The G protein-coupled estrogen receptor 1 (GPER-1) contributes to the proliferation and survival of mantle cell lymphoma cells

Mantle cell lymphoma (MCL) is an aggressive subtype of B-cell lymphoma with a poor prognosis. In recent years, critical subsets of genes and signaling pathways that are of prognostic relevance have been identified.[1,2] Given that the alternative estrogen receptor, the G protein-coupled receptor 1 (GPER-1) can activate a variety of signaling pathways, such as the PI3K/AKT pathway, which is critical for the pathogenesis of MCL, and that the role of the estrogen receptor in cell growth and survival of lymphoma cells is well documented,[3] we wanted to determine whether GPER-1 might be involved in the pathogenesis of MCL. In the present study, we show that GPER-1 is expressed in the majority of MCL samples and MCL cell lines, and contributes to cell survival and proliferation by regulation of critical signaling pathways. Moreover, inhibition of GPER-1 leads to microtubulus stabilization and is highly synergistic with paclitaxel treatment, opening potential new therapeutic options for the treatment of MCL.

GPER-1 is expressed in a variety of normal tissues. The subcellular localization of GPER-1 can vary, since GPER-1 trafficking from the cell membrane to the nucleus has been well documented.[4] In breast and ovarian cancer, a predominant nuclear staining pattern of GPER-1 has been shown to correlate with poor differentiation, chemotherapy resistance and inferior survival.[5] In human B cells, GPER-1 is expressed in germinal center B cells, as demonstrated in normal tonsils by immunohistochemistry (Figure 1A). To analyze GPER-1 expression in various lymphoma subtypes, we initially studied GPER-1 expression *via* immunohistochemistry in 20 diffuse large B-cell lymphomas, 20 follicular lymphomas (WHO grade 1-2), and 20 MCL. A predominant nuclear expression was observed with only weak cytoplasmic staining in 5% of follicular lymphomas, 30% of diffuse large B-cell lymphomas, and the majority of MCLs (Figure 1A). We then expanded our analysis to MCL tumors from two randomized trials of the German Low-Grade Lymphoma Study Group (GLSG19966 and GLSG20007). For inclusion in this study, all cases were reviewed by experienced hematopathologists (MR, GO, AR) and were required to be Cyclin D1-positive. Patients' characteristics are summarized in *Online Supplementary Table S1*. Of 157 included MCL cases, 60% (n=94) exhibited high nuclear GPER-1 expression with only slight variation in staining intensity (Figure 1B). However, GPER-1 expression in this series did not correlate with the histological subtype (typical/blastoid; P=0.86) or with the proliferation index assessed by Ki-67 staining of the tumor cells (P=0.79). There was also no difference in expression of GPER-1 between male and female patients (P=0.21). Thus, it is unlikely that GPER-1 expression might contribute to the male predominance among MCL patients.

Ten out of 13 MCL cell lines tested expressed GPER-1 at high levels, including REC-1, GRANTA-519, MINO, JEKO, HBL-2, NCEB-1, UPN-1, UPN-2, L128 and Z138C. In contrast, JVM-2, JVM-13 and MAVER showed little or no expression of GPER-1, as assessed by western blot analysis. GPER-1 expression was found to be nuclear and constitutive, as serum deprivation did not alter GPER-1 expression levels (Figure 1C and D).

Since in breast and ovarian cancer GPER-1 can enhance survival and proliferation of tumor cells, we set out to test the functional role of GPER-1 in MCL. To inhibit GPER-1, we used G36, a highly specific inhibitor of the alternative estrogen receptor that does not cross-react with estrogen receptor alpha or beta.[8] Thus, it represents

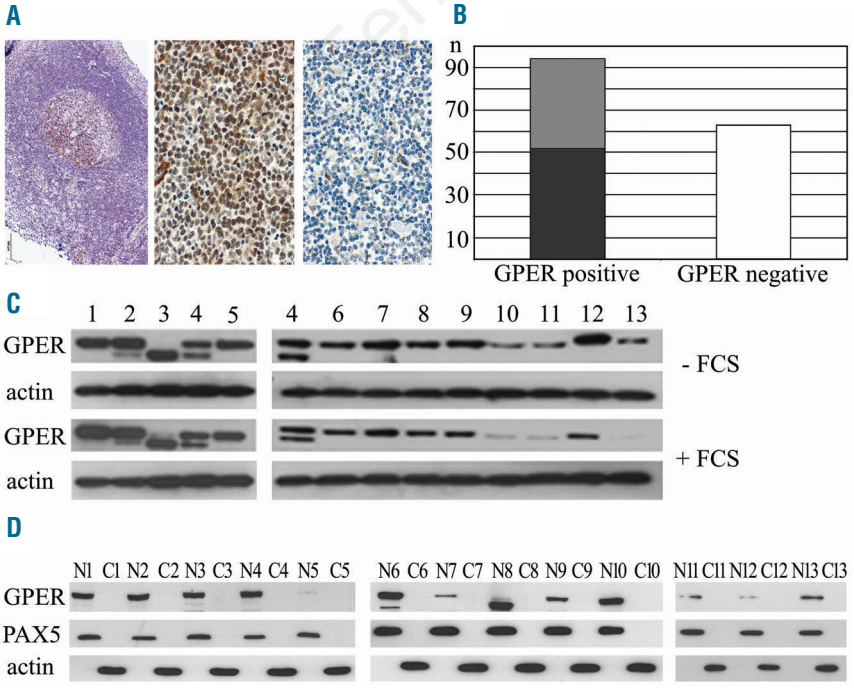

Figure 1. GPER-1 is expressed in the majority of mantle cell lymphoma (MCL) cases and MCL cell lines. (A) Immunohistochemistry of a normal tonsil proves staining of germinal center B cells (left). An example of a GPER-1 positive (middle) and a GPER-1 negative (right) MCL are displayed. (B) The range of GPER-1 positivity is illustrated with 53 MCL showing strong nuclear staining (black column), 41 MCL showing moderate nuclear staining (gray column) and 63 MCL without nuclear staining (white column). (C) The majority of MCL cell lines shows constitutive expression of GPER-1 as analyzed by western blot of cells cultured for 24 h with serum (+FCS) or under serum deprivation (-FCS); (1:REC-1, 2:GRANTA-519, 3:MINO, 4:JEKO, 5:HBL-2, 6:NCEB-1, 7:UPN-1, 8:UPN-2, 9:L128, 10:JVM-2, 11:MAVER, 12:Z138C, 13:JVM-13). (D) GPER-1 expression in MCL cell lines is nuclear as assessed by western blot of the nuclear (N) or cytoplasmic (C) fractions (1:REC-1, 2:GRANTA-519, 3:HBL-2, 4:UPN-1, 5:JVM-2; 6:JEKO, 7:NCEB, 8:MINO, 9:UPN-2, 10:L128, 11:JVM-13, 12:MAVER, 13:Z138C).

a valuable tool to assess the biological function of GPER-1 in MCL cell lines. Inhibition with G36 reduced cell proliferation in MCL cell lines with high GPER-1 expression (REC-1, GRANTA-519, MINO, JEKO, HBL-2, NCEB-1, UPN-1, UPN-2, L128 and Z138C) as assessed by the MTT assay after 48 h showed IC50 values of 1.4-8.9 μM, whereas MCL cell lines with low GPER-1 expression (JVM-2, JVM-13 and MAVER) did not respond to the treatment (Figure 2A). GPER-1 inhibition induced apoptosis in GRANTA-519 and MINO (high GPER-1 expression), but not in MAVER (low GPER-1 expression), as shown by western blot analysis demonstrating cleavage of caspase 3 after 48 h (Figure 2B). We then validated our results of the inhibition experiments with siRNA-mediated knockdown of GPER-1 to rule out non-specific side-effects of G36 and we observed similar effects after 24 and 48 h following transfection (Figure 2C).

Given that the constitutive activation of AKT and MAPK has been reported in MCL,[2,9,10] and that the AKT and MAPK pathways are well-established downstream targets of GPER-1, we determined whether the activation of these pathways is dependent upon GPER-1 activity. Therefore, we analyzed the specific downstream targets after G36 inhibition or functional knockdown by western blot analysis. We show that GPER-1 expression correlates with AKT and MAPK phosphorylation as well as Cyclin D1 expression. Moreover, the G36-mediated inhibition of GPER-1 or functional knockdown of GPER-1 reduced or abrogated the phosphorylation of the two kinases in MINO and GRANTA-519 after 1 h of treatment or 24 h knockdown (Figure 2B and C). Interestingly, GPER-1 inhibition or downregulation also reduced the expression level of Cyclin D1. This result is particularly interesting, given that Cyclin D1 overexpression *via* the t(11;14)(q13;q32) translocation is one of the major characteristic oncogenic events in MCL. Reduced Cyclin D1 expression following GPER-1 downregulation can be explained by the inhibition of the AKT/mTOR pathway, shown by reduction of phosphorylation of AKT, as well as p70S6K and the dephosphorylation, and thereby reactivation of GSK-3β. Active GSK3-β can then phosphorylate Cyclin D1 and thereby target Cyclin D1 for degrada-

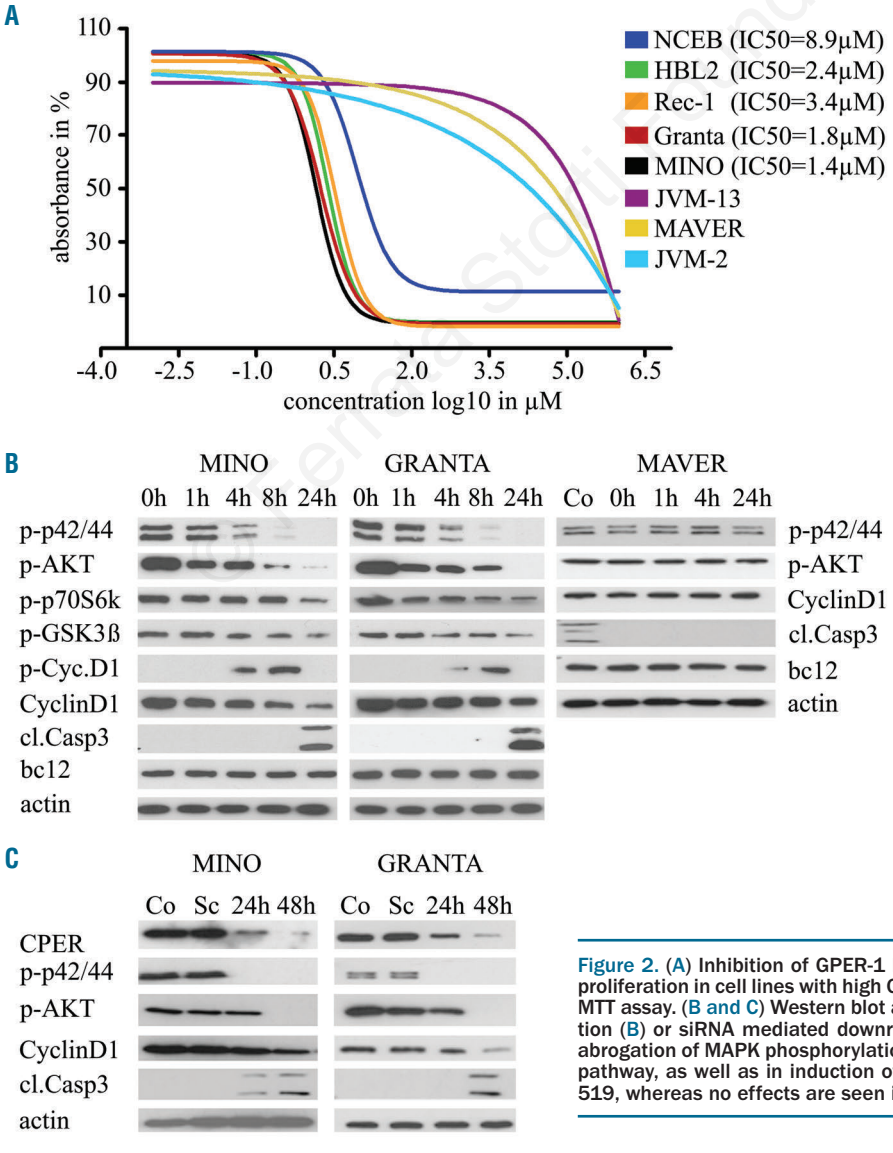

Figure 2. (A) Inhibition of GPER-1 by G36 results in reduction of cell proliferation in cell lines with high GPER-1 expression as shown by the MTT assay. (B and C) Western blot analysis demonstrating that inhibition (B) or siRNA mediated downregulation (C) of GPER-1 results in abrogation of MAPK phosphorylation and inhibition of the AKT/mTOR pathway, as well as in induction of apoptosis in MINO and GRANTA-519, whereas no effects are seen in MAVER.

tion[11] (Figure 2B and C). The expression of BCL-2, a further downstream target of GPER-1, was independent of GPER-1 expression and activity, which is not surprising because BCL-2 is constitutively over-expressed in MCL.

It has been well established that G protein-coupled receptor-associated G protein (Gα) binds to tubulin with high affinity, thereby modulating microtubulus dynamics,[12] and *in vivo* GPER-1 activation results in microtubulus destabilization in filopodial structures.[13] Therefore, we tested whether GPER-1 increases microtubulus dynamics in MCL cell lines. We incubated two MCL cell lines (MINO and GRANTA-519) with G36 (GPER-1 inhibitor), G1 (GPER-1 agonist) or vincristine separately and visualized the microtubulus apparatus using immunofluorescence after 48 h. After treatment with G36, well-defined microtubulus apparatuses were observed (Figure 3A1). However, the microtubules had clearly shrunk after activation of GPER-1 by G1 or after treatment with vincristine at cytotoxic doses (Figure 3A2 and A3) demonstrating that inhibition of GPER-1 clearly reduces microtubulus dynamics and leads to microtubulus stabilization.

Microtubules, the major components of the mitotic spindle, are a target of numerous anti-cancer drugs. Cells with increased microtubulus dynamics are more resistant to polymer-binding drugs, such as paclitaxel, and, on the other hand, more sensitive to dimer-specific drugs, such as vincristine.[14,15] Interestingly, this is also the case for MCL. As inhibition of GPER-1 could stabilize the microtubules, we wanted to test if inhibition of GPER-1 could potentially sensitize cells to paclitaxel treatment. To determine whether GPER-1 inhibition in MCL potentiates the effects of paclitaxel, we combined GPER-1 inhibition (G36) with paclitaxel treatment. After 48 h, cell viability and proliferation were assessed in two cell lines (MINO and Granta-519) using the MTT assay, and combination index (CI) values were calculated. A strong synergistic effect between G36 and paclitaxel was observed in both cell lines with CI values less than 0.7, as shown in Figure 3B (ED50, ED75, ED90, and average CI).

Given the functional role of GPER-1 in MCL cell lines, and the highly synergistic effect of combination of GPER-1 inhibition and paclitaxel, we wanted to assess if GPER-1 expression is of prognostic value in MCL. We analyzed 72 patients of the GLSG cohorts for event-free and overall survival (OS). Of the 72 included patients, 65 had a treatment failure and 58 died, resulting in a 5-year failure-free survival of 14% and a 5-year OS of 41%. No significant differences were noted in OS (median 3.8 *vs.* 4.4 years; *P*=0.83) or event-free survival (median 1.4 *vs.* 1.5 years; *P*=0.7) between the GPER-1-positive or -negative cases (Figure 3C and D). Although this result might appear disappointing, it does not negate the hypothesis that inhibition of GPER-1 and paclitaxel treatment could be beneficial in a subset of MCL patients, since this pathway is not affected by current treatment approaches.

In conclusion, we demonstrate GPER-1 expression and activation in the majority of MCL tumors and MCL cell lines. The functional knockdown of GPER-1 inhibited cell proliferation, induced apoptosis, and stabilized microtubules. Importantly, the combination of GPER-1 inhibi-

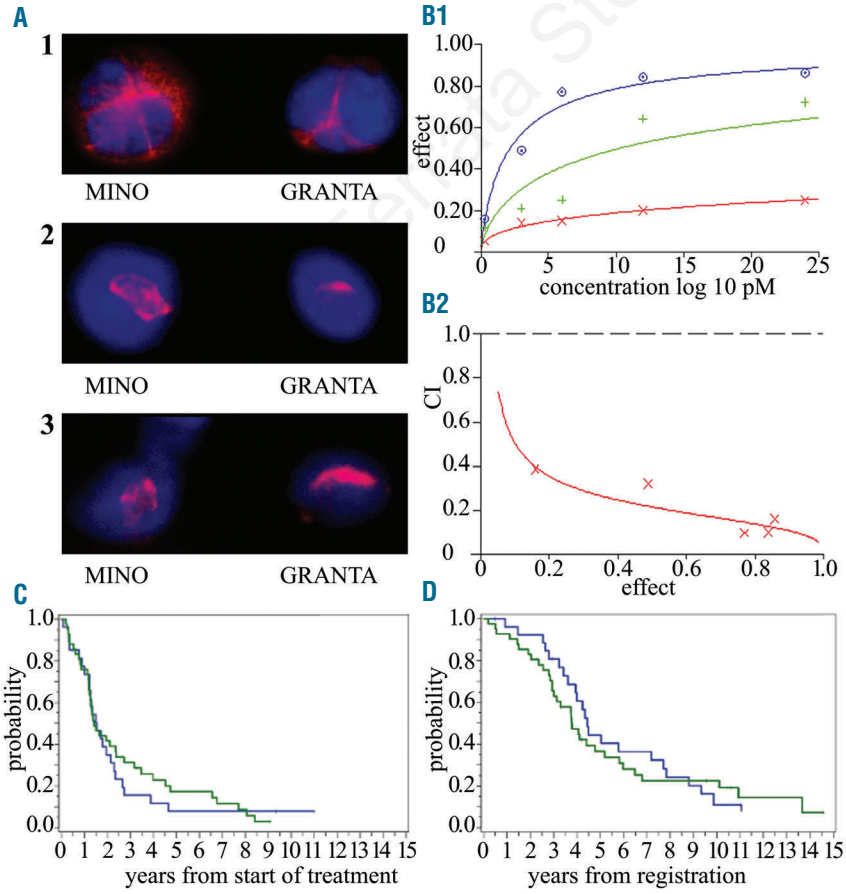

Figure 3. (A) Inhibition of GPER-1 by G36 treatment for 48 h stabilized the microtubulus apparatus (3A1), whereas activation of GPER-1 by G1 treatment for 48 h (3A2) and cytotoxic doses of vincristine (3A3) lead to destabilization of the microtubulus apparatus, shown by immunofluorescence for β-tubulin. (B) GPER-1 inhibition potentiates the effects of paclitaxel. GPER-1 inhibition (G36) was combined with paclitaxel treatment and after 48 h, cell viability/proliferation was assessed in MINO using the MTT assay, and CI values were calculated (3B1) (red curve: paclitaxel; green curve: G36, blue curve: paclitaxel + G36). Equal doses of both drugs were used (2 pM, 1 nM, 100 nM, 100 μM, 100 M). A strong synergistic effect between G36 and paclitaxel was observed with CI values (Fa-CI plot) less than 0.7, as shown in Figure 3B2. There was no significant difference between failure-free (3C) or overall (3D) survival between GPER-1 positive (green curve) *versus* GPER-1 negative (blue curve) MCL cases. Failure was defined as failure to achieve a remission during induction chemotherapy, progression, or death from any cause.

tion with well-known chemotherapeutics, such as paclitaxel, was highly synergistic. This result suggests that GPER-1 may serve as a potential therapeutic target in MCL, which exhibits relatively poor OS in response to current chemotherapy regimens.

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