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# Lead optimization of isocytosine-derived xanthine oxidase inhibitors

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ABSTRACT

We report our attempts at improving the oral efficacy of low-nanomolar inhibitors of xanthine oxidase from isocytosine series through chemical modifications. Our lead compound had earlier shown good in vivo efficacy when administered intraperitoneally but not orally. Several modifications are reported here which achieved more than twofold improvement in exposure. A compound with significant improvement in oral efficacy was also obtained.



Purine oxidation in eukaryotes is catalyzed by enzymes called xanthine oxidoreductases (XOR) that catabolize physiological substrate hypoxanthine to xanthine and then to uric acid (UA) by hydroxylation.[1] Hyperuricemia results from increased production or reduced elimination of UA from the body, or both.[2] It is associated with gout and other systemic disorders like cardiovascular and metabolic diseases.[3] Gout, a painful and potentially disabling form of arthritis that has been recognized since ancient times, has seen an increase in prevalence in recent years.[4]

Treatment regimes for gout include uric acid lowering agents, which are recommended along with lifestyle changes like weight loss, reduction in intake of alcohol and purine-rich foods, etc.[5] Anti-gout drugs include XOR inhibitors which reduce production, uricosurics which enhance excretion (e.g., probenecid, sulfinpyrazone, benzbromarone and isobromindione), and urate oxidase which mops up excess UA (pegloticase, approved by US FDA for severe treatment-refractory chronic gout). Allopurinol, a hypoxanthine analog that is a mechanism-based inhibitor of XOR,[3] and febuxostat, a non-purine inhibitor of XOR,[6] are the approved first and second lines of UA production inhibitors in the market. Colchicine, a neutrophil motility and activity inhibitor, is often administered along with other anti-gout therapies to control treatment-related gout flares. New generation small molecules like RDEA594, a uricosuric[7] and BCX4208, a purine nucleoside phosphorylase inhibitor that works upstream of XOR,[8] are currently in phase II/III clinical trials in combination with allopurinol or febuxostat. These compounds showed inadequate efficacy or response as single agents in clinical trials indicating the continuing importance of XOR inhibitors in the treatment of gout.

We had earlier reported the discovery of a novel scaffold with isocytosine moiety, compound **1**, whose in vitro XOR inhibitory activity was better than that of allopurinol (Fig. 1).[9] Docking studies showed important interactions of this molecule in the active site of XOR and provided directions for structure-based modifications leading to **2**, with significant improvement in in vitro activity. Further optimization efforts resulted in **3** with significantly higher in vitro activity and in vivo efficacy by intraperitoneal (ip) administration (Fig. 1).[10] However, this compound had oral bioavailability of only 8–12% in rats. To obtain good oral efficacy, equivalent or better in vitro potency and much higher oral exposure was required.

*Abbreviations:* XOR, xanthine oxidoreductase; XO, xanthine oxidase; PK, pharmacokinetics; AUC, area under curve; XDH, xanthine dehydrogenase; ip, intraperitoneal; po, per oral; SAR, structure–activity relationship; UA, uric acid; sUA, serum UA.

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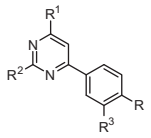


Compound **1**
$R^1$=OH, $R^2$=$NH_2$
$R^3$=H, R=$OCH_3$
In vitro $IC_{50}$ = 9.4 μM
In vivo reduction =
84% (100 mg/kg, po)

Compound **2**
$R^1$=OH, $R^2$=$NH_2$
$R^3$=H, R=$OCH_2CH(CH_3)_2$
In vitro $IC_{50}$ = 0.6 μM
In vivo reduction=
24% (100 mg/kg, po)

Compound **3**
$R^1$=OH, $R^2$=$NH_2$
$R^3$=CN, R=$OCH_2CH(CH_3)_2$
In vitro $IC_{50}$ = 0.02 μM
In vivo reduction =
12% (200 mg/kg, po)
124% (100 mg/kg, ip)

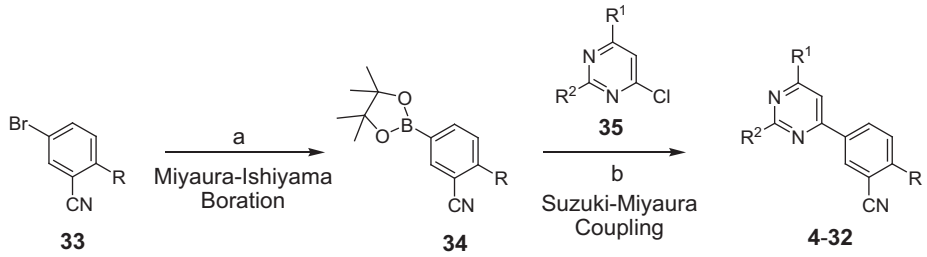
**Figure 1.** Milestone compounds obtained with isocytosine scaffold. In vivo efficacy was assessed by percentage reduction in UA levels in hyperuricemic rats treated with compound vs vehicle.

Towards this end, the R position of **3** (Fig. 1), which approaches the solvent front, was explored (Scheme 1)[9,10] to obtain a range of molecules with equivalent or enhanced potencies (Table 1). These molecules were docked to xanthine dehydrogenase (XDH) co-crystallized with piraxostat (PDB code 1VDV[11]) using XP docking protocol within GLIDE module[12] of Schrodinger Suite.[13] All of these structures docked well, with better scores than allopurinol, and some equivalent to febuxostat. In vitro xanthine oxidase (XO) inhibition assay was carried out on a high throughput system as described earlier.[9,14] Many of these substitutions resulted in compounds with low nanomolar in vitro activity, equivalent to febuxostat.

When the isobutoxy group on **3** was replaced by the smaller and less hydrophobic methoxy group (**4**), activity was reduced. Branched alkoxy groups gave very potent compounds such as **5**, **6** and **7**, the last being the most potent with an $IC_{50}$ of 4 nM. Benzyl substitution at R (**8**) maintained potency at nanomolar levels. Fluorination, substitutions like $-CF_3$ and *t*-butyl (**9**–**12**), and cyclic saturated rings (**13**, **14**) also gave nanomolar $IC_{50}$ values (Table 1).

Having obtained a plethora of potent compounds, subsequent designs were aimed at getting better oral exposure. Our aim was to obtain $C_{max}$ of at least 10 times the $IC_{50}$ of the compound and sufficiently high area under the curve (AUC) of plasma concentration vs. time profile to be able to show efficacy in the 5 h time period of the in vivo assay.[9,10] Precipitation at concentrations over 30 μM of several compounds in this series and the presence of the highly polar isocytosine group led us to suspect that both solubility and permeability could be issues. Depending on the assessed cause of the problem with oral absorption, conventional medicinal chemistry approaches include increasing polarity to improve solubility and hydrophobicity to enhance permeability.

Hence, acid and ester functionalities were introduced (Scheme 1)[9,10] on some of the potent compounds like **7**, **8** and **14** (Table 2). All the derivatives performed well in docking, retained




**Scheme 1.** Synthetic scheme for compounds in Tables 1–3: Reagents and conditions: (a) $Pd(dppf)Cl_2{\cdot}CH_2Cl_2$, dioxan, bis-pinacolato diborane, potassium acetate, 120 °C, 8 h. (b) $Pd(PPh_3)_4$, DMF, $Na_2CO_3$, microwave, 140 °C, 1–2 h.

**Table 1**
Potent analogs of compound **3** with modifications at R position

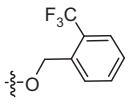
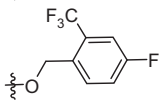
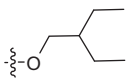
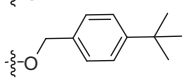
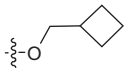
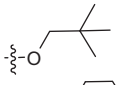
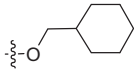
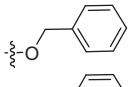
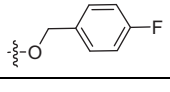
| Compound ID | R | Docking scores (kcal/mol) | In vitro $IC_{50}$ (μM) | Compound ID | R | Docking scores (kcal/mol) | In vitro $IC_{50}$ (μM) |
|---|---|---|---|---|---|---|---|
| **3** |  | −10.98 | 0.018 ± 0.029 | **10** |  | −10.89 | 0.009 ± 0.001 |
| **4** |  | −10.26 | 0.160 ± 0.040 | **11** |  | −10.79 | 0.011 ± 0.003 |
| **5** |  | −10.96 | 0.010 ± 0.001 | **12** |  | −10.72 | 0.019 ± 0.030 |
| **6** |  | −11.28 | 0.01 3 ± 0.001 | **13** |  | −10.63 | 0.018 ± 0.024 |
| **7** |  | −11.06 | 0.004 ± 0.001 | **14** |  | −11.15 | 0.010 ± 0.007 |
| **8** |  | −11.19 | 0.017 ± 0.035 | Aflopurinol | | −8.47 | 4.500 ± 0.060 |
| **9** |  | −11.13 | 0.007 ± 0.077 | Febuxostat | | −12.05 | 0.020 ± 0.003 |

**Table 2**
Analogs of compound **3** with polar groups to improve PK

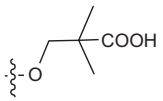
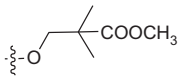
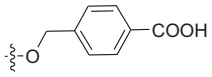
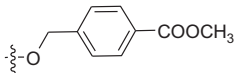
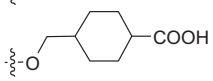
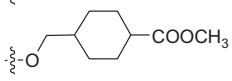
| Compound ID | R | Docking scores (kcal/mol) | In vitro $IC_{50}$ (μM) |
|---|---|---|---|
| **15** |  | −12.65 | 0.005 ± 0.001 |
| **16** |  | −12.12 | 0.015 ± 0.003 |
| **17** |  | −11.01 | 0.005 ± 0.000 |
| **18** |  | −11.18 | 0.009 ± 0.001 |
| **19** |  | −12.37 | 0.009 ± 0.000 |
| **20** |  | −10.74 | 0.019 ± 0.002 |
| Allopurinol | | −8.47 | 4.200 ± 0.200 |
| Febuxostat | | −12.05 | 0.020 ± 0.006 |

**Table 3**
PK studies at 10 mg/kg oral dose in rats of selected compounds with potent in vitro activities

| PK parameters | **3** | **3** (2 mg/kg, iv) | **7** | **15** | **16** | Released **15** | **14** | **19** | **20** | Released **19** | **23** | **25** | **25** (2 mg/kg, iv) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $C_{max}$ (ng/mL) | 65.72 | 1191.85 | 49.15 | 2168.70 | 14.03 | 2779.68 | 13.76 | 19.06 | ND[a] | 126.24 | 308.40 | 1124.20 | 2666.17 |
| $C_{max}$ (μM) | 0.23 | 4.19 | 0.16 | 6.60 | 0.04 | 8.46 | 0.04 | 0.05 | — | 0.34 | 1.15 | 4.19 | 9.94 |
| $T_{max}$ (h) | 0.19 | 0.03 | 0.50 | NC[b] | 0.75 | 4.00 | 1.00 | 4.00 | — | 2.00 | 0.25 | 0.75 | 0.03 |
| Elimination half-life (h) | 11.45 | 0.63 | 10.69 | 4.00 | 2.04 | 11.60 | 17.96 | NC | — | 3.90 | 9.88 | 1.35 | 5.96 |
| $AUC_{last}$ (ng* h/mL) | 202.69 | 471.19 | 296.53 | 19469.00 | 44.81 | 22699.83 | 109.35 | 119.41 | — | 795.24 | 415.81 | 1999.82 | 2991.35 |
| %F | 8.60 | — | — | — | — | — | — | — | — | — | — | 13.37 | — |
| $C_o$ (ng/mL) | — | 1360.78 | — | — | — | — | — | — | — | — | — | — | 3716.38 |
| Vd (mL/kg) | — | 3825.58 | — | — | — | — | — | — | — | — | — | — | 5498.68 |
| CL (mL/h/kg) | — | 4279.22 | — | — | — | — | — | — | — | — | — | — | 639.64 |
| Plasma stability[c] | 96.00 | — | 99.68 | 33.87 | 30.24 | — | 94.00 | 106.13 | 0.00 | — | 90.52 | 88.42 | — |
| Liver microsomal stability[c] | 91.50 | — | — | 90.22 | 95.88 | — | 97.30 | 90.57 | 0.00 | — | 49.58 | 21.20 | — |
| Liver S9 fraction stability[c] | 65.60 | — | — | 98.86 | 93.58 | — | 86.80 | 101.17 | 0.00 | — | 13.49 | 71.30 | — |

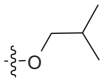
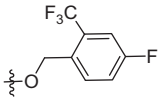
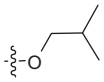
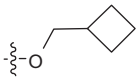
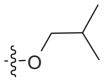
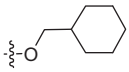
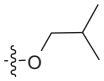
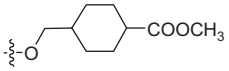
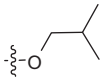
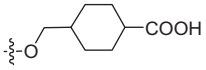
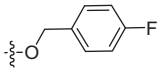
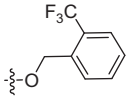
[a] ND: not detected.
[b] NC: not calculated.
[c] % Parent remaining after 60 min in in vitro assay.
* indicating multiplication sign needs to be added between ng and h.

$IC_{50}$ values in nanomolar range and hence were suitable for further investigation. Plasma pharmacokinetics (PK) studies[10,15] were performed at 10 mg/kg per oral (po) dose in Sprague Dawley rats (Table 3). Compound **7** was more potent than **3**, but did not show much gain in AUC. The acid derivative of **7** (**15**) showed significant improvement in PK over both **7** and **3**. The ester derivative, **16**, released **15** in vivo with higher $C_{max}$ and AUC than **15** administered directly. Compound **14** was as active as **3** but failed to achieve better $C_{max}$. Compound **19**, the acid derivative of **14** did not achieve better $C_{max}$. This may be due to low permeability of **19**. Its ester derivative, **20**, released **19** in sufficient quantities so that its $C_{max}$ and AUC were much better than those of **3**. Thus, the approach of increasing polarity using acid and ester groups provided compounds with better PK profile in terms of both $C_{max}$ and AUC. At $R^1$ position the acid derivative (**21**) docked well but resulted in a drop in activity to 400 nM while the amide derivative (**22**) performed poorly in both docking and in vitro assay (Table 4).

The other approach was to add hydrophobic groups or remove polar groups to enhance permeability. Removal of –CN from **3** would result in **2** and not serve our purpose. When $–NH_2$ was replaced by $–CH_3$ at $R^2$ position (**24**, Table 4), the compound did not dock in the correct pose and was inactive. Removal of –OH and $–NH_2$ groups, tried earlier when SAR was done for **1** and **2**, had resulted in complete loss of activity as loss of H-bonds prevented the molecules from anchoring well in the active site.[9,10] However, in the presence of –CN which compensated for this loss, correct docked poses were obtained (Fig. 2) albeit with a decrease in dock scores (**23** and **25** in Table 4). As we hoped, the loss of potency could be compensated by improved PK. $C_{max}$ of **23** and **25** was about five and 18 times better, respectively, than that of **3** (Fig. 3, Table 3). $AUC_{last}$ for **23** and **25** was, respectively, about two and 10 times better than for **3**. Thus, **25** led to greater improvement in oral exposure than **23**, with $C_{max}$ being about 52 times the $IC_{50}$ value.

Since the –OH group had a greater effect on PK than the $–NH_2$ group, deshydroxyl forms of some of the other potent compounds in Tables 1 and 2 were synthesized (**26**–**32**, Scheme 1) and tested (Table 4). In general, reduction in in vitro potency was observed for all the deshydroxylated compounds while $C_{max}$ and AUC increased.

**Table 4**
Analogs of compound **3** with modifications at $R^1$, $R^2$ and R positions

| Compound ID | $R^1$ | $R^2$ | R | Docking scores (kcal/mol) | In vitro $IC_{50}$ (μM) | Compound ID | $R^1$ | $R^2$ | R | Docking scores (kcal/mol) | In vitro $IC_{50}$ (μM) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **21** | COOH | $NH_2$ |  | −12.74 | 0.400 ± 0.300 | **28** | H | $NH_2$ |  | −8.44 | 0.080 ± 0.007 |
| **22** | $CONH_2$ | $NH_2$ |  | −6.23 | 3.900 ± 0.700 | **29** | H | $NH_2$ |  | −8.56 | 0.400 ± 0.007 |
| **23** | OH | H |  | −10.45 | 0.060 ± 0.010 | **30** | H | $NH_2$ |  | −8.38 | 0.050 ± 0.007 |
| **24** | OH | $CH_3$ |  | −7.53 | >30 | **31** | H | $NH_2$ |  | −8.83 | 0.010 ± 0.001 |
| **25** | H | $NH_2$ |  | −8.22 | 0.080 ± 0.003 | **32** | H | $NH_2$ |  | −8.26 | 0.010 ± 0.000 |
| **26** | H | $NH_2$ |  | −8.63 | 0.150 ± 0.020 | Allopurinol | | | | −8.47 | 4.200 ± 0.200 |
| **27** | H | $NH_2$ |  | −8.67 | 0.060 ± 0.004 | Febuxostat | | | | −12.05 | 0.020 ± 0.010 |

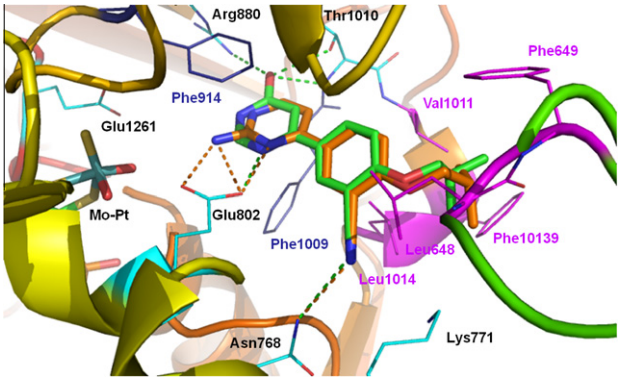



**Figure 2.** Docked poses of compounds **23** (green) and **25** (orange). H-bond of –CN group with Asn768 anchors the compounds in the active site even though **23** loses H-bonds with Glu802 and **25** with Thr880. This figure has been generated using Pymol.[16]

In vivo efficacy studies[9,17] were carried out for a few selected compounds (Fig. 4). Compound **25** showed 49% as much reduction in serum uric acid (sUA) level[17] as allopurinol (Fig. 4A) at 10 mg/kg oral dose. At 30 mg/kg po dose **25** showed almost 70% of the efficacy of allopurinol and greater decrease in sUA than higher doses of **16**, **20** and **23** (Fig. 4B and C). Thus **25** shows trend for dose dependent efficacy profile, with oral $ED_{50}$ between 10 and 30 mg/kg, which is a vast improvement on **3**. **31** and **32** were tested in vivo but no reduction was seen in sUA levels (data not shown). This is consistent with the low efficacy seen for the hydroxyl analog, **20**.

Major metabolic liabilities anticipated were conjugation reactions on hydroxyl group at $R^1$, O-dealkylation at R and hydroxylation reactions. However, most compounds in this series were found to be stable in in vitro metabolic stability studies. Some changes introduced to improve PK, nevertheless, enhanced the liabilities. Compound **20** was not detected in plasma, liver microsomes and liver S9 fraction while the de-esterified form, **19** was found. Likewise **31** showed poor stability (plasma 28%, liver microsomes 0% and liver S9 fraction 11% remaining at 60 min) but its de-esterified form, **32** was seen. Both **20** and **32** may additionally undergo other phase I and II biotransformations. Reduced stability of **23** in both liver microsomes and S9 fraction (Table 3) suggests increase in phase I and II metabolism, which may be responsible for its poor efficacy. Removal of –OH in **25** seems to have reduced liability for phase II metabolism (better S9 fraction stability) as compared to **3** and **23**, but increased phase I metabolism, possibly to generate active metabolites.

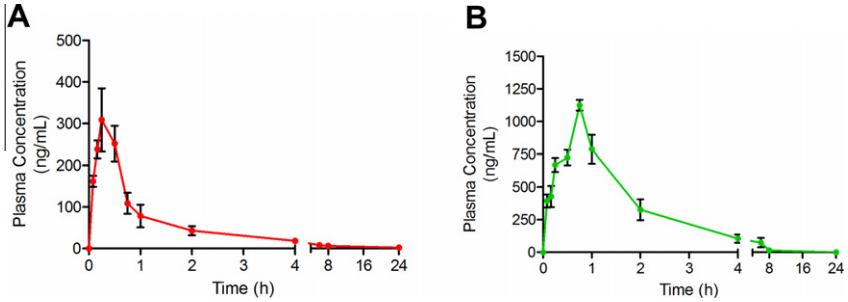



**Figure 3.** PK of compounds (A) **23** and (B) **25**.

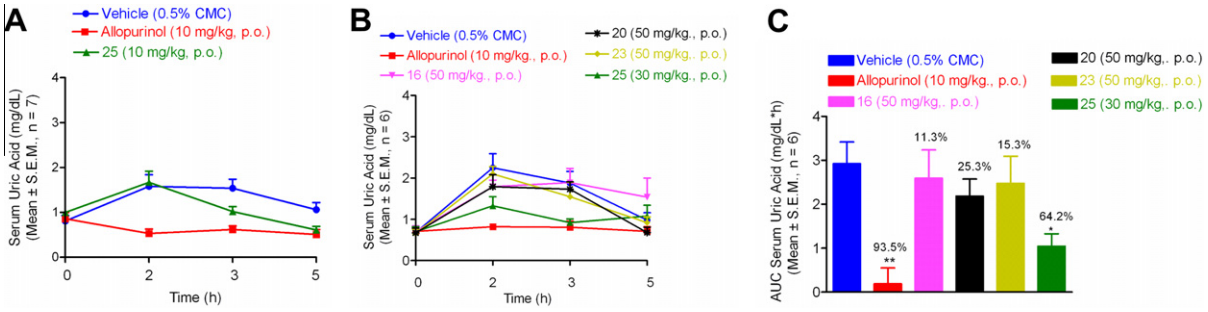



**Figure 4.** Antihyperuricemic effect of **16**, **20**, **23** and **25** along with allopurinol in hyperuricemic rats. sUA levels after treatment by (A) 10 mg/kg of **25**, (B) higher doses of **16**, **20**, **23** and **25**, (C) AUC of sUA levels over 5 h time period of study. Percent values indicate decrease in sUA levels with respect to vehicle.

It is observed (Fig. 4) that **25** is equally efficacious as allopurinol at 3 h time point, suggesting that the compound may act more slowly than allopurinol. This could be due to slower absorption but more importantly, it could be due to metabolism by liver enzymes (Table 3). One of the possible metabolites of **25** is **3**, which is also an active compound and may be generated in sufficient quantities after a time lag. This slow onset of activity of **25** suggests that a study in chronic hyperuricemic rat model could lead to a better understanding of its efficacy potential in a chronic disease like gout.

In summary, using virtual screening and enzymatic assay, we identified a novel isocytosine-containing scaffold for xanthine oxidase inhibition with **3** compounds showing micromolar $IC_{50}$s.[9] By structure-based design methods, we obtained compounds with improved in vitro potency in low nanomolar range and potent activity in hyperuricemic rat model by ip route but not by oral route.[10] Further medicinal chemistry approaches to lead optimization along with structural considerations resulted in a potent compound (**25**) with good oral activity.

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## Supplementary data

Supplementary data (supplementary table) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.11.057.

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14. Detailed in vitro assay protocol was described earlier.[9] Briefly, XO was added to the test compound or vehicle control and reaction was initiated by addition of xanthine. The UV absorbance indicating the formation of UA was measured at 30 min at ambient temperature. The $IC_{50}$ values were calculated for test compounds. Allopurinol or febuxostat was used as positive control.
15. Oral PK parameters of the compounds were assessed using a suspension [1 mg/mL] in 0.5% CMC and Tween 80. Sprague Dawley rats were weighed and the compounds were administered orally (10 mg/kg, $n = 8$). Blood samples were drawn at 0.08, 0.17, 0.25, 0.5, 0.75, 1.0, 2.0, 4.0, 6.0, 8.0 and 24 h after dosing following alternate bleeding protocol (four rats per time point). Concentrations of the compounds in plasma were determined using an ESI-LC/MS/MS method developed at Piramal Healthcare Ltd PK parameters were determined by non-compartmental analysis using WinNonlin Professional (Version 4.1).
16. The PyMOL Molecular Graphics System, Version 1.2r2, Schrödinger, LLC.
17. Hyperuricemic rat model was generated by administering potassium oxonate at a dose of 250 mg/kg ip to overnight fasted rats, as described earlier. Test compounds were administered 1 h later and sUA level was measured at 0, 2, 3 and 5 h time points. Percent reduction in sUA level was computed as AUC over the time period of observation for treated animals with respect to vehicle controls.[9] Reference compound (allopurinol) was administered at 10 mg/kg po while other compounds were tested at different doses by po route.