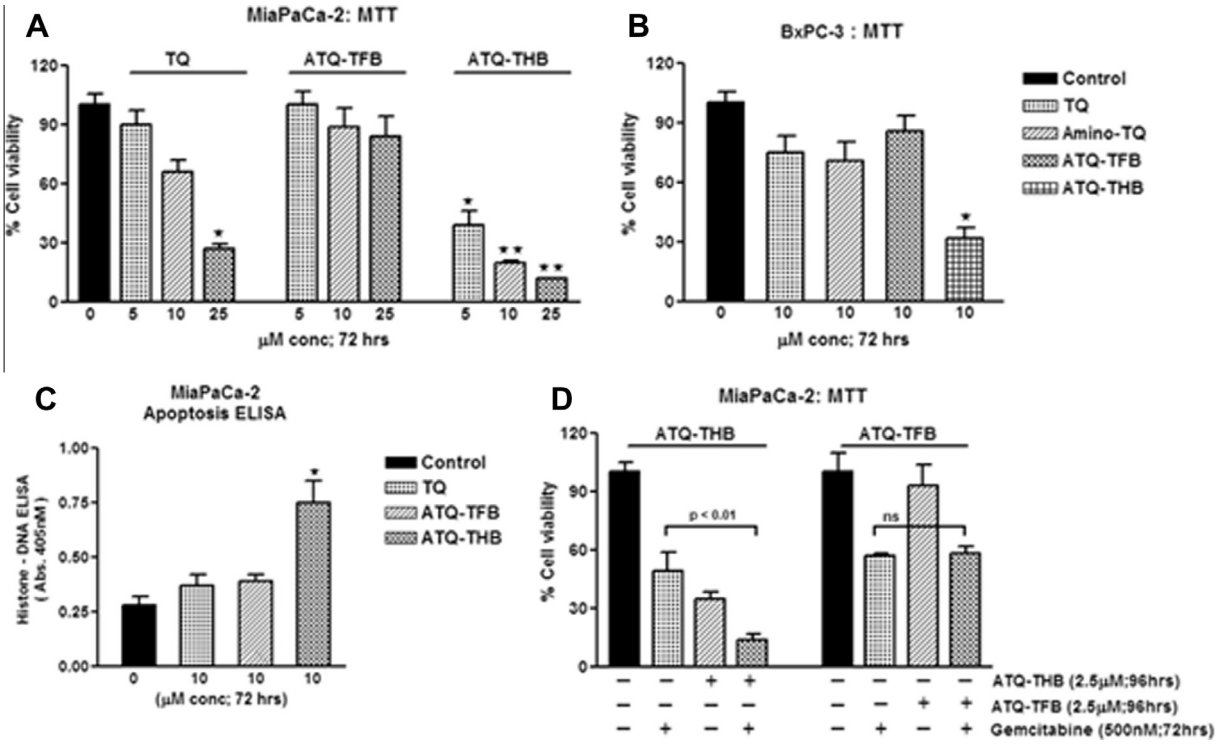

Figure 4. Cytotoxic activity of **ATQTHB** and **ATQTFB** against (A) MiaPaCa-2 and (B) BxPC-3 cell lines (*p <0.05), (C) histone DNA–ELISA for apoptosis detection in MiaPaCa-2 cells (*p <0.05), (D) Chemosensitisation to Gemcitabine by the analogs—**ATQTHB** and **ATQTFB** in MiaPaCa-2 cells.

The anticancer potency of the synthesized analogs was evaluated in two pancreatic MiaPaCa-2 and BxPC-3 cancer cell lines. The results obtained revealed differential sensitivity of the compounds towards cell viability. **ATQ** and **ATQTFB** exhibited either no significant effect or an effect similar to **TQ** at equimolar concentrations in BxPC-3 cell line that we investigated (Fig. 4B). In contrast, **ATQTHB** was promising amongst all; at equimolar concentration of **TQ**, against MiaPaCa-2 cell line and it showed >50% enhancement in the loss of cell viability compared to parental **TQ** (Fig. 4A). Similar results were also noted in BxPC-3 cells (Fig. 4B). This loss in cell viability, paralleled with apoptosis induction which was confirmed by Histone DNA ELISA in MiaPaCa-2 cells using **ATQTFB** and **ATQTHB** (Fig. 4C). Results revealed a trend similar to cell viability results showing a significant increase in apoptotic cells which was more pronounced in case of **ATQTHB** treated cells than **TQ** at equivalent concentration (Fig. 4C). To examine our hypothesis whether the Gemcitabine resistant MiaPaCa-2 cells when pre-treated with the synthesized **ATQTHB** or **ATQTFB** analogs could be more sensitive to the cytotoxic effect of Gemcitabine, we followed schedule of **ATQTHB** and **ATQTFB** pre-treatment protocol wherein the effect of only **ATQTHB** (2.5 μM), Gemcitabine alone (0.5 μM),[33] and **ATQTHB**/**ATQTFB** pre-treatment (2.5 μM; 24 h) followed by Gemcitabine treatment (72 hrs) on viability of MiaPaCa-2 cells evaluated by MTT (Fig. 4D). Based on our data we conclude that sub-toxic dose of **ATQTFB** did not exert any significant chemosensitizing effect on the investigated cell lines. On the contrary we found treatment of cells with Gemcitabine alone (for 72 h) caused >50% (p <0.05) loss of MiaPaCa-2 cells viability (Fig. 4D). However, pre-treatment of cells with **ATQTHB** for 24 h followed by treatment with the cytotoxic agent Gemcitabine for 72 h resulted in a significant loss of viable cells (<80%; p <0.001) in the MiaPaCa-2 cell line indicating that **ATQTHB** sensitizes resistant MiaPaCa-2 cells to the cytotoxic effect of Gemcitabine.

Over the years, the quest to explore and improve existing traditional phytochemicals for the treatment and prevention of cancer has driven the development of novel analogs with improved anticancer therapeutic effecacy without toxicity to normal cells. Several pre-clinical studies that have been reported reveal that **TQ** exhibits multi-targeted pleiotropic effects associated with chemoprevention and chemosensitisation of cancer. Previously, we have demonstrated that **TQ** exhibited chemosensitisation effects in Gemcitabine and Oxaliplatin.[2] The effect was attributed to the inactivation of DNA binding activity of NF-kB, resulting in the inactivation of multiple downstream survival molecules. In the present study we have synthesized new **TQ** analogs that reduce cell viability of pancreatic cancer cells by stimulating tumor cells to undergo apoptosis. Of interest, compared to parental compound **TQ**, one of the synthesized analog (**ATQTHB**) presents a much superior and significant inhibition of viable cells at equimolar concentration of **TQ**. Although yet to be explored, we propose that our analogs sensitize tumor cells to Gemcitabine by suppressing the pro-survival and pro-angiogenic molecule COX-2.

In conclusion, we have synthesized and structurally characterized novel analogs of thymoquinone which exhibit potent anti-proliferative activities against pancreatic cancer cell lines which open up the possibilities of optimizing application of these analogs for translational research. Amongst these analogs, **ATQTHB** showed superior sensitization of pancreatic cancer under in vitro system. Further studies are in progress to delineate precise molecular mechanism relating to their anti-proliferative and pro-apoptotic effects.

Acknowledgments

S.P. would like to acknowledge Mr. P. A. Inamdar, President, MCES, Pune, for the encouragement. We are thankful to NMR Research Centre, Indian Institute of Science, Bangalore for providing NMR spectral data.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.03.003.

References

1. Siegel, R.; Naishadham, D.; Jemal, A. *CA Cancer J. Clin.* **2012**, *62*, 10.
2. Banerjee, S.; Padhye, S.; Azmi, A.; Wang, Z.; Philip, P. A.; Kucuk, O.; Sarkar, F. H.; Mohammad, R. M. *Nutr. Cancer* **2010**, *62*, 938.
3. Padhye, S.; Banerjee, S.; Ahmad, A.; Mohammed, R.; Sarkar, F. H. *Cancer Ther.* **2008**, *6*, 495.
4. Woo, C. C.; Kumar, A. P.; Sethi, G.; Tan, K. H. *Biochem. Pharmacol.* **2012**, *83*, 443.
5. Tageja, N.; Padhye, S.; Dandawate, P.; Al-katib, A.; Mohammed, R. *J. Hematol. Oncol.* **2009**, *2*, 50.
6. Dandawate, P. R.; Vyas, A. C.; Padhye, S. B.; Singh, M. W.; Baruah, J. B. *Mini-Rev. Med. Chem.* **2010**, *10*, 436.
7. Banerjee, S.; Azmi, A. S.; Padhye, S.; Singh, M. W.; Baruah, J. B.; Philip, P. A.; Sarkar, F. H.; Mohammad, R. M. *Pharm. Res.* **2010**, *27*, 1146.
8. Effenberger, K.; Breyer, S.; Schobert, R. *Chem. Biodivers.* **2010**, *7*, 129.
9. Breyer, S.; Effenberger, K.; Schobert, R. *Chem. Med. Chem.* **2009**, *4*, 761.
10. Padhye, S.; Yang, H.; Jamadar, A.; Cui, Q. C.; Chavan, D.; Dominiak, K.; McKinney, J.; Banerjee, S.; Dou, Q. P.; Sarkar, F. H. *Pharm. Res.* **2009**, *26*, 1874.
11. Padhye, S.; Ahmad, A.; Oswal, N.; Dandawate, P.; Rub, R. A.; Deshpande, J.; Swamy, K. V.; Sarkar, F. H. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 5818.
12. Moore, H. W.; Shelden, H. R. *J. Org. Chem.* **1968**, *33*, 4019.
13. Sadowski, J.; Gasteiger, J.; Klebe, G. *J. Chem. Inf. Comput. Sci.* **1994**, *34*, 1000.
14. Trott, O.; Olson, A. J. *J. Comput. Chem.* **2010**, *31*, 455.
15. The PyMOL Molecular Graphics System, Schrödinger, LLC.
16. Mahadevan, D.; Periandy, S.; Ramalingam, S. *Spectrochim. Acta, Part A* **2011**, *84*, 86.
17. Rozwadowski, Z.; Ambroziak, K.; Szypa, M.; Jagodzińska, E.; Spychaj, S.; Schilf, W.; Kamieński, B. *J. Mol. Struct.* **2005**, *734*, 137.
18. Ding, X. Z.; Hennig, R.; Adrian, T. E. *Mol. Cancer* **2003**, *2*, 10.
19. Rioux, N.; Castonguay, A. *Carcinogenesis* **1998**, *19*(8), 1393.
20. Shureiqi, I.; Lippman, S. M. *Cancer Res.* **2001**, *61*, 6307.
21. Steele, V. E.; Holmes, C. A.; Hawk, E. T.; Kopelovich, L.; Lubet, R. A.; Crowell, J. A.; Sigman, C. C.; Kelloff, G. J. *Cancer Epidemiol. Biomarkers Prev.* **1999**, *8*, 467.
22. Subbaramaiah, K.; Dannenberg, A. J. *Trends Pharmacol. Sci.* **2003**, *24*, 96.
23. Dannenberg, A. J.; Altorki, N. K.; Boyle, J. O.; Dang, C.; Howe, L. R.; Weksler, B. B.; Subbaramaiah, K. *Lancet Oncol.* **2001**, *2*, 544.
24. Okami, J.; Yamamoto, H.; Fujiwara, Y.; Tsujie, M.; Kondo, M.; Noura, S.; Oshima, S.; Nagano, H.; Dono, K.; Umeshita, K.; Ishikawa, O.; Sakon, M.; Matsuura, N.; Nakamori, S.; Monden, M. *Clin. Cancer Res.* **1999**, *5*, 2018.
25. Kokawa, A.; Kondo, H.; Gotoda, T.; Ono, H.; Saito, D.; Nakadaira, S.; Kosuge, T.; Yoshida, S. *Cancer (Philadelphia)* **2001**, *91*, 333.
26. Tucker, O. N.; Dannenberg, A. J.; Yang, E. K.; Zhang, F.; Teng, L.; Daly, J. M.; Soslow, R. A.; Masferrer, J. L.; Woerner, B. M.; Koki, A. T.; Fahey, T. J. *Cancer Res.* **1999**, *59*, 987.
27. El-Rayes, B. F.; Ali, S.; Sarkar, F. H.; Philip, P. A. *Mol. Cancer Ther.* **2004**, *3*, 1421.
28. Arjona-Sánchez, A.; Ruiz-Rabelo, J.; Perea, M. D.; Vázquez, R.; Cruz, A.; Muñoz Mdel, C.; Túnez, I.; Muntané, J.; Padillo, F. J. *Pancreatology* **2010**, *10*, 641.
29. Lipton, A.; Campbell-Baird, C.; Witters, L.; Harvey, H.; Ali, S. *J. Clin. Gastroenterol.* **2010**, *44*, 286.
30. Dragovich, T.; Burris, H., 3rd.; Loehrer, P.; Von Hoff, D. D.; Chow, S.; Stratton, S.; Green, S.; Obregon, Y.; Alvarez, I.; Gordon, M. *Am. J. Clin. Oncol.* **2008**, *31*, 157.
31. Chehl, N.; Chipitsyna, G.; Gong, Q.; Yeo, C. J.; Arafat, H. A. *HPB (Oxford)* **2009**, *11*, 373.
32. El Mezayen, R.; El Gazzar, M.; Nicolls, M. R.; Marecki, J. C.; Dreskin, S. C.; Nomiyama, H. *Immunol. Lett.* **2006**, *106*, 72.
33. Li, Y.; VandenBoom, T. G.; Kong, D.; Wang, Z.; Ali, S.; Philip, P. A.; Sarkar, F. H. *Cancer Res.* **2009**, *69*, 6704.