L'ATX-101, UN PEPTIDE CHE BERSAGLIA PCNA, POSSIEDE ATTIVITA' ANTITUMORALE E RADIOSENSIBILIZZANTE IN MODELLI MURINI GI GLIOBLASTOMA MULTIFORME UMANO

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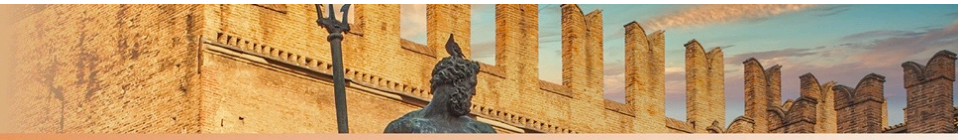


DICHIARAZIONE

Relatore: Prof. Giovanni Luca Gravina

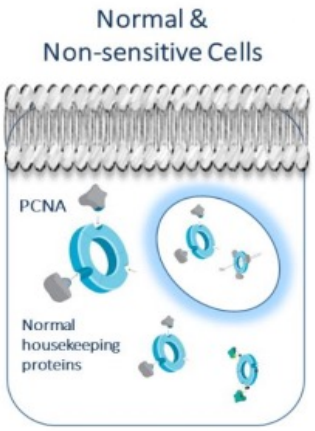
Come da nuova regolamentazione della Commissione Nazionale per la Formazione Continua del Ministero della Salute, è richiesta la trasparenza delle fonti di finanziamento e dei rapporti con soggetti portatori di interessi commerciali in campo sanitario.

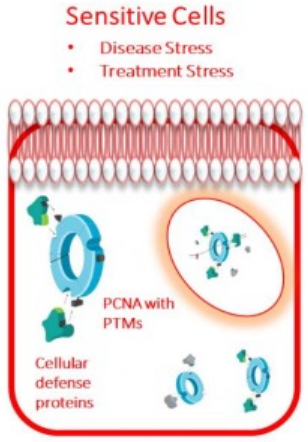
- Posizione di dipendente in aziende con interessi commerciali in campo sanitario (NIENTE DA DICHIARARE)
- Consulenza ad aziende con interessi commerciali in campo sanitario (NIENTE DA DICHIARARE)
- Fondi per la ricerca da aziende con interessi commerciali in campo sanitario (NIENTE DA DICHIARARE)
- Partecipazione ad Advisory Board (NIENTE DA DICHIARARE)
- Titolarità di brevetti in compartecipazione ad aziende con interessi commerciali in campo sanitario (NIENTE DA DICHIARARE)
- Partecipazioni azionarie in aziende con interessi commerciali in campo sanitario (NIENTE DA DICHIARARE)
- Altro

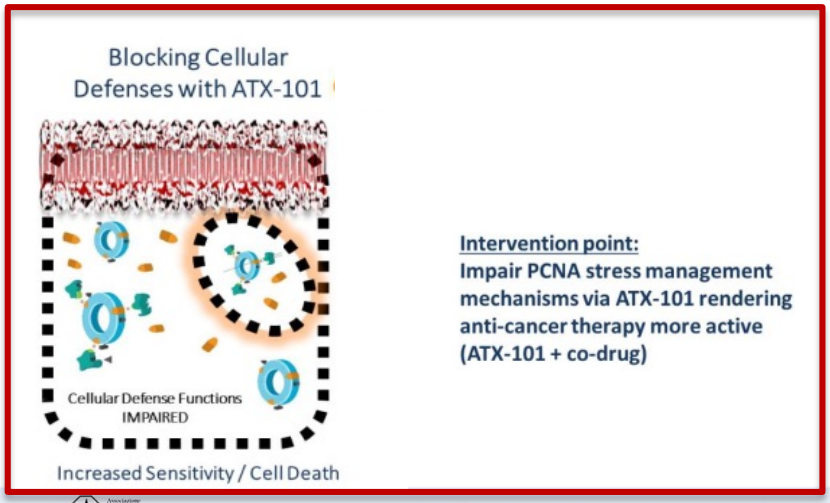
Background

Proliferating cell nuclear antigen (PCNA) and ATX-101

- PCNA coordinates the DNA replication machinery with key cellular functions.
- Two binding motifs interact with PCNA:
  1. PIP-box: under normal conditions.
  2. APIM: during stress e.g. upon treatment with anti-cancer therapy.
  .
- ATX-101, a PCNA-APIM-Protein interaction blocking peptide:
  1. impairs PCNA scaffold functions
  2. affects several cellular mechanisms involved in stress management.
  3. leads to decreased survival and enhancement of the action of multiple anti-cancer drugs.







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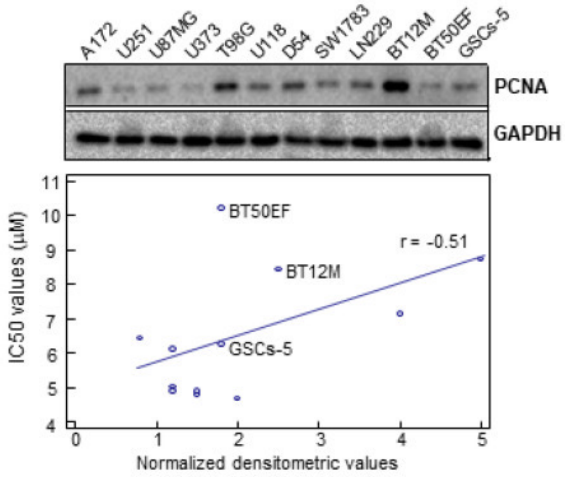
Cell line	IC50 (μM) ± SD
GBM cell lines	
U87MG	4.3 ± 0.3
U251	4.9 ± 0.3
A172	6.4 ± 0.4
T98G	7.1 ± 0.2
U373	5.0 ± 0.2
U118	6.8 ± 0.2
D54	8.4 ± 0.1
SW1783	4.8 ± 0.2
LN229	4.7 ± 0.2
U138	7.5 ± 0.3
SF268	4.8 ± 0.1
SNB19	12.0 ± 0.6
Patient derived GICs	
BT12M	8.7 ± 0.5
BT48EF	6.1 ± 0.2
BT50EF	10.2 ± 0.7
GSCs-5	4.9 ± 0.2
GSCs-7	6.3 ± 0.2

C



ATX-101 inhibits cell proliferation of GBM and GICs cells in vitro

(**A**) Twelve GBM cell lines and 5 GICs were examined for viability after treatment with ATX-101 (0.1–10 μM). IC50 values are calculated for 12 GBM cell lines and 5 GICs at 72 h.

(**C**) Upper panel: Western blots showing PCNA and GAPDH expression levels in 9 GBM and 3 GICs cell lines; however, linear regression of PCNA levels plotted against IC50s did not suggest a correlation between PCNA levels and ATX-101 sensitivity (**Figure 1**C, r = –0.51)).

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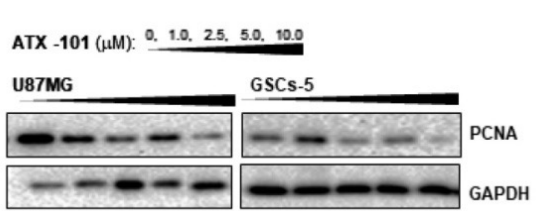

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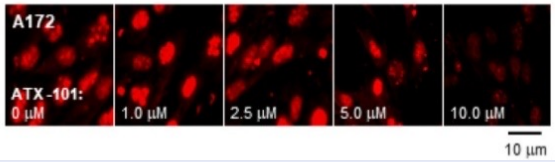
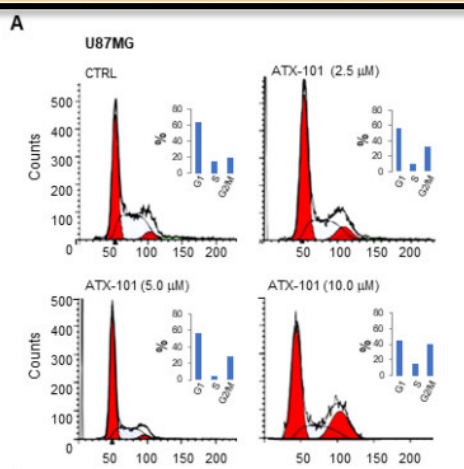
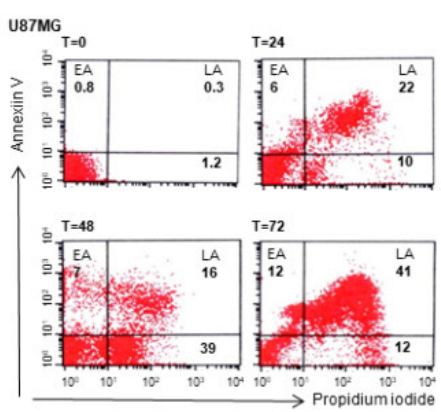


ATX-101 inhibits total PCNA expression in GBM cell models

(A) Representative Western blots performed on cell lysates collected from U87MG and GSCs-5 cells treated with ATX-101 (1–10 μM) for 24 h. ATX-101 dose dependently inhibited the expression of total PCNA protein at doses below IC50 values in all the cell lines examined

(**C**) Immunofluorescence staining confirms a reduction of PCNA levels in both the cytoplasm and in the nucleus upon ATX-101 treatment

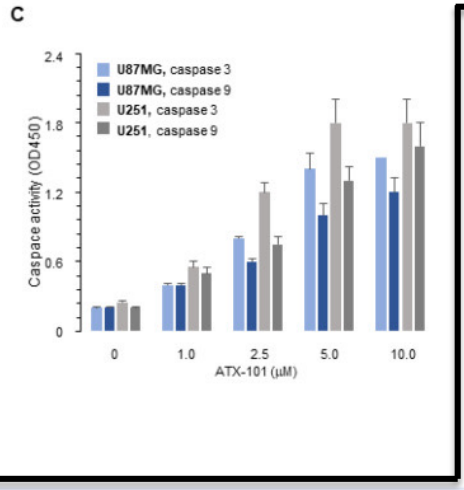


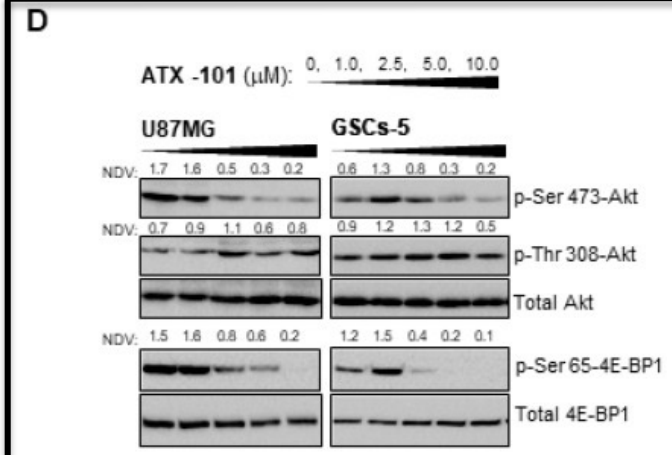


ATX-101 induces G2/M cell arrest and mediates apoptosis.

(**A**) Cell cycle analysis of U87MG cells after treatment with ATX-101 (2.5–10 μM) for 24 h. **ATX-101 treatment was found to affect the cell cycle distribution, i.e., an increased number of cells in S and G2/M** were found in the four cell lines U87MG, U251, A172, and T98G

(**C**) Enzymatic caspase 9 and 3 activity and Annexin V and necrosis (by propidium iodide) FACS analyses in U87MG and U251 cells treated with ATX-101 (2.5–10 μM) for 24 h. **ATX-101 treatment showed that ATX-101 efficiently induced caspase-3/9-dependent apoptosis**





ATX-101 inhibits Akt activity in glioma cells.

(**D**) **ATX-101** dose dependently **inhibited the phosphorylation of Ser 473 and Thr 308 in Akt and** Phosphorylation in Ser 65 in the eukaryotic translation initiation factor 4E-binding protein 1 (**p-Ser 65-4E-BP1), a member of the family of translation repressor proteins and a well-known substrate of mechanistic target of rapamycin (mTOR).**

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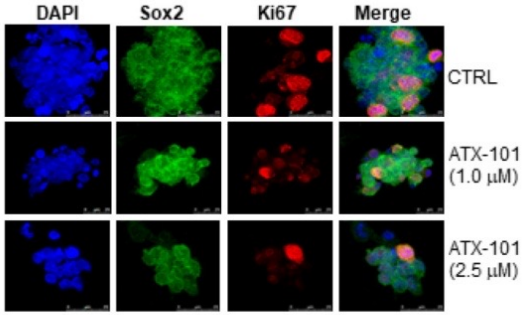
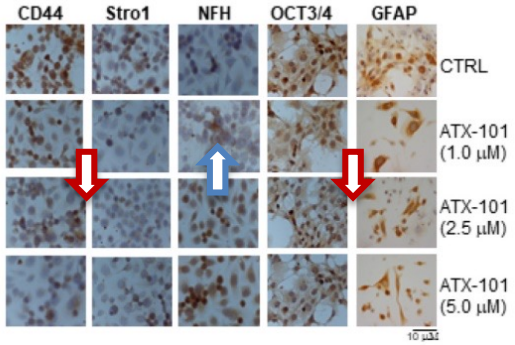


Marker	CTRL	ATX-101 1.0 µM	ATX-101 2.5 µM	ATX-101 5.0 µM
CD44	71.8 %	31.6 %	26.9 %	19.0 %
CD90	70.0%	28.9 %	26.9 %	1.6 %
GAP43	1.7 %	2.3 %	2.3%	1.4 %
βIII tubulin	3.5%	2.5 %	6.7%	5.7 %

C



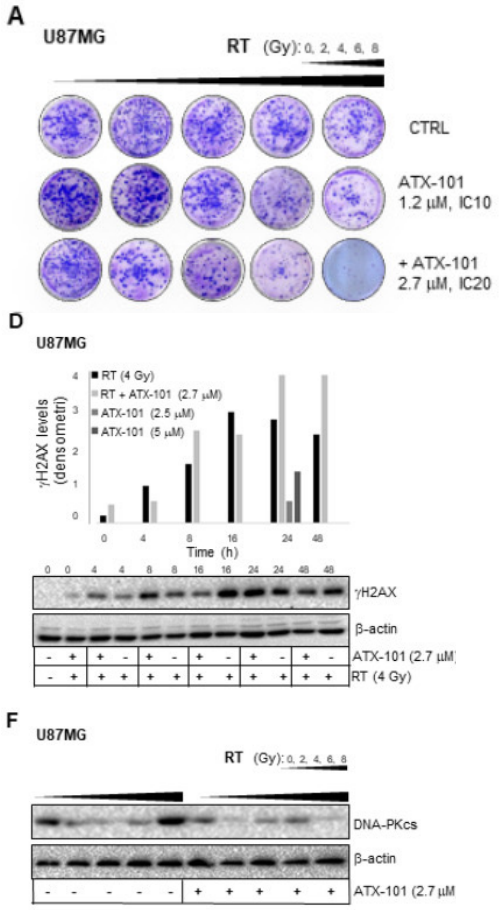
ATX-101 inhibits stemness phenotype and induces a reversion of Neural/proneural to mesenchymal phenotype.

It has been demonstrated that the activity of Akt/TORC1 pathways modulates the stemness of several cancer stem cells

(**A**) *Confocal analyses* of Ki67- and Sox2-stained GSCs-5 cells treated with ATX-101 (1.0 and 2.5 µM) for 48 h. **ATX-101 reduced the expression of Ki67 and Sox2 in GSCs-5 cells, thus reducing both cell proliferation and stemness**

(**B**) FACS analyses for mesenchymal markers CD44 and CD90 in GSCs-5 cells after treatment with ATX-101 (1.0, 2.5, and 5 µM) for 48 h. **FACS analysis showed that the percentage of mesenchymal markers, CD44 and CD90, was reduced by ATX-101 treatment**

(**C**) ICC analyses. These analyses showed **that the expression of mesenchymal and stem cell markers (CD44, Stro1, OCT3/4 and GFAP) was dose-dependently reduced by ATX-101 treatment, while NFH was significantly increased** and that fewer cells were stained for GFAP



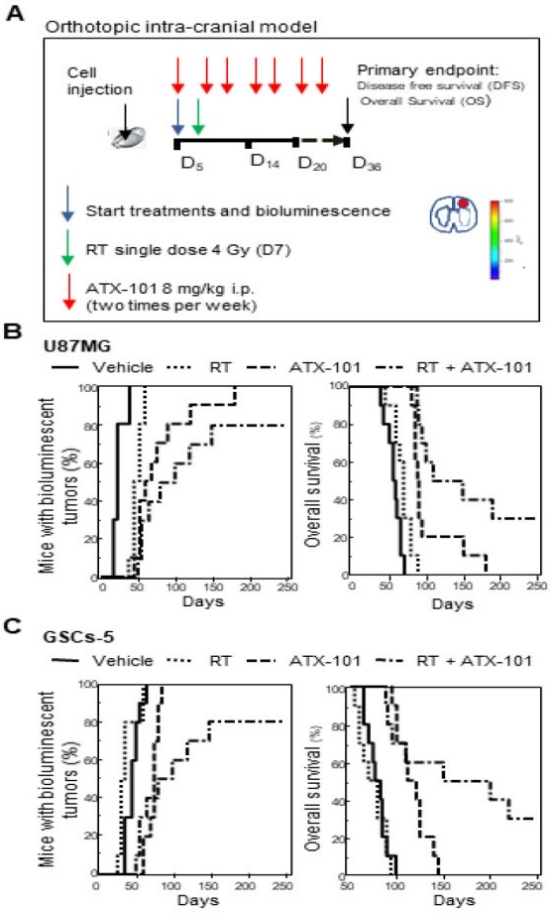
ATX-101 has radiosensitizing effects.

Multiple proteins involved in DNA repair and damage tolerance must interact with PCNA in order to be fully active and ATX-101 is shown to increase the activity of multiple DNA-damaging agents

(**A**) Representative images of crystal-violet-stained U87MG colonies (clonogenic assay) treated with ATX-101 IC10 (1.2 µM) and IC20 (2.7 µM) in combination with RT (2, 4, 6, and 8 Gy). **ATX-101 enhanced the effect of RT in U87MG cell line**

(**D**) Western blots showing γH2AX levels in U87MG treated with RT (4 Gy) alone or in combination with ATX-101 IC20 (2.7 µM) for 0–48 h (lower panel). **An increased level of γH2AX was found in the combination-treated cells (ATX-101 + RT) after 24 h than RT or ATX-101 treatments suggesting increased levels of DNA double-strand breaks (DSBs)**

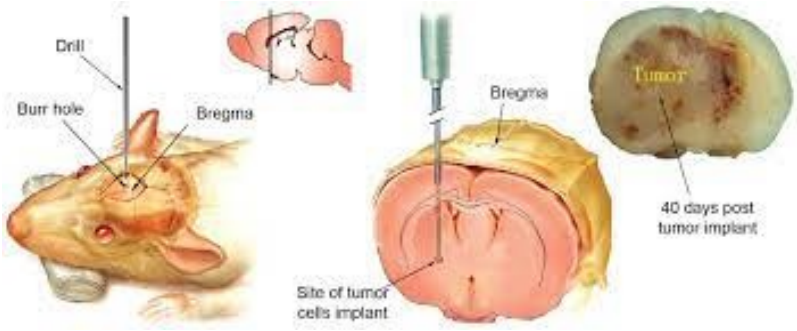
(**F**) Western blots showing the levels of activated DNA-PKcs in U87MG cells treated with ATX-101 IC20 (2.7 µM) in combination with RT (2, 4, 6, and 8 Gy) for 24 h. **ATX-101 reduced activation of DNA-PKcs**. Because **DNA-PKcs is required for the nonhomologous end-joining (NHEJ) pathway of DSBs repair, this suggests that ATX-101 reduces these cells' ability to repair DSBs**

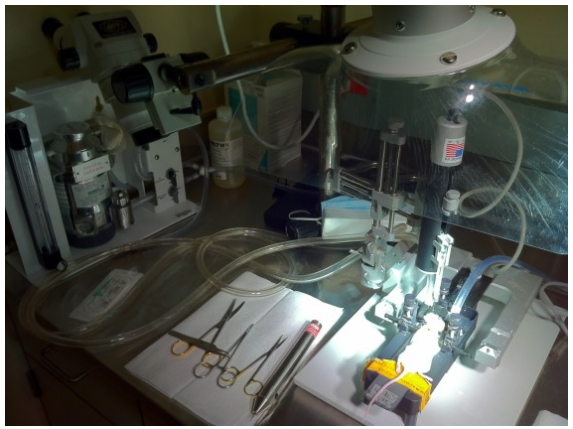


ATX-101 reduces the growth of intrabrain U87MG and GSCs-5 tumors.

(**A**) Treatment scheme: 10 animals in each group received intrabrain injection of 1 × 10^3 luciferase-tagged U87MG or GSCs-5 cells. Vehicle or ATX-101 (8 mg/kg) were administered intraperitoneally (i.p.) two times per week (D5, D8, D12, D15, D19, D22, D26, D29, and D33); radiotherapy (RT) (4 Gy) was given as a single administration on D7. Primary endpoint was set at D36, but the mice were followed for up to 250 days.

(**B**,**C**) Kaplan–Meier curves of percentage of mice with bioluminescent tumors, i.e., mice with progression, and overall survival, U87MG (**B**), and GSCs-5 (**C**).





Conclusions

In this report, we demonstrated that;

1. ATX-101 has good activity against GBM in both in vitro and in vivo experiments.

1. ATX-101 was shown to reduce tumor growth in several animal models, including an intracranial tumor model, and potentiate the efficacy of RT.

2. The mode of action of ATX-101 was verified to include dysregulation of cellular signaling and apoptosis both in the absence and presence of DNA damage via inhibition of both nuclear and cytosolic roles of PCNA.

3. These results warrant further studies of ATX-101 for use in GBM therapies