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Zebrafish based strategy for the identification of a potential pharmacophore for apoptosis: a greener CuAAC approach for novel 1,2,3-triazoles derived from mefenamic acid†

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Prompted by the potential of a screening strategy in zebrafish for the identification of valuable pharmacophores, a series of triazole substituted mefenamic acid derivatives were designed and synthesized *via* a CuAAC under green conditions. A variety of terminal alkynes were reacted with the azide obtained from mefenamic acid to give the expected products in good to excellent yields. When screened for apoptosis, teratogenicity and hepatotoxicity in zebrafish embryos, one of these compounds showed encouraging apoptotic properties and safety profiles and seemed to have medicinal value.

The synthesis of a library of novel small molecules and their screening in zebrafish for early indications of their possible utility in therapeutic regimes and potential toxic side-effects is of immense importance. Apart from identifying useful chemical probes, this strategy can also help in identifying valuable pharmacophores for the design and discovery of potential drugs to combat deadly diseases.

Zebrafish (or *Danio rerio*), a small pet-shop fish, has attracted considerable interest as an emerging model for understanding human biology and is being pursued as a tool for enhancing interdisciplinary studies in biology and chemistry as well as in drug discovery.[1] For example, zebrafish provide an inexpensive, reliable and efficient first-level screening model for testing toxicity, efficacy, and tissue-targeting for a large number of new chemical entities (NCEs). The use of zebrafish embryos is also being considered as a one-step strategy to perform developmental screens to identify compounds with bioactivities relevant to vertebrates.[2,3] Indeed, pharmacologically active African plant extracts and subsequently their active components, possessing anti-angiogenic properties, were identified using this strategy.[4] The structure activity relationship (SAR) studies centered on pharmacologically active scaffolds have also been reported using zebrafish embryos.[5] Our continued interest[6–11] in zebrafish as a screening model for NCEs prompted us to design and synthesise a library of triazole based small organic molecules derived from mefenamic acid to assess their potential pharmacological effects in zebrafish and/or their embryos.

Triazoles, one of the extensively studied 5-membered nitrogen heterocycles, have found a wide range of applications especially as drugs and agrochemicals. Besides anti-HIV,[12] antiallergic,[13] antifungal,[14] and antimicrobial[15] activities, the 1,2,3-triazole derivatives (**A**, Fig. 1) have shown p38 MAP kinase and PfPK7 protein kinase inhibitory properties.[16] These moieties can also be tuned to form valuable pharmacophores[17] and play an important role in bio-conjugation. Mefenamic acid (**B**, Fig. 1) on the other hand is a well known non-steroidal anti-inflammatory drug (NSAID) used to treat pain and is a non-selective inhibitor of COX-1 and 2.[18] The presence of the $-CO_2H$ moiety is known to be responsible for its gastrointestinal side effects in addition to COX-1 inhibition. We therefore designed the basic template **C** by replacing the $-CO_2H$ group of **B** with a $-CH_2$-linked triazole moiety and introducing an "R" group to create diversity around this framework. In view of the reported apoptotic activities of both 1,2,3-triazoles[19] and mefenamic acid,[20] we anticipated that a library of molecules generated based on **C** would show similar pharmacological properties.

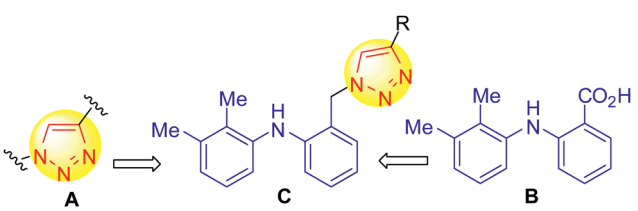

Fig. 1 The design of the new template **C** based on 1,2,3-triazoles (**A**) and mefenamic acid (**B**).

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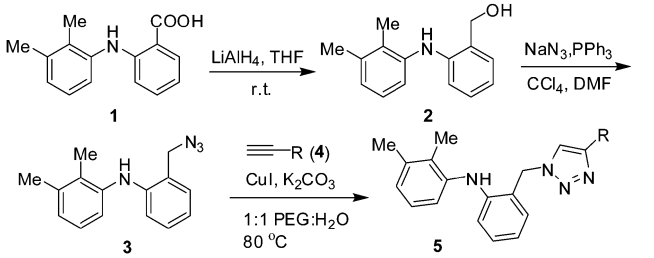
1,2,3-Triazoles are generally prepared using a click chemistry approach due to its specificity, efficiency, simple reaction workup procedure, and quantitative yield of products.[21,22] We adopted a similar but greener approach to prepare our target compounds **C** (or **5**, Scheme 1).

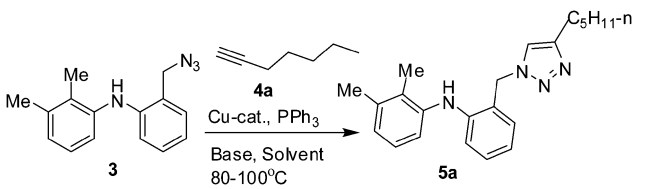
The key starting material *i.e.* the azide **3** was prepared *via* the reduction of the carboxylic acid moiety of mefenamic acid (**1**) to the corresponding alcohol **2**, which was then converted to the azide **3** (Scheme 1). The Cu-catalysed azide–alkyne cycloaddition (CuAAC) of the azide **3** and alkyne **4a** was performed initially with a catalytic amount of CuI in the presence of diisopropyl ethyl amine (DIPEA) in DMF. After 2 h, the desired triazole **5a** was obtained in 95% yield (entry 1, Table 1). The use of an inorganic base *e.g.* K_2CO_3 (entry 2, Table 1) decreased the product yield. The reaction did not proceed in the absence of CuI (entry 3, Table 1) or DIPEA (entry 4, Table 1), indicating the key role played by the catalyst and the base. The reaction however proceeded well in the presence of other copper catalysts *e.g.* CuCl (entry 5, Table 1) or CuBr (entry 6, Table 1). While all these reactions were performed in DMF, the use of other solvents such as 1,4-dioxane, acetonitrile, DMSO and PEG400 were also successful (entries 7–10, Table 1). To make this method more economic, we replaced PEG with 1 : 1 PEG400–H_2O (entry 11, Table 1) and to make it more environmentally benign we replaced DIPEA with K_2CO_3 (entry 12, Table 1). To our delight **5a** was obtained in 96 and 95% yield in both the cases. In order to avoid the use of hazardous organic amine bases we opted for the reaction conditions of entry 12 of Table 1 to perform further reactions.

We then prepared a range of 1,2,3-triazole derivatives (**5**) using this greener methodology (Table 2). A variety of terminal alkynes containing alkyl, hydroxyl alkyl, aryl, cyclic alkyl and



Scheme 1 Synthesis of 1,2,3-triazoles derived from mefenamic acid *via* a greener CuAAC approach.

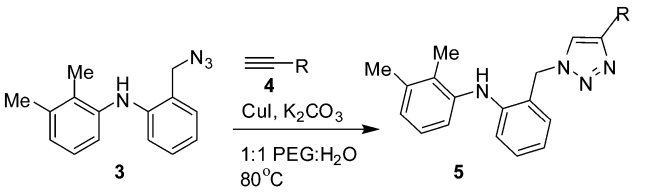
Table 1 Effect of reaction conditions on CuAAC of **3** and **4a**[a]

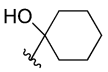
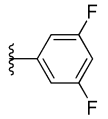
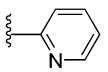


Entry	Solvent	Catalyst	Base	Time (h)	Yield[b] (%)
1	DMF	CuI	DIPEA	2	95
2	DMF	CuI	K_2CO_3	2.5	82
3	DMF	No catalyst	DIPEA	3	No product
4	DMF	CuI	No base	4	No reaction
5	DMF	CuCl	DIPEA	2	89
6	DMF	CuBr	DIPEA	2	85
7	1,4-Dioxane	CuI	DIPEA	2.5	90
8	Acetonitrile	CuI	DIPEA	2.5	92
9	DMSO	CuI	DIPEA	2.5	93
10	PEG 400	CuI	DIPEA	1.5	98[c]
11	1 : 1 PEG–H_2O	CuI	DIPEA	1.5	96[c]
12	1 : 1 PEG–H_2O	CuI	K_2CO_3	1.5	95[c]

[a] Reaction was carried out using **3** (1 mmol) and **4a** (1 mmol) in presence of catalyst, base and a solvent at 80–100 °C. [b] Isolated yield. [c] PPh_3 was not added.

Table 2 Synthesis of mefenamic acid based 1,2,3-triazole derivatives **5**[a]



Entry	Alkyne; R = (**4**)	Product[b] (**5**)	Yield[b] (%)
1	$-(CH_2)_4CH_3$ (**4a**)	**5a**	88
2	$-(CH_2)_2CH_3$ (**4b**)	**5b**	98
3	$-(CH_2)_3CH_3$ (**4c**)	**5c**	90
4	$-(CH_2)_5CH_3$ (**4d**)	**5d**	85
5	$-(CH_2)_7CH_3$ (**4e**)	**5e**	98
6	$-(CH_2)_9CH_3$ (**4f**)	**5f**	80
7	$-(CH_2)_3CN$ (**4g**)	**5g**	95
8	$-(CH_2)_3Cl$ (**4h**)	**5h**	97
9	$-CH_2OH$ (**4i**)	**5i**	95
10	$-(CH_2)_2OH$ (**4j**)	**5j**	80
11	$-(CH_2)_3OH$ (**4k**)	**5k**	98
12	$-C(CH_3)_3$ (**4l**)	**5l**	95
13	$-C(CH_3)_2OH$ (**4m**)	**5m**	85
14	$-CH(CH_3)OH$ (**4n**)	**5n**	80
15	–Ph (**4o**)	**5o**	98
16	$-C_6H_4CH_3$-*p* (**4p**)	**5p**	97
17	 **4q**	**5q**	91
18	–CH(OH)Ph (**4r**)	**5r**	90
19	 **4s**	**5s**	96
20	 **4t**	**5t**	95

[a] Reaction was carried out using **3** (1 mmol), **4** (1 mmol), CuI (0.05 mmol) and K_2CO_3 (3 mmol) in 1 : 1 PEG–H_2O (5 mL) at 80 °C. [b] Isolated yield.

heterocyclic moieties were reacted with the azide **3** to give the corresponding products **5** in good to excellent yields.

All the mefenamic acid based 1,2,3-triazole derivatives **5** prepared were characterized using NMR, IR and MS spectroscopy. In most cases the appearance of a singlet at ~7.40 δ (this singlet was merged with another aromatic proton in some cases) in the ^{1}H NMR and a signal at 122.6 ppm in the ^{13}C NMR confirmed the presence of a triazole ring.

These compounds (only solids) were tested for their apoptotic activities initially at 30 μM using zebrafish embryos. Apoptosis[23] is an ordered and orchestrated cellular process that occurs under physiological and pathological conditions. Its complex mechanism involves many pathways. Defects in apoptotic pathways are thought to contribute to a number of human diseases, ranging from neurodegenerative disorders to malignancy.[23] For example, cancer is associated with a lower degree of apoptosis and most cytotoxic anticancer agents are known to induce apoptosis. The most active compounds *i.e.* **5d**, **5p** and **5q** were tested at 1, 3, 10 and 30 μM along with a known drug methotrexate[24] at 30 μM. The percentage induction of apoptosis caused by these three compounds at different concentrations is shown in Fig. 2 and the representative images of embryos are also shown in Fig. 3. It is evident from Fig. 2 that all these compounds showed considerable effects in the present apoptosis assay. While compound **5d** showed milder apoptotic activities at 1 and 3 μM, a substantial increase in activity was observed when tested at 10 μM. Notably, the embryos were found dead at a higher concentration *i.e.* 30 μM. The compound **5p** showed a consistent increase in activity with a dose that reached a maximum value at 30 μM. Like **5d**, the compound **5q**

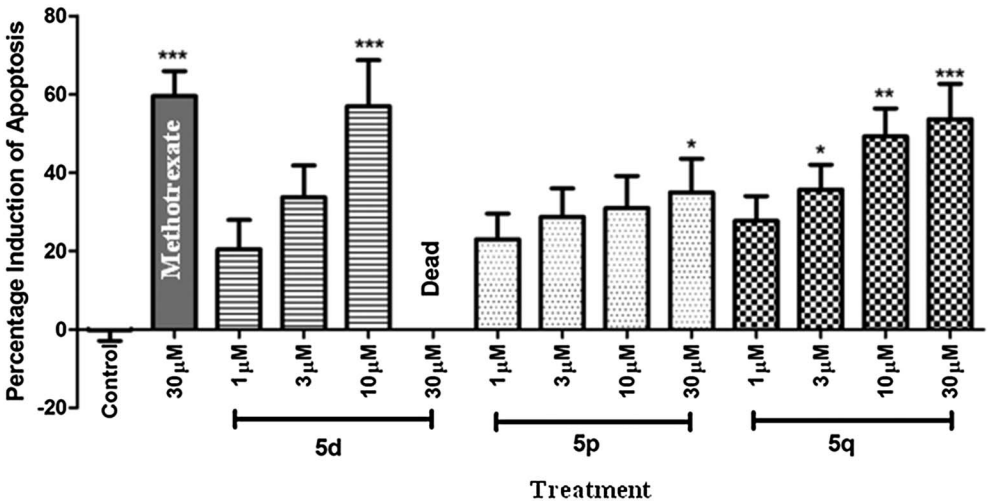


Fig. 2 The percentage induction of apoptosis caused by compounds **5d**, **5p** and **5q** at different concentrations along with methotrexate. All the statistical analysis was performed using GraphPad Prism® software.

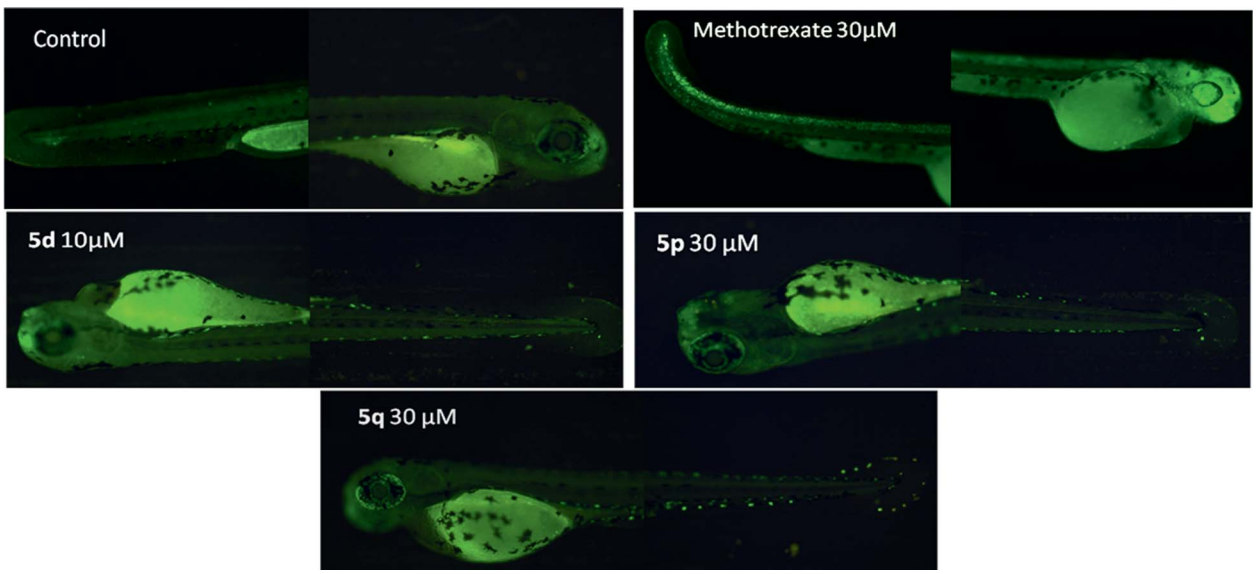


Fig. 3 Representative images of the embryos treated with compounds assayed for apoptosis.

also showed minimal apoptotic activities at 1 and 3 μM that increased at 30 μM. The EC_{50} values of **5d**, **5p** and **5q** were found to be 8.23, 2.28 and 3.64 μM, respectively.

In view of their promising apoptotic activities the safety profile of **5d**, **5p** and **5q** were evaluated in zebrafish embryos in the range of 1.0–30 μM. The transparency of the developing zebrafish embryos and their similar toxicity profiles to humans made it a promising model for various toxicity assessments like teratogenicity, hepatotoxicity *etc.* In the teratogenicity assay (Fig. 4 and 5, see also Table S-1 in ESI†), compound **5p** was found to be safe up to 30 μM with No Observed Adverse Effect Level (NOAEL) ≥ 10 μM, while compounds **5d** and **5q** were non-toxic at 10 μM with NOAEL ≥ 10 and 3 μM, respectively. However, in the hepatotoxicity assay (Fig. 6 and 7), the NOAEL for **5d**, **5p** and **5q** was found to be ≤3 μM, ≤10 μM and ≤1 μM, respectively. The overall results of the zebrafish based pharmacological evaluation of compounds **5d**, **5p** and **5q** are

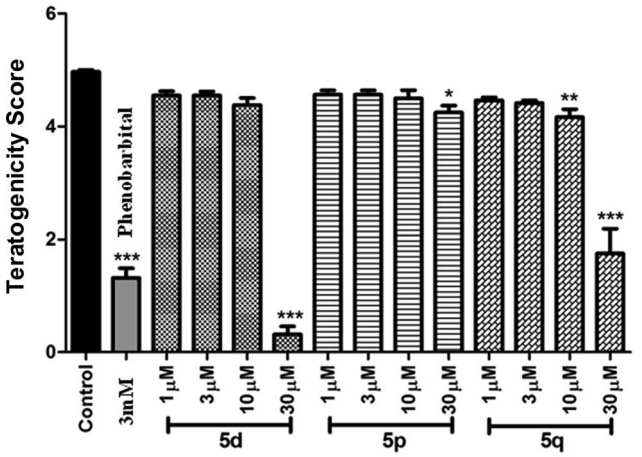


Fig. 4 Teratogenicity assay: each embryo was scored based on their level of toxicity from 5, being non toxic, to 0.5, being highly toxic. The graph represents the teratogenic scoring compared to the positive control phenobarbital.

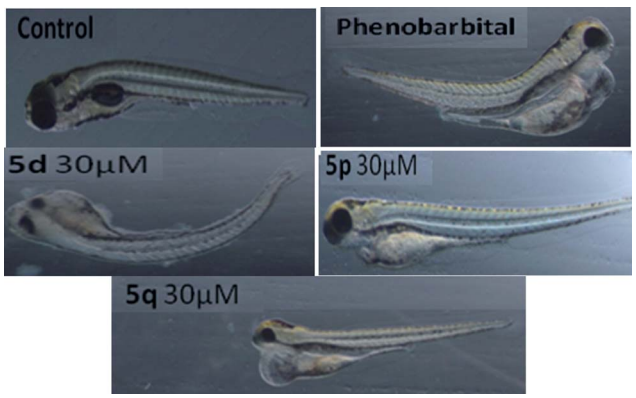


Fig. 5 Representative images obtained from the teratogenicity assay: control embryo showing normal body; embryo treated with phenobarbital (positive control) at 3 mM showing severe abnormalities, body bent; compound **5d** showing severe abnormalities at 30 μM; compound **5p** and **5q** showing slight abnormalities at 30 μM.

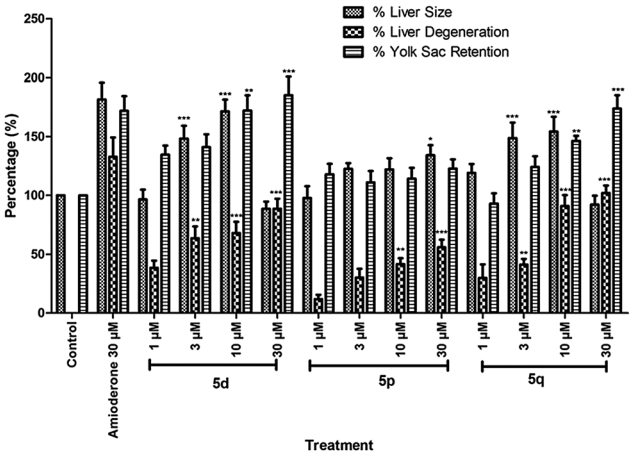


Fig. 6 Hepatotoxicity assay: the graph represents the qualitative data of percentage liver size, percentage liver degeneration & percentage yolk sac retention of three compounds at different concentrations compared to the positive control amiodarone.

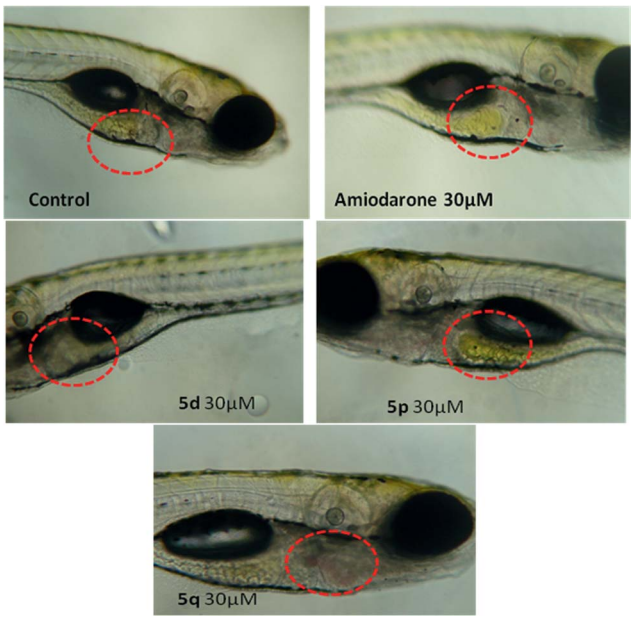


Fig. 7 Representative images obtained from the hepatotoxicity assay.

summarized in Table 3 (see also Fig. S-1 in the ESI†). It is evident from Table 3 that compound **5p** showed encouraging apoptotic properties and safety profiles with a superior therapeutic index (ratio of NOAEL/EC_{50}) of 4.38, compared to other compounds *e.g.* **5d** (therapeutic index = 0.12) and **5q** (therapeutic index = 0.27).

In conclusion, we have demonstrated the potential of a screening strategy in zebrafish for the identification of a new pharmacophore for apoptosis. A series of novel triazole substituted mefenamic acid derivatives were designed and subsequently synthesized *via* a greener Cu-catalyzed azide–alkyne cycloaddition (CuAAC). A variety of terminal alkynes containing alkyl, hydroxyl alkyl, aryl, cyclic alkyl and heterocyclic moieties were reacted with the azide obtained from

Table 3 Summary of pharmacological evaluation of compounds **5d**, **5p** and **5q** in zebrafish

Pharmacological evaluations				Test compounds data		
Tests	Endpoint	Positive control	Parameters	**5d**	**5p**	**5q**
Apoptosis	Acridine orange staining of apoptotic cells	Methotrexate	EC_{50} (μM)	8.231	2.278	3.642
Hepatotoxicity	% Liver size, % liver degeneration, % yolk sac retention	Amiodarone	NOAEL (μM)	1	10	1
Teratogenicity	Morphological assessment of phenotypic changes	Phenobarbital	NOAEL (μM)	10	10	3
Overall therapeutic index	Ratio of NOAEL/EC_{50} (overall NOAEL = lowest NOAEL)	—	Therapeutic index	0.121	4.389	0.274

mefenamic acid to give the expected products in good to excellent yields. When screened for apoptosis, teratogenicity and hepatotoxicity in zebrafish embryos, compound **5p** showed encouraging apoptotic properties and safety profiles. In view of the fact that most cytotoxic anticancer agents are known to induce apoptosis, compound **5p** seemed to have medicinal value. The present class of compounds therefore represents a new pharmacophore for the design and discovery of potential new drugs.

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