RATIONALLY DESIGNED SEMI-SYNTHETIC ANALOGUES OF OLEUROPEIN EXHIBIT IMPROVED ANTICANCER ACTIVITY

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Oleuropein-rich products of *Olea europaea* are well-known for their beneficial anticancer activity. In this study, we designed and synthesized a series of novel semisynthetic analogues of oleuropein and evaluated their antitumor properties *in vitro* and preclinically. Twenty-two semisynthetic oleuropein analogues were tested against various human cancer cell lines using the MTT assay and their half-maximal inhibitory concentration (IC_{50}) was determined. To evaluate their cytotoxicity against normal cells, healthy donor-derived peripheral blood mononuclear cells (PBMCs) were used. The mode of action of the most active compounds was investigated by flow cytometry (FC), as for the type of cell death induced, their cytostatic effects and cell cycle alterations, using Annexin V/PI, CFSE and PI staining, respectively. Immunocompetent BALB/c and C57BL/6J mice bearing CT26 colon cancer and B16.F1 melanoma tumors, respectively, were administered 8 doses (1-100 μg/dose) of the most active analogue (GS32) every other day for 15 days. Tumor growth was monitored for 29 days. Immunohistochemical (IHC) analyses were performed on tumor sections, using antibodies against immune-cell markers (CD3, CD4, CD8, Mac-3). Spleen cells from treated mice were isolated, co-incubated with the syngeneic B16.F1 or CT26, YAC-1 (NK-sensitive) and WEHI-164 (LAK-sensitive) mouse cell lines, and the expression of CD107 (degranulation marker) was quantified *ex vivo* via FC. The initial screening highlighted two analogues, GS32 and GS36, with significant differential antitumor activity (IC_{50} values 0.17-2.54 and 10-19 μM, respectively). Both analogues showed marginal toxicity against PBMCs, induced cancer cell apoptosis, exhibited high cytostaticity and inhibited cell cycle progression at the phase of DNA synthesis. In both therapeutic mouse models, administration of GS32 retarded tumor growth in a dose-dependent manner. IHC revealed higher infiltration of the tumors by $CD3^+$, $CD4^+$ and $CD8^+$ T cells in treated animals compared to controls. The higher expression of CD107 assessed *ex vivo,* indicated that GS32 stimulated the *in vivo* expansion of tumor-reactive T cells, and, to a lesser extent, the cytotoxicity of NK and LAK cells. Overall, oleuropein analogues GS32 and GS36 demonstrated potent and consistent cytotoxicity across all cancer lines tested *in vitro*. Neither analogue was toxic against normal cells. Their anticancer activity can be, at least in part, attributed to their cytostatic properties. *In vivo* administration of GS32 retarded both colon and melanoma tumor growth and prolonged mouse survival*,* likely through the induction of specific (T cell-mediated) and non-specific (NK and LAK cell-mediated) antitumor immune responses. Structure-activity relationship studies verified the importance of the addition of long carbon chains on the scaffold used. The dual

effect of GS32, both on tumor progression and immune-cell stimulation, shows promise for the further design and synthesis of improved small molecules with potent anticancer activity and minimum toxic side effects.