

# RSC Advances



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

*Accepted Manuscripts* are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. This *Accepted Manuscript* will be replaced by the edited, formatted and paginated article as soon as this is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

# Synthesis, biological evaluation and *in silico* and *in vitro* mode-of-action analysis of novel dihydropyrimidones targeting PPAR- $\gamma$

H Bharathkumar<sup>a</sup>, Shardul Paricharak<sup>b,c</sup>, K. R. Dinesh<sup>a</sup>, Kodappully Sivaraman Siveen<sup>d</sup>, Julian E. Fuchs<sup>b</sup>, Shobith Rangappa<sup>e</sup>, C. D. Mohan<sup>f</sup>, Nima Mohandas<sup>d</sup>, Alan Prem Kumar<sup>d,g,h</sup>, Gautam Sethi<sup>d</sup>, Andreas Bender<sup>b,\*</sup>, Basappa<sup>a,\*</sup> and K. S. Rangappa<sup>f,\*</sup>

Received (in XXX, XXX) Xth XXXXXXXXX 200X, Accepted Xth XXXXXXXXX 200X

First published on the web Xth XXXXXXXXX 200X

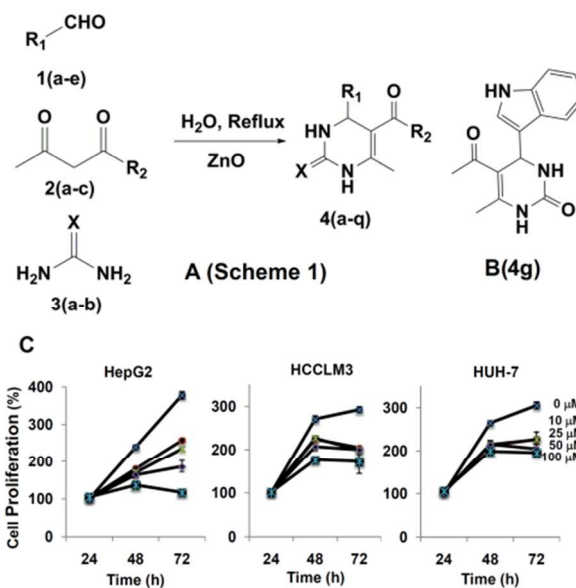
DOI: 10.1039/b000000000x

Hepatocellular carcinoma, a fatal liver cancer, affects 600,000 people annually and ranks third in cancer-related lethality. In this work we report the synthesis and related biological activity of novel dihydropyrimidones. Among the tested compounds, 5-acetyl-4-(1H-indol-3-yl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**4g**) was found to be most active towards the HepG2 cell line ( $IC_{50}$ =17.9  $\mu$ M), being at the same time 7.6-fold selective over normal (LO2) liver cells ( $IC_{50}$ =136.9  $\mu$ M). Subsequently, we identified peroxisome proliferator-activated receptor  $\gamma$  as target of compound **4g** using an *in silico* approach, and confirmed this mode-of-action experimentally.

The peroxisome proliferator-activated receptor (PPAR) belongs to the nuclear hormone receptor superfamily of transcription factors that includes 48 human transcription factors. Its activity is regulated by direct binding of steroid and thyroid hormones, vitamins, lipid metabolites, and xenobiotics.<sup>1</sup> PPARs heterodimerize with retinoid X receptors, which get activated and bind to specific response elements in the target DNA of various target genes.<sup>2,3</sup> PPARs consist of three different isoforms, PPAR- $\alpha$ , PPAR- $\beta/\delta$ , and PPAR- $\gamma$ . Due to their central function in a variety of physiological processes, PPARs are important targets in drug discovery.<sup>4</sup> Small molecules frequently interact with more than a single PPAR isoform leading to unique profiles of their biological effects.<sup>5</sup>

In particular, PPAR- $\gamma$  has been established as a key regulator for inflammation,<sup>6</sup> proliferation,<sup>7</sup> metabolism,<sup>8</sup> and differentiation,<sup>9</sup> and it is also upregulated by many tumor suppressor genes.<sup>10</sup> PPAR- $\gamma$  is overexpressed in several types of human cancers, including breast, colon, bladder, and prostate cancer, and it also induces apoptosis in several malignant cell lineages.<sup>11</sup> Furthermore, PPAR- $\gamma$  ligands such as 15d-PGJ2 as well as antidiabetic thiazolidinediones (TZDs) function as anti-proliferative and proapoptotic agents, suggesting that PPAR- $\gamma$  could be a promising therapeutic target for the treatment of cancer.<sup>12-14</sup> Non-thiazolidinedione derivatives, such as 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid, GW-7845, JTT-501, KRP-297, L-764406, MCC-555 and GW-0207 are further known synthetic ligands of PPAR- $\gamma$ .<sup>15</sup> On the other hand, the natural product ectoine, a tetrahydropyrimidine derivative, offers protection against the

effects of ischemia-reperfusion injury in intestinal transplantation *in vivo*.<sup>16</sup> ( $\pm$ )-Aplicyanin analogs bearing a pyrimidone moiety showed significant anti-tumor activity *via* targeting p38 MAP kinase.<sup>17-19</sup>



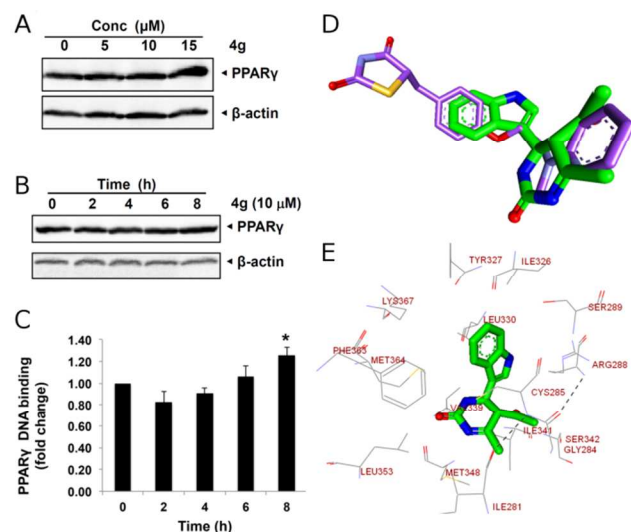
**Fig. 1** Synthetic scheme of novel dihydropyrimidones and cytotoxicity studies for the lead compound **4g** against various HCC cells.

Similar small molecules, dihydropyrimidines (DHPs), have also been reported as anti-cancer agents.<sup>20</sup> Recently, pyrimidine-tethered curcumins showed anticancer activity by targeting EGFR tyrosine kinase.<sup>21</sup>

In continuation of our interest to synthesize novel bioactive heterocycles,<sup>22,23,24</sup> we herein report the synthesis of DHP tethered to various functional heterocycles like indole, flavone and benzofuran. Furthermore, and conceptually rather novel, we rationalized the mode-of-action for the lead DHP as PPAR- $\gamma$  *via* an *in silico* cheminformatics approach, followed by experimental validation. Details on synthesis, compound characterization, biological assays, and *in silico* methods are described in more detail in the Supplementary Information.

For synthesis, multi-component reactions (MCRs) have been employed which constitute an efficient synthetic strategy for rapid and effective laboratory organic transformations,

because products are prepared in a one-pot and single step approach and the diversity can be obtained directly by changing the reacting components.<sup>25</sup> Here, we synthesized the dihydropyrimidone bearing small molecules via multi-component Biginelli reaction.<sup>26</sup> The mechanism for the

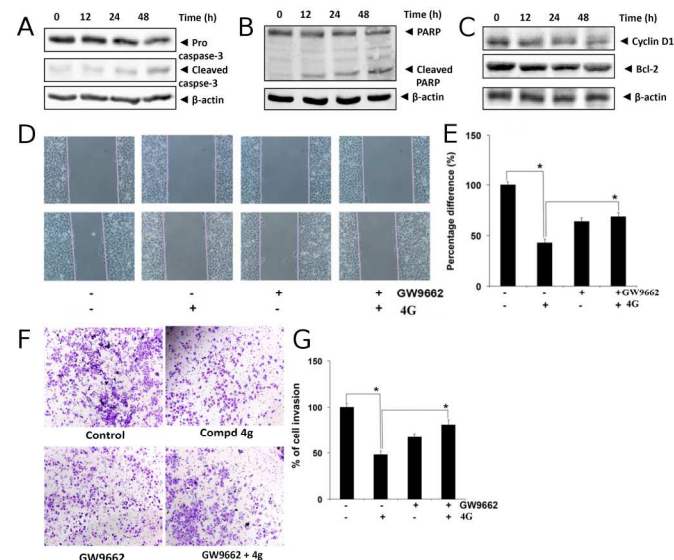


**Fig. 2** *In silico* and *in vitro* rationalization of the mode-of-action analysis for the lead compound 4g that targets PPAR- $\gamma$  in HepG2 cells.

formation of the title compounds involves the condensation between the aldehyde and urea to form an iminium intermediate, which acts as electrophile for the nucleophilic addition of the ketoester enol, and the ketone carbonyl of the resulting adduct undergoes condensation with the amine group of urea to give the cyclized product (Figure 1A). Also further heterocycles such as indole, flavone and benzofuran moieties were successfully employed for the preparation of novel dihydropyrimidones (see Supplementary Table 1). The protocol was effective with aromatic amines having electron donating groups, and products were identified based on IR, LCMS, <sup>1</sup>H NMR, and <sup>13</sup>C NMR spectra (see Supplementary Material). In the next step, the anti-proliferative effect of dihydropyrimidones was investigated in HepG2 cell lines using the MTT assay. The cells were treated with 0, 10, 25, 50, and 100  $\mu$ M concentrations of dihydropyrimidones for 72 h. DHPs were found to inhibit the proliferation of HepG2 cells in a dose- and time-dependent manner. Compound 4g, 4k and 4p were found to be most effective with IC<sub>50</sub> values less than 50  $\mu$ M. In addition, we were able to identify that all the dihydropyrimidone series of compounds inhibited the proliferation of immortalized normal human liver cells, LO2, at higher concentrations. Compound 4g was found to have an IC<sub>50</sub> value of 17.9  $\mu$ M against HepG2 and has the highest, 7.6-fold selectivity over the normal LO2 liver cell line (IC<sub>50</sub> = 136.93  $\mu$ M; see Supplementary Table 2 for more details). Therefore, we considered 4g (Figure 1B) in more detail and studied its mode-of-action in HepG2 cells. Human protein targets were predicted for the most bioactive

compound 4g, and of all predicted targets (Supplementary Table 3), PPAR- $\gamma$  was the only target with score of 26.02, exceeding the significance cut-off of 10. Given the involvement of PPAR- $\gamma$  in the induction of apoptosis and cancer development, as described in previous studies,<sup>4-6</sup> this target was selected for further analysis.

PPAR- $\gamma$  has been extensively shown to be associated with anti-cancer effects in a variety of cancer types including HCC. HepG2 cells were treated with different concentrations of compound 4g for 8 h and then analyzed for the expression of PPAR- $\gamma$  by western blot analysis. It was found that compound



**Fig. 3** Apoptotic induction, anti-migration, and anti-invasive activity of the compound 4g in HCC cells.

4g could significantly increase PPAR- $\gamma$  expression in a dose-dependent manner, with maximum effect at 15  $\mu$ M concentration (Figure 2A). Compound 4g also increased PPAR- $\gamma$  expression in a time-dependent manner, with maximum activity at 8 h (Figure 2B). Evidently, the DNA-binding assay for PPAR- $\gamma$  in nuclear extracts showed that compound 4g significantly enhanced PPAR- $\gamma$  activation in a time dependent manner (Figure 2C).

DHPs are well known ligands for PPAR- $\gamma$ . In order to determine the interaction of dihydropyrimidones towards PPAR- $\gamma$ , the co-crystal structure of human PPAR- $\gamma$  in complex with rosiglitazone was considered for our studies.<sup>27</sup> Using Discovery Studio (DS) default tools and settings, the ligand binding domain (LBD) of PPAR- $\gamma$  was identified and ligands were prepared for docking utilizing the CDOCKER programme of the Ligand-Receptor interaction module of Discovery Studio version 2.5, the dihydropyrimidones were docked into the LBD of PPAR. The CDOCKER energies for all docked ligands (Supplementary Table 4) indicate that compound 4g bound to the LBD region with the lowest (and thus most favorable) score of -24.0 kcal/mol (Supplementary Table 3). Furthermore, the binding pose of compound 4g was found to overlap with the hydrophobic tail of TZDs

(rosiglitazone; Figure 2D). Compound 4g forms mainly hydrophobic contacts with residues at the LBD region of PPAR- $\gamma$  including Ile281, Met348, Ile353, Val339, Cys285, Ile341, Ser342, Gly284, Tyr321, Ile326, Lys367, Phe363, Met364, Leu330, Ser289, and Arg288 (Figure 2E). Thereby, CH- $\pi$  interactions with Leu-330 and van der Waals contacts with the alkyl chain of Arg-288 are proposed as main driving forces of the ligand. This molecular docking study revealed that compound 4g could be a suitable non-TZD ligand for PPAR- $\gamma$ .

We investigated whether activation of PPAR- $\gamma$  in HepG2 cells by compound 4g leads to apoptosis. In HepG2 cells treated with compound 4g, there was a time-dependent reduction in the levels of procaspase-3 (Figure 3A). This suggests that cleavage events had occurred, indicating the activation of caspase-3, the levels of which were shown to be increased after 48 h. Activation of caspase-3 led to the cleavage of a 118 kDa Poly (ADP-ribose) polymerase (PARP) protein into an 85 kDa fragment (Figure 3B). This result clearly suggests that compound 4g induces caspase-3-dependent apoptosis in HepG2 cells. In addition, compound 4g was found to downregulate the anti-apoptotic and proliferative proteins, Bcl-2 and Cyclin D1 in a time dependent manner (Figure 3C), with maximum effect observed at 48 h. Pronounced expression of PPAR- $\gamma$  was demonstrated in HCC cells treated with rosiglitazone and such induction markedly suppressed the migration of HCC cells.<sup>28</sup> The effect of compound 4g on the migration of HepG2 cells was investigated using a wound healing assay. We found that compound 4g treatment significantly suppressed the migration of HepG2 cells, and the pretreatment with GW9662, a pharmacological PPAR- $\gamma$  inhibitor, reversed the anti-migratory effects of compound 4g as shown in Figure 3D and 3E. It is well known that PPAR- $\gamma$  ligands may have therapeutic value for the treatment of highly invasive breast cancer by targeting invasion.<sup>29</sup> An *in vitro* Bio-Coat Matrigel invasion assay was performed (BD Biosciences, San Jose, CA, USA), and it was found that upon treatment with compound 4g there was a significant reduction in the number of cells that could invade the Matrigel coated polycarbonate membrane, indicating that compound 4g could indeed significantly inhibit the invasive property of HepG2 cells (Figure 3F and 3G). Moreover, we found that pretreatment with the PPAR- $\gamma$  antagonist GW9662 reversed the anti-invasive potential of compound 4g in HCC cells, confirming that the activity is mediated through inhibition of PPAR- $\gamma$  pathway.

In conclusion, we herein report the synthesis of novel dihydropyrimidones and found 5-acetyl-4-(1H-indol-3-yl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (4g) to be most active against HCC cells. Furthermore, compound 4g down-regulated anti-apoptotic and proliferative proteins such as Bcl-2 and Cyclin D1 in a time-dependent manner. *In silico* studies predicted PPAR- $\gamma$  as mode-of-action of compound 4g, which could be validated by *in vitro* studies. Molecular docking furthermore suggested the binding pose of 4g within the ligand binding domain of PPAR- $\gamma$ , which overlaps with the tail of rosiglitazone. Compound 4g inhibited the invasive property of HepG2 cells and the pretreatment with GW9662

reversed the anti-invasive potential of compound 4g in HCC cells *in vitro*, thereby confirming that the activity is mediated through inhibition of PPAR- $\gamma$  pathway. Hence, both *in silico* and experimental methods agree on the mode-of-action of compound 4g *via* PPAR- $\gamma$ .

#### Acknowledgement

This research was supported by the Department of Science and Technology (NO. SR/FT/LS-142/2012), University Grants Commission (41-257-2012-SR), and Vision Group Science and Technology to Basappa. Basappa thanks Karnataka University, INDIA for DC PAVATE Fellowship and IISc, Bangalore for providing access to the NMR Facility. Bharathkumar thanks UGC for a BSR fellowship. SP thanks the Netherlands Organisation for Scientific Research (NWO, grant number NWO-017.009-065) and the Prins Bernhard Cultuurfonds for funding. CDM thanks DST for INSPIRE fellowship.

#### Notes and references

- <sup>a</sup>Laboratory of Chemical Biology, Department of Chemistry, Bangalore University, Palace Road, Bangalore-560001, India.
- <sup>b</sup>Unilever Centre for Molecular Science Informatics, Department of Chemistry, University of Cambridge, Lensfield Road, CB2 1EW, Cambridge, United Kingdom.
- <sup>c</sup>Division of Medicinal Chemistry, Leiden Academic Centre for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands.
- <sup>d</sup>Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597.
- <sup>e</sup>Interdisciplinary Research Group of Infectious Diseases, Singapore-MIT Alliance for Research & Technology Centre (SMART), Singapore-138602. Cancer Science Institute of Singapore, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117599.
- <sup>f</sup>Department of Chemistry, University of Mysore, Manasagangotri, Mysore-560001, India.
- <sup>g</sup>School of Biomedical Sciences, Faculty of Health Sciences, Curtin University, Western Australia.
- <sup>h</sup>Department of Biological Sciences, University of North Texas, Denton, Texas, USA.
- † Electronic Supplementary Information (ESI) available: Chemical structures of all educts and products as well as synthesis protocols and spectroscopic characterization of the compounds are available as Supplementary Information. See DOI: 10.1039/b000000x/.
- 1 M. Lehrke and M. A. Lazar, *Cell*, **2005**, 123, 993-999.
- 2 F. S. Celi and A. R. Shuldiner, *Curr. Diabetes Rep.*, **2002**, 2, 179.
- 3 C. Grommes, G. E. Landreth and M. T. Heneka, *Lancet Oncol.*, **2004**, 5, 419-429.
- 4 T. M. Willson, P. J. Brown, D. D. Sternbach and B. R. Henke, *J. Med. Chem.*, **2000**, 43, 527-550.
- 5 M. Dietz, P. Mohr, B. Kuhn, H. P. Maerki, P. Hartman, A. Ruf, J. Benz, U. Grether and M. B. Wright, *ChemMedChem*, **2012**, 7, 1101-1111.
- 6 C. Jiang, A. T. Ting and B. Seed, *Nature*, **1998**, 391, 82-86.
- 7 H. P. Koeffler, *Clin. Cancer Res.*, **2003**, 9, 1-9.
- 8 J. M. Lehmann, L. B. Moore, T. A. Smith-Oliver, W. O. Wilkinson, T. M. Willson and S. A. Sliewer, *J. Biol. Chem.*, **1995**, 270, 12953-12956.
- 9 S. A. Kliewer, J. M. Lenhard, T. M. Willson, I. Patel, D. C. Morris and J. M. Lehmann, *Cell*, **1995**, 83, 813-819.
- 10 L. Patel, I. Pass, P. Coxon, C. P. Downes, S. A. Smith and C. H. Macphee, *Curr. Biol.*, **2001**, 11, 764-768.
- 11 E. Elstner, C. Muller, K. Koshizuka, E. A. Williamson, D. Park, H. Asou, P. Shintaku, J. W. Said, D. Heber and H. P. Koeffler, *Proc. Natl. Acad. Sci. USA*, **1998**, 95, 8806-8811.
- 12 G. Venkatachalam, A. P. Kumar, K. R. Sakharkar, S. Thangavel, M. V. Clement and M. K. Sakharkar, *J. Drug Target.*, **2011**, 19, 781-796.
- 13 M. Pignatelli, J. Sanchez-Rodriguez, A. Santos and A. Perez-Castillo, *Carcinogenesis*, **2005**, 26, 81-92.
- 14 I. W. Campbell, *Curr. Mol. Med.*, **2005**, 5, 349-363.
- 15 E. H. Kim and Y. J. Surh, *Biochem. Pharmacol.*, **2008**, 76, 1544-



- 1553.
- 16 T. Pech, I. Ohsawa, M. Praktijnjo, M. Overhaus, S. Wehner, M. von Websky, K. Abu-Elmagd, G. van Echten-Deckert, J. C. Kalff and N. Schaefer, *Pathobiology*, **2013**, 80, 102-110.
- 17 Sisa, D. Pla, M. Altuna, A. Francesch, C. Cuevas, F. Albericio and M. Alvarez, *J. Med. Chem.*, **2009**, 52, 6217-6223.
- 44, 1106-1118.
- 20 B. R. Prashantha Kumar, G. Senkar, R. B. Nasir Baig and S. Chandrashekar, *Eur. J. Med. Chem.*, **2009**, 44, 4192-4198.
- 21 M. J. Ahsan, H. Khalilullah, S. Yasmin, S. S. Jadav and J. Govindasamy, *Biomed. Res. Int.*, **2013**, 239354.
- 22 Basappa, M. P. Sadashiva, K. Mantelingu, S. N. Swamy and K. S. Rangappa, *Bioorg. Med. Chem.*, **2003**, 11, 4539-4544.
- 23 Basappa, S. Murugan, C. V. Kavitha, A. Purushothaman, K. G. Nevin, K. Sugahara and K. S. Rangappa, *Cancer Lett.*, **2010**, 297, 231-243.
- 24 Basappa, K. Sugahara, K. N. Thimmaiah, H. K. Bid and K. S. Rangappa, *PLoS ONE*, **2012**, 7, e39444.
- 18 T. Asano, H. Yamazaki, C. Kasahara, H. Kubota, T. Kontani, Y. Harayama, K. Ohno, H. Mizuhara, M. Yokomoto, K. Misumi, T. Kinoshita, M. Ohta and M. Takeuchi, *J. Med. Chem.*, **2012**, 55, 7772-7785.
- 19 S. Cesarini, A. Spallarossa, A. Ranise, S. Scenone, C. Rosano, P. La Colla, G. Sanna, B. Busonera and R. Loddo, *Eur. J. Med. Chem.*, **2009**, 25, 1189-1218.
- 20 P. Slobbe, E. Ruijter and R. V. A. Orru, *Med. Chem. Commun.*, **2012**, 3, 1189-1218.
- 26 C. O. Kappe, *Tetrahedron*, 1993, 49, 6937-6963.
- 27 M. V. Liberato, A. S. Nascimento, S. D. Ayers, J. Z. Lin, A. Cvor, R. L. Silveira, L. Martinez, P. C. Souza, d. Saidemberg, T. Deng, A. A. Amato, M. Togashi, W. A. Hsueh, K. Phillips, M. S. Palma, F. A. Neves, M. S. Skaf, P. Webb and I. Polykarpov, *PLoS ONE*, 2012, 7, e36297.
- 28 B. Shen, E. S. Chu, G. Zhao, K. Man, C. W. Wu, J. T. Cheng, G. Li, Y. Nie, C. M. Lo, N. Teoh, G. C. Farrell, J. J. Sung and J. Yu, *Br. J. Cancer*, 2012, 106, 1486-1494.
- 29 H. Liu, C. Zang, M. H. Fenner, K. Possinger and E. Elstner, *Breast Cancer Res. Treat.*, 2003, 79, 63-74.