



Novel smoothed antagonists as anti-neoplastic agents for the treatment of osteosarcoma

This is a pre print version of the following article:

Original:

Bernardini, G., Geminiani, M., Gambassi, S., Orlandini, M., Petricci, E., Marzocchi, B., et al. (2018). Novel smoothed antagonists as anti-neoplastic agents for the treatment of osteosarcoma. JOURNAL OF CELLULAR PHYSIOLOGY, 233(6), 4961-4971 [10.1002/jcp.26330].

Availability:

This version is available <http://hdl.handle.net/11365/1034671> since 2018-02-27T16:42:46Z

Published:

DOI:10.1002/jcp.26330

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(Article begins on next page)



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Journal:	<i>Journal of Cellular Physiology</i>
Manuscript ID	JCP-17-0965.R1
Wiley - Manuscript type:	Original Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Bernardini, Giulia; University of Siena, Dpt Biotecnologie, Chimica e Farmacia Geminiani, Michela; Università degli Studi di Siena, Dipartimento di Biotecnologie, Chimica e Farmacia Gambassi, Silvia; Università degli Studi di Siena, Dipartimento di Biotecnologie, Chimica e Farmacia Orlandini, Maurizio; Università degli Studi di Siena, Biotecnologie, Chimica e Farmacia Petricci, Elena ; Università degli Studi di Siena, Dipartimento Biotecnologie, Chimica e Farmacia Marzocchi, Barbara; Università degli Studi di Siena, Dipartimento di Biotecnologie, Chimica e Farmacia Laschi, Marcella; Università degli Studi di Siena, Dipartimento di Biotecnologie Taddei, Maurizio; Università degli Studi di Siena, Dipartimento Biotecnologie, chimica e farmacia Manetti, Fabrizio; Università degli Studi di Siena, Dipartimento di Biotecnologie, Chimica e Farmacia Santucci, Annalisa; Università degli Studi di Siena, Dipartimento di Biotecnologie, Chimica e Farmacia
Key Words:	Osteosarcoma, SMO inhibitors, Apoptosis, Hedgehog pathway, GLI-1

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Novel smoothed antagonists as anti-neoplastic agents for the treatment of osteosarcoma

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Running head: Hedgehog pathway inhibition in osteosarcoma

Keywords:

- Osteosarcoma
- Hedgehog pathway
- SMO inhibitors
- Apoptosis
- Cancer
- Research Resource Identifiers – RRID
- CyQuant
- GLI-1

Total number of text figures: 6

Total number of text tables: 2

Abstract

Osteosarcoma (OS) is an ultra-rare highly malignant tumor of the skeletal system affecting mainly children and young adults and it is characterized by an extremely aggressive clinical course. OS patients are currently treated with chemotherapy and complete surgical resection of cancer tissue. However, resistance to chemotherapy and the recurrence of disease, as pulmonary metastasis, remain the two greatest challenges in the management and treatment of this tumour. For these reasons, it is of primary interest to find alternative therapeutic strategies for OS.

Dysregulated Hedgehog signalling is involved in the development of various types of cancers including OS. It has also been implicated in tumor/stromal interaction and cancer stem cell biology, and therefore presents a novel therapeutic strategy for cancer treatment.

In our work, we tested the activity of five potent Smoothed (SMO) inhibitors, four acylguanidine and one acylthiourea derivatives, against an OS cell line. We found that almost all our compounds were able to inhibit OS cells proliferation and to reduce Gli1 protein levels. Our results also indicated that SMO inhibition in OS cells by such compounds, induces apoptosis with a nanomolar potency. These findings suggest that inactivation of SMO may be a useful approach to the treatment of patients with OS.

Introduction

Osteosarcoma (OS) is the most frequent malignant primary bone sarcoma characterized by the presence of mesenchymal cells depositing immature osteoid matrix. It has a bimodal age distribution with a major peak in children and adolescents and a second significant peak in older adults (over forty years). Primary OS usually originates in the metaphysis of the long bones of the extremities (distal femur, proximal tibias and proximal humerus), adjacent to the most proliferative growth plates (Alfranca et al., 2015) and arises as a palpable mass, with progressive pain. It is characterized by an extremely aggressive clinical course; at the time of diagnosis, about 15-30% of the patients already present metastasis, with the lung and the bone the most common sites of distant diseases (Bernardini et al., 2012; Broadhead et al., 2011; Geller and Gorlick, 2010). The percentage of 5-years survival rate was 60-78% for patients with localized disease and only 20-30% for those with metastatic disease (Friebele et al., 2015). OS possesses high genetic instability and variability, even if no characteristic chromosomal translocations have been associated to the development of the tumor. At the molecular level, several genetic conditions have been associated to the development of the tumor such as Li-Fraumeni syndrome, Rothmund-Thomson syndrome and hereditary Retinoblastoma. Moreover, OS is characterized by an immature phenotype expressing primitive osteoblast markers or dysregulated signaling pathways such as Hedgehog or Src pathways (Bernardini et al., 2014a; Bernardini et al., 2014b; Laschi et al., 2015; Laschi et al., 2017) (Urciuoli et al., 2018). The most commonly used regimen for OS treatment is defined by the acronym MAP [methotrexate (MTX, M), adriamycin or doxorubicin (A) and platinum-derived drugs such as cisplatin (P)] and has become standard in North America and Europe (Ferrari and Serra, 2015; Isakoff et al., 2015). Unfortunately, the addition of other therapeutic agents, such as ifosfamide and/or etoposide, or the increase of the individual dose and the length of the treatment have not led to the desired results, with complications and toxicities in various organs, some of which are fatal (Chou et al., 2008; Ferrari et al., 2012). Despite a number of preclinical and clinical agents are currently under investigation (Bernardini et al., 2014a; Grignani et al., 2015; Hattinger et al., 2015; Whelan et al., 2015), new therapies are urgently needed to improve survival rate and quality of life for OS patients (Indovina et al., 2017).

The Hedgehog (Hh) signaling pathway is essential for the regulation of proliferation and differentiation during early embryogenesis (Varjosalo and Taipale, 2008), whereas in adulthood, it is usually involved in the maintenance of tissues homeostasis and repair, besides other important cellular responses such as proliferation, self-renewal ability, differentiation and migration (McMahon et al., 2003). Hh signaling pathway has a pivotal role also in skeleton development and its activation has been associated to degenerative pathologies such as osteoarthritis (Gambassi et al.,

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3 2017) (Mundy et al., 2016; Wang et al., 2017). Moreover, aberrant activation of Hh signaling
4 contributes to tumor aggressiveness, affecting key tumorigenic processes, such as proliferation,
5 invasion and progression of cancer cells (Ruiz i Altaba et al., 2007) and it has been described in a
6 wide variety of human tumors, including OS (Lo et al., 2014, Marini, 2011 #9; Tiet et al., 2006;
7 Yang et al., 2010). For these reasons, in recent years most of the studies have focused on Hh
8 pathway and its involvement in the treatment of several types of cancers.
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13 Three ligands have been identified in Hh pathway: Sonic Hedgehog (SHh), Indian Hedgehog (IHh)
14 and Desert Hedgehog (DHH). In the absence of Hh ligand Patched (PTCH), a 12-transmembrane
15 protein, inhibits the localization of Smoothened (SMO), a 7-transmembrane protein, to the primary
16 cilium preventing the activation of downstream signaling. On the contrary, in the presence of Hh
17 ligand, it binds to the receptor PTCH preventing PTCH-mediated inhibition of SMO, thus allowing
18 SMO to localize in the primary cilium and promote downstream activation of the 5-zinc-finger
19 transcription factors family: GLI1, GLI2 and GLI3 (glioma-associated oncogene homologs) (Scales
20 and de Sauvage, 2009).
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27 The receptor SMO is the central transducer of the Hh signaling pathway and is considered the most
28 druggable target. Cyclopamine, a plant Veratrum alkaloid, is the first SMO antagonist molecule
29 discovered but it is chemically unstable (Tremblay et al., 2009a) and it is not suitable for clinical
30 development because of limited potency and significant adverse effects (Warzecha et al., 2012).
31 Among the other compounds under investigation GDC-0449 (Vismodegib), a second generation
32 cyclopamine, is the first drug approved by the Food and Drug Administration (FDA) for the
33 treatment of locally advanced basal cancer carcinoma (BCC). Vismodegib lacks for the fast
34 resistance phenomena observed after the treatment. Nevertheless, in 2015 another Smo inhibitor,
35 LDE-225 (Sonidegib), has been approved for the treatment of BCC and skin cancers as well.
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43 Members of our research group have recently discovered a new family of acylguanidine and
44 acylthiourea derivatives acting as SMO antagonists. Several cell-based assays were used to evaluate
45 the ability of such compounds to inhibit the Hh pathway: the GLI-dependent luciferase report assay,
46 the alkaline phosphatase assay and the Bodipy-Cyclopamine binding assay (Roudaut et al., 2011;
47 Solinas et al., 2012). In addition, several of these compounds, namely MRT86 and MRT92,
48 demonstrated to maintain their activity against the most common mutant form of SMO (D473H)
49 leading to chemoresistance induced by the treatment with Vismodegib or Sonisegib (Hoch et al.,
50 2015).
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The aim of this work was to test five of these new SMO antagonists for their anti-proliferative and pro-apoptotic activity in MNNG OS cell line in order to evaluate the inhibition of the Hh pathway as a potential therapeutic approach to treat OS.

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Materials and Methods

Drugs

Cyclopamine was purchased from Sigma-Aldrich (Milan, Italy); MRT61, MRT86, MRT89, MRT92 and MRT94 were synthesized by members of our research group as previously reported (Hoch et al., 2015; Manetti et al., 2010; Roudaut et al., 2011; Solinas et al., 2012) (Table 1). All tested compounds were dissolved in DMSO at a concentration of 10 mM and diluted in culture medium respectively with $\leq 0.1\%$ concentration of DMSO. The same concentration of DMSO was used in control cells.

Cell cultures

Human primary osteoblasts (HOB) were isolated and cultured as described (Spreafico et al., 2006). Once isolated, HOBs were used until the 3rd generation to prevent the use of de-differentiated cells. A human OS commercial cell line namely MNNG (ATCC-CRL-1547, RRID: CVCL_0439) was obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured as described (Spreafico et al., 2008).

Cell viability assay

The effect of five new SMO antagonist compounds (MRT61, MRT86, MRT89, MRT92 and MRT94) and cyclopamine on the viability of MNNG cells was evaluated by MTT assay. Briefly, cells were seeded in 96-well plate at a density of 2×10^3 cells/well and cultured until sub-confluence (70-75% confluence). Cells were serum starved (FCS 0.1%) for 24 h and then treated with SMO antagonists and cyclopamine at different concentrations (1 nM, 10 nM, 100 nM and 10 μ M) for 24 hours. At the end of the treatment cells were washed with sterile PBS and MTT (Sigma-Aldrich, Milan, Italy) was added to each well with a final concentration of 1 mg/ml. After a 3 h incubation, cells were lysed with 100 μ l DMSO to dissolve the crystals. The absorbance was measured at 550 nm and percentage of cell viability was calculated relative to control. The experiment was repeated three times.

Cell proliferation assay

Proliferation of MNNG cells was measured by means of CyQUANT® Cell Proliferation assay kit (Molecular Probes; Invitrogen Corp., USA). This assay is based on the use of a green fluorescent dye which shows a strong fluorescent enhancement when bound to cellular nucleic acids.

MNNG cells were seeded in 96-well black-well/clearbottom plates (Greiner Bio-One, Baden-Württemberg, Germany) at a density of 2×10^3 cells/well and cultured until sub-confluence (70-

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3 75% confluence). Cells were serum starved (FCS 0.1%) for 24h and then treated with SMO
4 antagonist compounds and cyclopamine at different concentrations (1 nM, 10 nM, 100 nM and 10
5 μ M) for 24 hours. At the end of the treatment cells were washed with sterile PBS and the plates
6 were subsequently frozen at -80°C . The plates were then thawed at room temperature and the cells
7 were lysed by addition of a buffer containing the CyQuant dye. The fluorescence intensity was
8 recorded by a fluorescence microplate reader with excitation at 485 nm and emission at 530 nm
9 (Molecular Devices, Sunnyvale, CA, USA).
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11 **Nuclear staining with 4', 6-diamidino phenylindole (DAPI)**

12 Cells were seeded at 2×10^3 cells/well and cultured until sub-confluence (70-75% confluence) in
13 sterile chamber slides (Ibidi®, Planegg/Martinsried, Germany). After treatment with SMO
14 antagonist compounds and cyclopamine for 24 hours, as previously described, the slides were
15 rinsed with PBS and fixed in 70% ethanol for 30 minutes. Finally, the slides were washed twice
16 with PBS and mounted with fluoroshield mounting medium with DAPI (Abcam, Cambridge, UK).
17 Images were captured by Leiz Aristoplan light microscope (Leica, Wetzlar, Germany).
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28 **Western Blot analysis**

29 Cells at 70-75% confluence were treated with SMO antagonist compounds and cyclopamine at
30 different concentrations (1 nM, 10 nM, 100 nM and 10 μ M) for 24 hours. After treatment, cells
31 were lysed with RIPA buffer and then disrupted by sonication for 5 minutes in an ice bath. Protein
32 concentration was assessed according to Bradford. Forty micrograms of cells protein lysate were
33 resolved by 8% SDS-PAGE and then proteins were electrotransferred onto nitrocellulose membrane
34 (0.45 mm pore size; Whatman). After blocking for 45 minutes with 5% not-fat dry milk in 0.05%
35 Tween-20 Tris buffer saline (TBS-T) at room temperature, membranes were incubated with anti-
36 GLI1 (1:1000), anti-GLI2 (1:1000) and anti-GAPDH (1:50.000) primary antibodies at 4°C
37 overnight. The membranes were then washed three times with TBS-T and then incubated with
38 1:80.000 dilution of anti-rabbit HRP- conjugated secondary antibodies. Immunoreactive bands were
39 detected using Luminata Crescendo (Millipore) and images were acquired using ImageQuant
40 LAS4000 (GE Healthcare). The optical densities of the bands were analysed by ImageQuant TM
41 TL analysis software (GE Healthcare, RRID: SCR_014246) using GAPDH as a loading
42 normalizing factor. The experiment was performed in triplicate.
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54 **Annexin V/Propidium iodide assay**

55 Detection of apoptosis was performed in MNNG cells treated with SMO antagonist compounds for
56 24 hours, using FITC Annexin V/Dead Cell Apoptosis Kit (Molecular Probes; Invitrogen Corp.,
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3 USA) following manufactures protocol. A total of 300 cells from each sample were scored by using
4 a fluorescence microscope (Zeiss AXIO LAB AI) and were assessed as viable cells (AnV-/PI-),
5 early apoptotic cells (AnV+/PI-), late apoptosis (AnV+/PI+) and necrotic cells (AnV-/PI+).
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8 9 **Statistical analyses**

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11 Statistical analyses were performed with GraphPad Prism 6.0 software (GraphPad Software, San
12 Diego, CA, USA, RRID:SCR_002798). The data were expressed as mean \pm SD and compared
13 using one-way ANOVA with Dunnett post hoc test. A P value of 0.05 or less was considered
14 significant. Each experiment was performed in triplicate.
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Results

Hedgehog pathway is activated in MNNG OS cells

To investigate the activation of the Hh signaling pathway in MNNG cells, we evaluated the expression of GLI1 and GLI2 proteins by Western blot analysis. Human primary osteoblasts (HOB) were used as a negative control. Both GLI1 and GLI2 were expressed in MNNG cells but not in HOB, suggesting that Hh pathway is activated in OS whereas silent in HOB (Figure 1).

Inhibition of Hedgehog pathway affects cell viability and proliferation in MNNG OS cells

To examine the effect of cyclopamine and SMO antagonists (MRT61, MRT86, MRT89, MRT92 and MRT94) on MNNG cells viability and proliferation, MTT and CyQUANT[®] Cell Proliferation assays were performed. Control cells were treated with DMSO at a concentration corresponding to the highest dose used. The final concentration of DMSO did not exceed 0.1% (v/v), in both treated and control samples, and did not adversely affect the analysed parameters. Results were reported as percentage of viability (Figure 2) or proliferation (Figure 3) respect to the control (DMSO).

The results of MTT assay indicated that all compounds tested induced a dose-dependent decrease of cell viability (Figure 2), although at different degrees. In particular, it was possible to calculate LD50 values only for cyclopamine (LD50 = 11 nM), MRT86 (LD50 = 4 μ M) and MRT89 (LD50 = 1 μ M) at 24 hours of treatment, while the other compounds slightly affected MNNG cells viability (Table 1).

CyQuant proliferation assay revealed that all compounds tested induced a dose-dependent decrease of cell proliferation at 24 hours of treatment (Figure 3). MRT61 showed a 56% inhibition at 10nM with a calculated IC50 = 5nM, MRT89 showed a 50% inhibition at 100nM with a calculated IC50 = 120nM, MRT92 showed a 52% inhibition at 10 μ M IC50 = 7 μ M, and MRT94 showed a 54% inhibition at 100 nM with a calculated IC50 = 50 nM. Finally, MRT86 reduced cell proliferation of less than 50% at the highest concentration tested (42.2% inhibition at 10 μ M) (Table 1) suggesting that three out of five compounds (MRT61, MRT89 and MRT94) have been demonstrated to be able to inhibit cell viability and proliferation in OS cells.

Novel SMO-antagonists inhibit GLI1 expression in MNNG OS cells

To further analyse the effect of SMO antagonist, the expression of the protein GLI1 in MNNG cells treated with SMO antagonist compounds was evaluated by Western blot analysis (Figure 4 B-D-F-H). Results were reported as percentage of expression of GLI1 in respect to the control, treated with the highest percentage of DMSO.

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3 Quantitative analysis of the immunoreactive bands revealed that the compounds MRT61, MRT89
4 and MRT94 decreased in a dose-dependent manner GLI1 expression in MNNG cells (Figure 4 A-C-
5 E-G). In particular, MRT61 (Figure 4C) and MRT89 (Figure 4E) inhibited GLI1 expression by
6 more than 50% at a concentration of 10 nM and MRT61 already at 1 nM. Importantly, cyclopamine
7 inhibited Hh pathway but only at the highest concentration used (10 μ M) (Figure 4A).
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10 11 **SMO antagonists affect MNNG OS cells nuclear morphology**

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14 To evaluate whether MRT61, MRT89 and MRT94 exhibited cytotoxicity in MNNG OS cells
15 through apoptosis, a DAPI staining analysis was undertaken in order to observe nuclear
16 morphological changes after treatment with SMO antagonist compounds previously listed and
17 cyclopamine at different concentrations (1 nM, 10 nM, 100 nM and 10 μ M) for 24 hours (Figure 5).
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21 DAPI staining demonstrated that the exposure of MNNG cells to MRT61, MRT89 and MRT94
22 induced apoptosis in cells; indeed, cells showed evident deep blue fluorescent nuclei, due to an
23 increase of the permeability of the membrane to the dye, with abnormal margins and condensed
24 chromatin in comparison to the control (Figure 5, panel A and B). MRT89 at the highest
25 concentration (10 μ M) induced the highest toxicity in MNNG OS cells (Figure 5, panel N).
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32 **SMO antagonists induce apoptosis in MNNG OS cells in a dose-dependent manner**

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34 To assess apoptosis, an Annexin V/PI assay was performed in MNNG cells treated with MRT61,
35 MRT89 and MRT94 at various concentrations (1 nM, 10 nM, 100 nM and 10 μ M) for 24h. The
36 results revealed that a marked dose-dependent induction of apoptosis occurred after 24h of
37 treatment with all anti-SMO compounds (Figure 6 and Table 1 SM). At the lower concentration
38 tested, MRT-61, MRT-94 and cyclopamine induced a marked increase of apoptosis (51.11 %,
39 53.33 % and 54.67 % respectively), whereas only a 26.44 % of apoptotic cells were detected after
40 treatment with 1 nM MRT89. In particular, the percentage of early apoptosis (AnV+/PI-) was more
41 elevated in MNNG cells treated with MRT61 ($32.00 \pm 2.85\%$) and MRT94 ($30.11 \pm 2.04\%$) than in
42 MRT-89 and cyclopamine treated cells ($4.67 \pm 1.33\%$ and $14.22 \pm 1.95\%$, respectively). On the
43 contrary, cells treated with 1 nM cyclopamine at the same concentration showed a marked increase
44 of late apoptosis (AnV+/PI+, $28.00 \pm 2.40\%$) and necrosis (AnV-/PI+, $12.44 \pm 1.95\%$), confirming
45 the data obtained in MTT assay (Figure 6 and Table 1 SM).
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Discussion

The role of Hh pathway in OS has not been investigated extensively. Hirotsu et al. (Hirotsu et al., 2010) have demonstrated that Hh pathway was activated in OS and that the inhibition of the 7-transmembrane receptor SMO by cyclopamine, the most commonly used SMO-antagonist, decreased the growth of OS cells *in vitro*. Other studies have shown that the SMO receptor itself should be the best target for novel cancer therapies providing a way to interfere with tumorigenesis and tumor progression (Tremblay et al., 2009b).

In this study, the effect of the steroidal alkaloid cyclopamine and of five new SMO antagonists (MRT61, MRT86, MRT89, MRT92 and MRT94) has been tested on MNNG OS cell line, chosen for the oncogenic potential and the elevated propensity to give lung metastasis.

We demonstrated that Hh signaling pathway was activated in MNNG cells (Figure 1), being the expression of the transcriptional factors GLI1 and GLI2 significantly greater than in normal osteoblast and accordingly to Yang et al. (Yang et al., 2013).

Among the five SMO-antagonist molecules tested in this study, only MRT86 (LD50=4 μ M) and MRT89 (LD50=1 μ M) revealed a cytotoxic activity on MNNG cells, whereas three out of five compounds showed a good proliferation inhibitory effect: MRT61 (IC50 = 5 nM), MRT89 (IC50 = 120 nM) and MRT94 (IC50 = 50 nM) (Figures 2 and 3, Table 1). Based on the results obtained, we decided to select three compounds with the best features for further experiments, namely MRT61, MRT89 and MRT94.

One of the major disadvantages of chemotherapy is the toxic action that affects both cancer and healthy tissues; for this reason, the fact that the three SMO-antagonist compounds selected showed a low toxicity against MNNG cells but, at the same time, slowed down their growth *in vitro* was of particular importance. In addition, the huge difference observed between cytotoxic and anti-proliferative activity has suggested a specific and target-mediated action, being cytotoxicity an adverse and off-target effect (Chan et al., 2013). Finally, since Hh pathway regulates endochondral ossification, coordinating chondrocytes and osteoblasts differentiation and proliferation and OS tends to form in areas of rapid bone growth or turnover, these drugs could be very important to restore impaired signaling pathway and to convert OS in a less aggressive and more differentiated tumor (Mohseny et al., 2012).

MRT61, MRT89 and MRT94 proved to inhibit the expression of the protein GLI1 in MNNG cells (Figure 4). Recent data reported that GLI1 is the direct transcriptional factor of Hh signaling pathway and that the silencing of the gene encoding for GLI1 in cells of chondrosarcoma stopped

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3 the proliferation and promoted G2/M cell cycle arrest (Sun et al., 2014). We demonstrated a dose-
4 dependent decrease in the expression of GLI1 by all tested compounds. In the overall, our data
5 confirmed that cyclopamine inhibitory effect on OS cell proliferation was probably off-target
6 (Mohseny et al., 2012) while the anti-proliferative activity of MRT61, MRT89 and MRT94 was
7 indeed due to a selective inhibition of Hh pathway. This could be related to the different mechanism
8 of action our compounds in respect to cyclopamine. Acylguanidine and acylthiourea derivatives
9 inactivate SMO directly in the cytoplasm and block its translocation into the primary cilium (Hoch
10 et al., 2015), whereas cyclopamine allow the translocation of the inactive SMO into the primary
11 cilium.

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19 In order to confirm that the inhibitory effect on cell proliferation was due to apoptosis, MNNG
20 nuclear morphology was analysed by means of the fluorescent dye DAPI, and the percentage of
21 apoptosis was calculated with Annexin V/PI Dead Cell assay (Figure 5). The examination of cell
22 morphology can provide important hints regarding the mechanism by which molecules could act,
23 especially for those affecting cell cycle and survival (Chan et al., 2013). MRT89, at the highest
24 concentration tried, induced a strong cytotoxic effect on MNNG cells, noticeable by nuclear
25 membrane destruction and loss of nucleotide material and confirmed by the high percentage of late
26 apoptosis ($26.77 \pm 4.55\%$) and necrosis ($33.89 \pm 3.50\%$), whereas MRT61 and MRT94 clearly did not
27 affect nuclear morphology at any concentrations tested with a greater percentage of cells in early
28 apoptosis ($27.22 \pm 5.67\%$ and $40.56 \pm 5.31\%$ respectively); results obtained let us assume a pro-
29 apoptotic activity on MNNG cells (Figure 6 and Table 2).

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37 In conclusion, we reported the evaluation of novel acylguanidine and acylthiourea derivatives, with
38 SMO antagonist activity, as anti-neoplastic agents towards OS cell line, namely MNNG. These
39 compounds possess an elevated pro-apoptotic activity and an elevated specificity for Hh signalling.

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43 Furthermore, our compounds, unlike other SMO antagonists such as cyclopamine and Vismodegib,
44 are known to bridge the two binding sites of the receptor (the upper and the bottom part of the 7-
45 transmembrane cavity) at the same time, and maintain their inhibitory activity towards the
46 chemoresistant mutated form of SMO (Hoch et al., 2015). These features together with the results
47 obtained in this work make compounds ideal candidates for further investigations.

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52 In this light, understanding the mechanism of action of such SMO inhibitors as well as delineating
53 their pharmacological and functional properties in OS, will allow for further development of new
54 classes of selective therapeutic molecules relevant for bone oncology and physiology.
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Acknowledgments:

The authors thank ITT (Istituto Toscano Tumori) [grant 2008] and Telethon [grant GGP10058].

Conflict of Interest: The authors have no disclosures or other conflicts of interest to report.

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Figure captions

Figure 1. Western blot analysis of GLI1 and GLI2 expression in MNNG OS cell line and human primary osteoblast (HOB). GAPDH was used as a loading control.

Figure 2. MTT assay to measure cell viability in MNNG cell line after treatment with cyclopamine and SMO antagonist compounds (MRT61, MRT86, MRT89, MRT92 and MRT94) at 1nM to 10 μ M concentrations. Results (mean \pm SD) were obtained from three different experiments in triplicate. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to DMSO by one-way ANOVA with post hoc Dunnett test.

Figure 3. Inhibition of MNNG cells proliferation after 24 hours of treatment with cyclopamine and SMO antagonist compounds (MRT61, MRT86, MRT89, MRT92 and MRT94) was determined using the CyQuant cell proliferation assay. Results (mean \pm SD) were obtained from three different experiments in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to DMSO by one-way ANOVA with post hoc Dunnett test.

Figure 4. (A, C, E, G) Graphic representation of relative expression of GLI1 normalized to GAPDH in MNNG cells in response to treatment with cyclopamine, MRT61, MRT89 and MRT94 at different concentrations (1nM, 10nM, 100nM, 10 μ M) for 24 hours. Results (mean \pm SD) were obtained from three different experiments in triplicate. ** $p < 0.01$, **** $p < 0.0001$ compared to DMSO by one-way ANOVA with post hoc Dunnett test. **(B, D, F, H)** Western blot analysis of GLI1 expression in MNNG cells in response to treatment with cyclopamine, MRT61, MRT89 and MRT94 at different concentrations (1nM, 10nM, 100nM, 10 μ M) for 24 hours. GAPDH was used as a loading control.

Figure 5. Cell morphology and DNA damage assessment by DAPI staining in MNNG OS cells treated with DMSO (control, A, B) or Cyclopamine (C, D, E, F), MRT61 (G, H, I, J), MRT89 (K, L, M, N) and MRT94 (O, P, Q, R) at various concentrations for 24 hours. Cells are shown at x40 magnification.

Figure 6. Apoptosis assessment using Annexin V-FITC/PI staining and fluorescent microscopy of MNNG OS cells treated with Cyclopamine, MRT61, MRT89 and MRT94 at various concentrations for 24 hours. Histograms show the percentage of non-apoptotic (AnV-/PI-), early apoptotic (AnV+/PI-), late apoptotic (AnV+/PI+) or necrotic (AnV-/PI+) cells in respect to control. Results (mean \pm SD) were obtained from three different experiments in triplicate. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to DMSO by one-way ANOVA with post hoc Dunnett test.

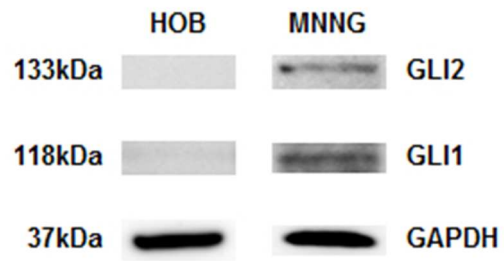


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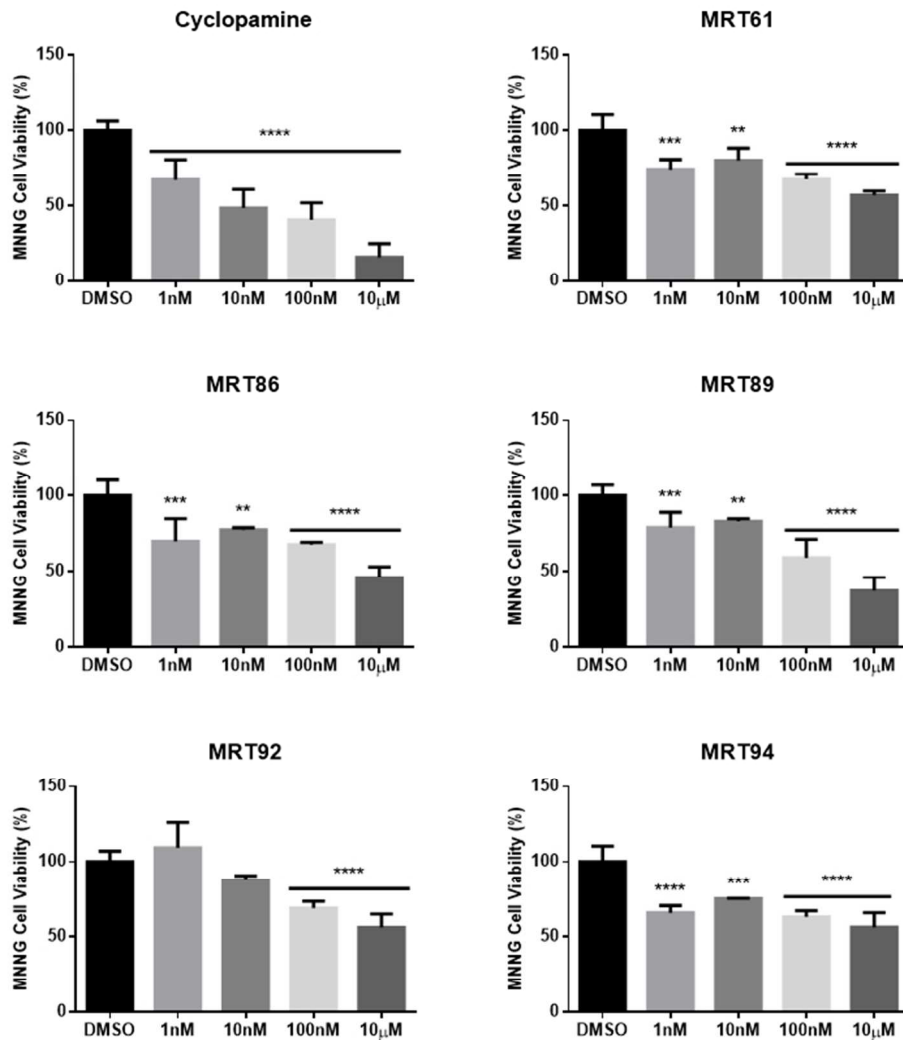


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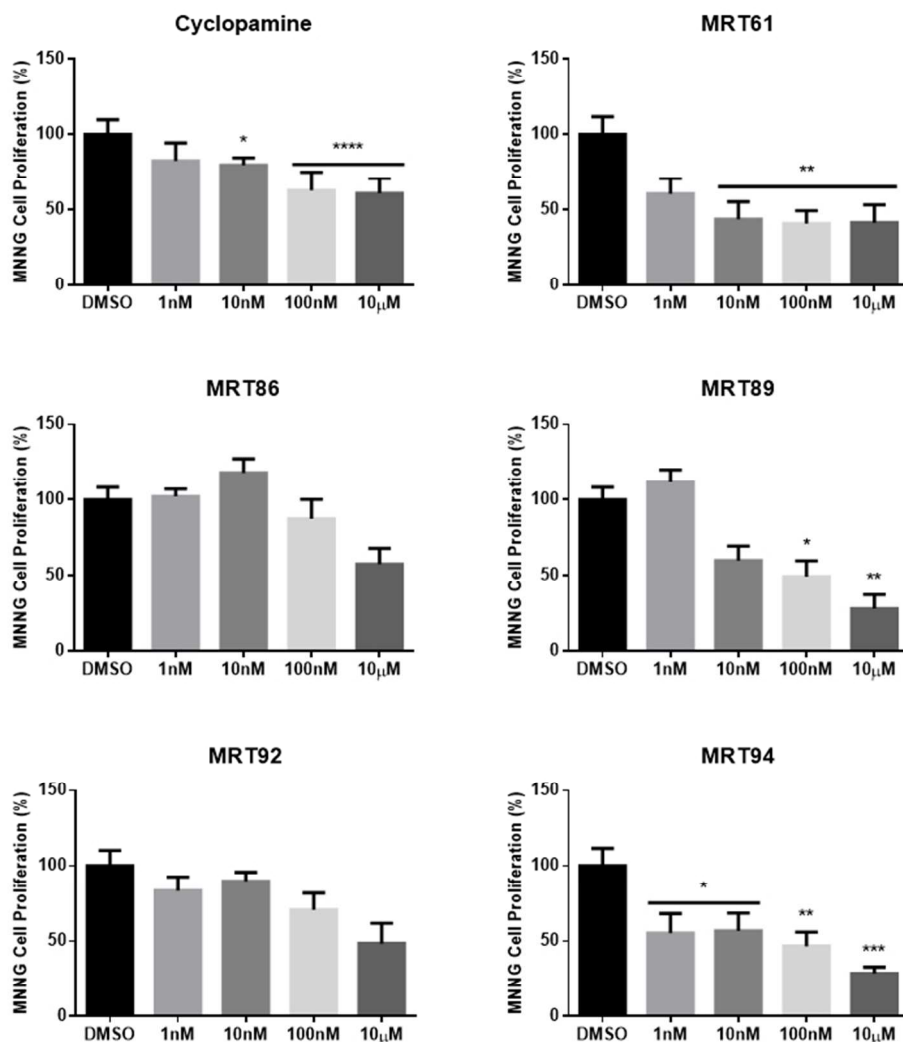


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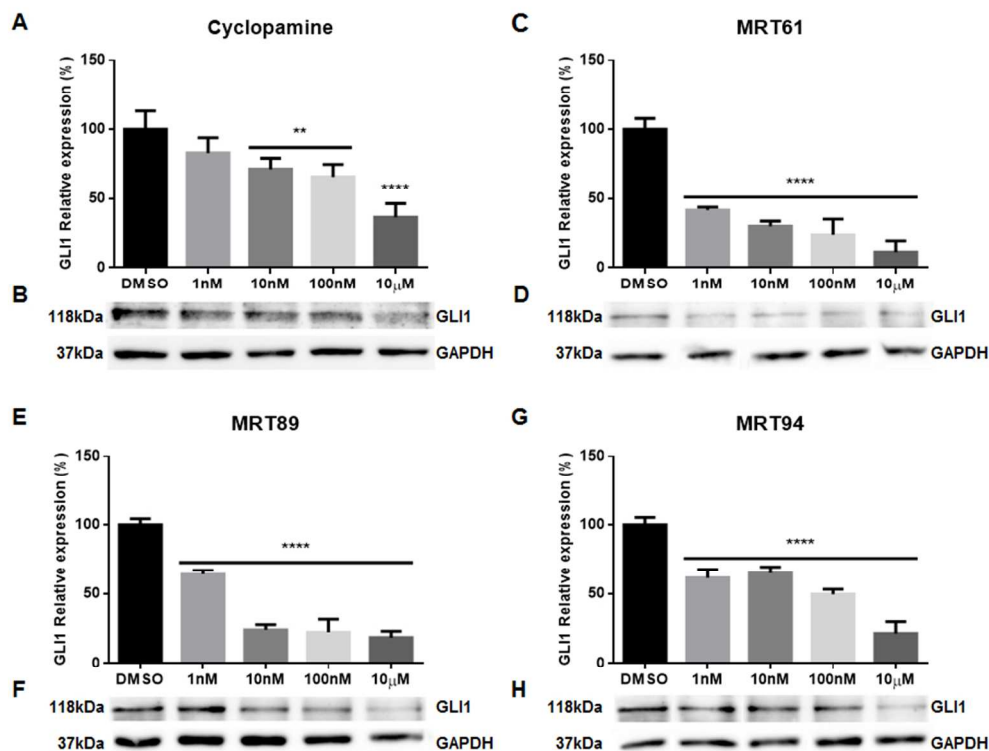


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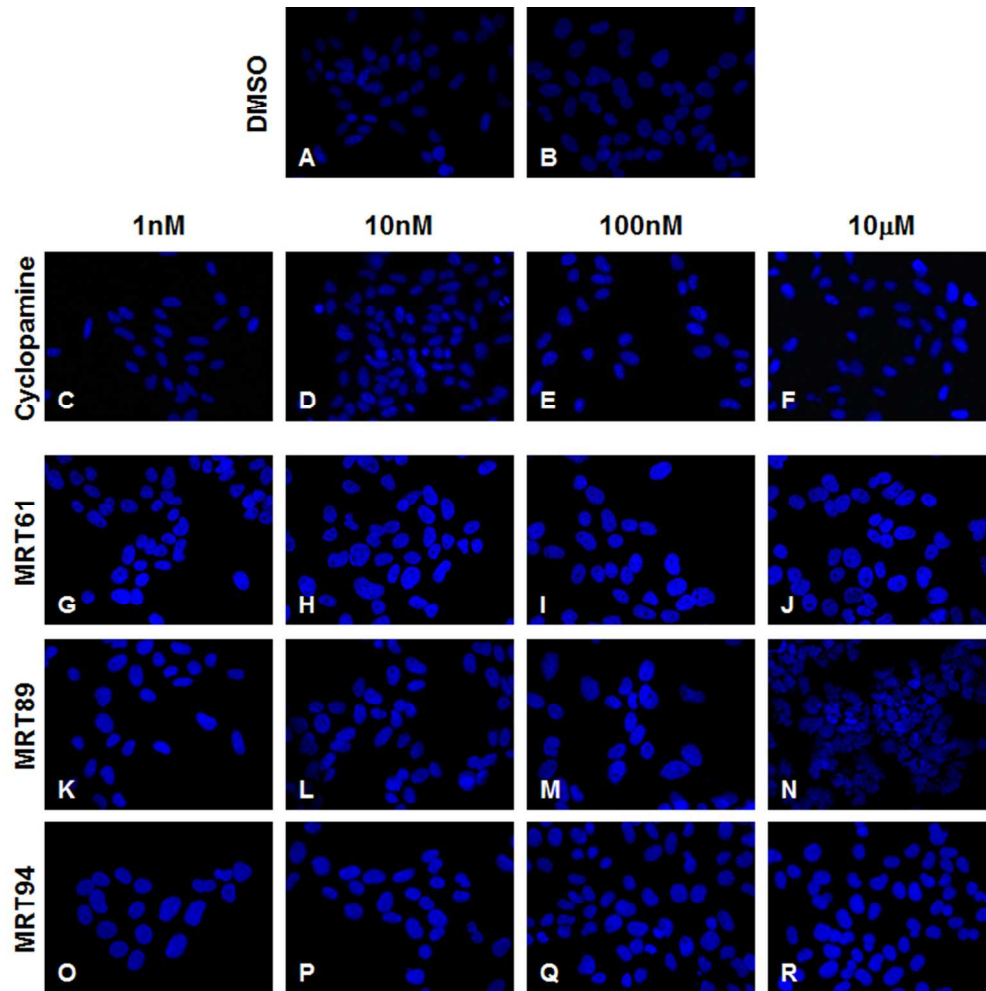


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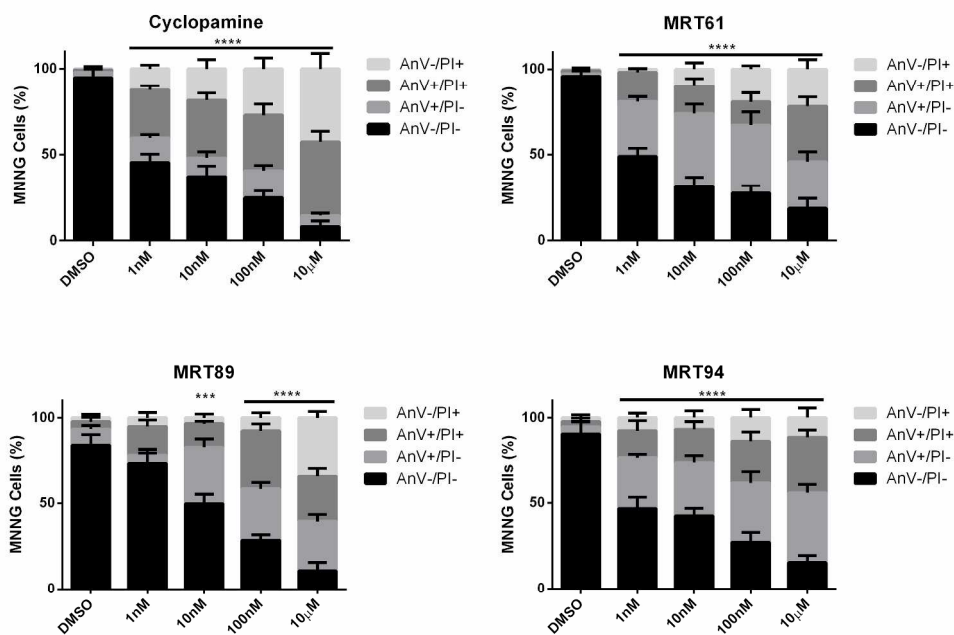
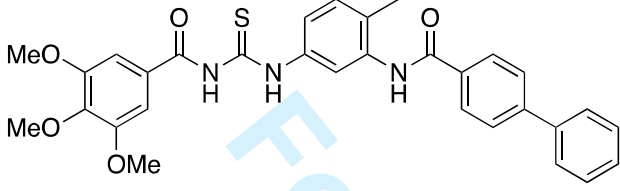
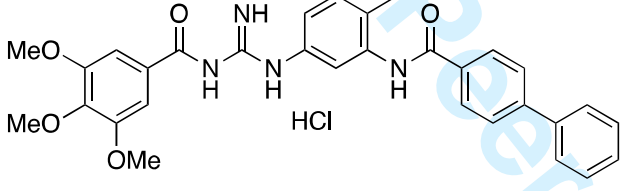
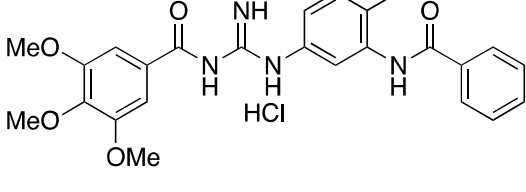
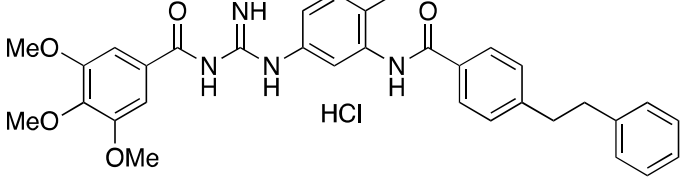
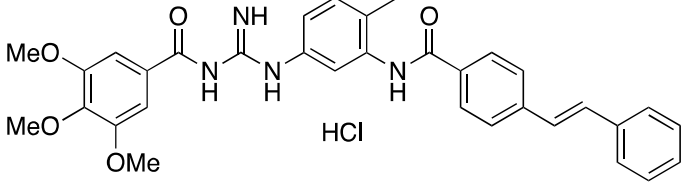


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Table 1. LD₅₀ and IC₅₀ values of Cyclopamine and anti-SMO compounds (MRT61, MRT86, MRT89, MRT92 and MRT94) at 24 hours of treatment on MNNG cells. ND not detectable. Letaal Dose 50 (LD₅₀) and Inhibitory Concentration 50 (IC₅₀) values were calculated with *Origin®-Data Analysis and Graphing Software* using a sigmoidal curve-fitting

Drug	LD ₅₀	IC ₅₀
Cyclopamine	11 nM	ND
 <p data-bbox="256 737 1015 829">MRT61-N-(2-Methyl-5-(3-(3,4,5-trimethoxybenzoyl)thioureido)phenyl)biphenyl-3-carboxamide</p>	ND	5 nM
 <p data-bbox="256 1052 1079 1144">MRT86-N-(2-Methyl-5-(3-(3,4,5-trimethoxybenzoyl)guanidino)phenyl)biphenyl-4-carboxamide, HCl</p>	4 μM	ND
 <p data-bbox="256 1356 1023 1449">MRT89-N-(N-(3-Benzamido-4-methylphenyl)carbamimidoyl)-3,4,5-trimethoxybenzamide, HCl</p>	1 μM	120 nM
 <p data-bbox="256 1671 998 1764">MRT92-3,4,5-trimethoxy-N-(N-(4-methyl-3-(4-phenethylbenzamido)phenyl)carbamimidoyl)benzamide, HCl</p>	ND	7 μM

 <p data-bbox="256 388 933 472">MRT94-(E)3,4,5-trimethoxy-N-(N-(4-methyl-3-(4-styrylbenzamido)phenyl)carbamimidoyl) benzamide, HCl</p>	ND	50 nM
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