

(51) International Patent Classification:  
A61K 31/4545 (2006.01) A61P 35/00 (2006.01)(21) International Application Number:  
PCT/IT2013/000281(22) International Filing Date:  
11 October 2013 (11.10.2013)

(25) Filing Language: Italian

(26) Publication Language: English

(30) Priority Data:  
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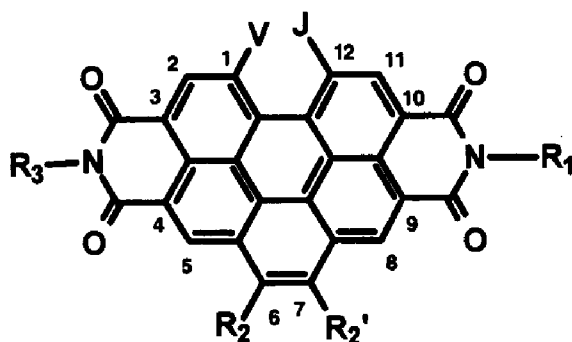
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kind of national protection available): AE, AG, AL, AM,  
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,  
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,  
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,  
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,  
KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,  
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,  
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,  
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM,  
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM,  
ZW.(84) Designated States (unless otherwise indicated, for every  
kind of regional protection available): ARIPO (BW, GH,  
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ,  
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,  
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,  
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,  
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,  
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,  
KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: USE OF EMICORONS AS SELECTIVE INDUCERS OF DAMAGE TO THE TELOMERE DNA



(I)

(57) Abstract: The hydrosoluble emicoron derivatives of the general formula (I) are particularly effective as inducers of selective telomere and non- telomere DNA damage in tumour and transformed cells. The damage is measured as the ability to cause a number of TIF foci in transformed cells that is equal to or higher than 4 at a 0.1  $\mu$ M dose of the compound of the formula (I). Such emicoron derivatives can be used in a kit together with other known anti-tumour drugs, such as, for example, topoisomerase I inhibitors, for the combined, simultaneous, delayed or sequential administration. The emicoron compounds of the formula (I) are particularly useful in the therapy of tumours that do not express p53 protein or express an inactive p53 protein and of tumours that maintain telomeres by mechanisms different from telomere maintenance by telomerase, and in the removal of cancer stem cells. Also, the method for the preparation of emicoron compounds of the formula (I), which envisages the use of intermediates such as the N,N'-bis [2-(1-piperidino)-ethyl] - 1-(1-piperidinyl)-7-[3-(1-piperidino)- butynyl]-perylene-3,4,9,10-tetracarboxyl diimide, is described.

"Use of emicorons as selective inducers of damage to the telomere DNA"

### Field of the Invention

The present invention relates to the use of emicoron and pharmaceutically acceptable water soluble salts and derivatives thereof as particularly selective ligands for quadruplex DNA and as particularly potent inducers of telomere DNA damage selective for tumour and transformed cells.

The present invention also relates to the use of emicoron and pharmaceutically acceptable water soluble salts and derivatives thereof in combination with known anti-tumour drugs in order to slow down and stop tumour progression and metastases formation.

The present invention further relates to a kit comprising one or more emicoron compounds of the general formula (I), in particular emicoron, and one or more anti-tumour compounds, for example a topoisomerase 1 inhibitor, for the combined, simultaneous, delayed or sequential administration.

The present invention also relates to the synthetic method for emicoron.

### Prior Art

For a review about DNA G-quadruplex structure, reference can be made to Burge, S. *et al.* (2006) *Nucleic Acid Res* **34**, 5402-5415. Here suffice to say that this structure is linked to the presence in DNA of guanine-rich sequences (guanine is represented by a G letter) which allow one single DNA strand to form complex three-dimensional structures, among which the structures called G-quadruplex can be mentioned.

G-quadruplexes are structures typical to the telomeres and their presence hinders telomerase action, thus being potentially very useful for inducing tumour cell death, where the keeping of telomeres by

telomerase is fundamental for their expansion (see Neidle and Parkinson (2002) *Nat Rev Drug Discov.*, **1**, 383-393).

The guanine-rich sequences forming G-quadruplex structures are also very much present in the gene sequences of important oncogene promoters, such as MYC (see Balasubramanian S. *et al.* (2011) *Nat Rev Drug Discov.*, **10**, 261-275). The excessive expression and amplification of MYC gene (better known as c-myc) are, for example, important indicators of melanoma progression (Kraehn GM *et al.* (2001) *British Journal of Cancer*, **84**, 72-79; Ramsden A.J. *et al.* (2007) *J. Plastic, Reconstructive and Aesthetic Surgery*, **60**, 626-630).

Even though research about small molecules that specifically interact with G-quadruplex present in cell cancers' genome is very active in order to find new active anti-tumour principles (see the review by Neidle S. (2010) *FEBS J.* **277**, 1118-1125, and Balasubramanian S. *et al.* (2011), *supra*), the problem of selectivity of the action of the molecules under study toward tumour cells with respect to non-tumour cells is still open and very present.

Thus, the need for new molecules with a selective toxicity for tumour cells only is very present, in order to overcome the therapeutic limits set by the toxicity of current tumour therapies also toward non-tumour cells. It is also very deep the interest regarding cancer stem cells (CSC) which have been isolated from different human tumours such as, for example, prostate (Collins A.T. *et al.* (2005) *Cancer Research* **65**, 10946-10951); breast (Ponti D. *et al.* (2005) *Cancer Research* **65**, 5506-5511); brain tumours, for example glioblastoma multiforme (Yuan X. *et al.*, (2004) *Oncogene* **23**, 9392-9400); ovarian tumours (Zhang S., *et al.* (2008) *Cancer Research* **68**, 4311-4320); pancreas (Li C., *et al.* (2007) *Cancer Research* **67**, 1030-7) and colon tumours (Ricci-Vitiani L. *et al.*, (2007) *Nature* **445**, 111-115). CSCs keep many of the normal stem cells features and, as such, are not sensitive to current chemotherapy treatment which, on the contrary, are able to kill differentiating and differentiated cancer cells that are present inside the tumours and are responsible for the existence of metastases. The rare CSCs inside the tumour are thus considered responsible for the resistance to current anti-tumour treatments and

for the spreading of metastases (Lobo N.A. et al., (2007) *Annual Reviews Cell Biology*, **23**, 675-699; McCubrey et al. (2012) *Curr Pharm Des*, **18**, 1784-95; e Malik & Nie (2012) *Front Biosci*, **4**, 2142-9).

Finally, mechanisms different from telomere maintenance by telomerase are of great clinical interest as they are of importance in the hystological typing and progression of some tumours, such as, for example, liposarcomas (Johnson J.E. et al., (2005) *Clinical Cancer Research*, **11**, 5347-5355; and Costa A. et al., (2006) *Cancer Research*, **66**, 8918-8924); sarcomas and astrocytomas (Henson J. et al. (2005) *Clinical Cancer Research*, **11**, 217-225).

In the international application WO 2008/126123 are described coronene derivatives that are soluble in water and are endowed with a telomerase-inhibiting activity thank to their ability to stabilize DNA G-quadruplex structures.

The derivatives, described in said application as "coronene derivatives", presented in the average a good ability to inhibit telomerase and a particular ability to bind to G-quadruplex form of DNA with respect to the duplex form (Casagrande, V. et al (2009) *J. Mass Spectrom.*, **44**, 530-540). Anyway, their action (Casagrande, V. et al (2011) *J. Med Chem*, **54**, 1140-56) was not particularly selective for tumour cells. As a consequence, these coron compounds, although presenting a very good activity as stabilizers of the DNA G-quadruplex form and, thus, as inhibitors of telomerase, do not have an advantageous anti-tumour action with respect to other known anti-tumour compounds, as they are not less toxic than the latter in non-tumour cells (lack of selectivity).

In the same application, a compound whose chemical name in the application was N,N'-bis[2-(1-piperidino-ethyl)-5-(1-piperinidyl)-12-[2-(1-piperidino)-ethyl]-benzoperylene-2,3,8,9-tetracarboxyl-diimide was described but not exemplified, to which the conventional name "emicoron" was given (see the structure formula further below in the "Detailed Description of the Invention" section).

The same compound can, more precisely, be described with the following chemical name: N,N'-Bis[2-(1-piperidino)-ethyl]-1-(1-piperidinyl)-6-[2-(1-piperidino)-ethyl]-benzo[ghi]perylene-3,4,9,10-

tetracarboxyl-diimide. All the names used above are to be considered as synonyms within the scope of the present invention.

It must be noted that in the application WO 2008/126123 neither the synthetic process of emicorons nor, in particular, of the "emicoron" molecule, is described. In addition, only the general use as an anti-tumour agent of coronenes and emicorononenes by means of the inhibition of the enzyme telomerase is described in this application.

### Summary of Invention

It has been now found and is an object of the present invention, that the benzo[ghi]perylene compounds (from here on conventionally called "emicorons" and "emicoronene derivatives") and in particular the N,N'-Bis[2-(1-piperidino)-ethyl]-1-(1-piperidinyl)-6-[2-(1-piperidino)-ethyl]-benzo[ghi]perylene-3,4;9,10-tetracarboxyl-diimide (conventionally called "emicoron") do not have a particularly marked telomerase inhibitory activity, but do have the peculiar activity of producing significant damage to the telomere DNA. In particular, it was found that this activity is highly selective toward cancer and transformed cells, while it is little or not present at all in non-tumour and non-transformed cells (healthy cells).

It was also unexpectedly found and is an object of the invention, that emicorons, in particular emicoron, show a particularly potent activity on p53<sup>-/-</sup> (p53-null) tumour cell growth. This effect was clearly greater than the one observed in the same cells wherein the p53 activity was still present. For a review about the role of p53 in cells, see Zilfou J.T. e. Lowe S.W. (2009) *Cold Spring Harb Perspect Biol*, 1, 1-12.

It has also unexpectedly been found and is an object of the invention, that emicorons are able to prevent growth and survival of Cancer Stem Cells (CSC), which are highly tumorigenic cells resistant to conventional chemotherapy treatment present inside tumours, as well as of ALT(Alternative Lengthening of Telomeres) cells which maintain telomeres also by alternative means with respect to telomerase. It has also been found that emicoron has an inhibitory effect on the expression of oncogene c-myc.

It is thus an object of the present invention the use of emicorons for the treatment of tumours containing CSC, such as, for example, prostate tumours, breast tumours, brain tumours and glioblastoma multiforme, ovarian tumours, pancreatic tumours and colon tumours; of tumours that maintain telomeres by means of mechanisms that are different from telomerase, such as, for example, liposarcomas, sarcomas and astrocytomas; and of tumours wherein c-myc expression has an important prognostic value, such as, for example, melanoma.

It is also an object of the present invention the use of emicoron compounds of the general formula (I) and derivatives and pharmaceutically acceptable salts thereof, preferably hydrosoluble ones, as selective ligands for quadruplex DNA and as effective inducers of telomere and non-telomere damage selective for tumour and transformed cells.

Further objects of the present invention are the synthetic method for emicorons of the general formula (I) and the reaction intermediates of the general formula (III).

Another object of the present invention is the use of emicorons and derivatives and salts thereof in combination with different active principles, in particular in association with other known anti-tumour drugs, such as, for example, taxol (paclitaxel), 5-fluorouracil, oxaliplatin and irinotecan, which are active in differentiated or differentiating cells.

It is a further object of the invention the administration schedule of emicoron in combination with topoisomerase I inhibitors, for example, irinotecan.

A further object of the invention is the use of the emicoron compounds of the general formula (I) and of the derivatives and pharmaceutically acceptable salts thereof, preferably water soluble ones, for the treatment of tumours which maintain telomeres by means of mechanisms that are different from telomerase (Alternative Lengthening of Telomeres, ALT), and for eradicating cancer stem cells (CSC), i.e., cells that are highly tumorigenic and resistant to conventional chemotherapy treatments, which are present inside tumours and are responsible for the appearance of metastases.

Still a further object of the invention is the use of emicoron compounds for the prevention of tumour metastases appearance.

Still a further object of the invention is the use of emicoron compounds of the general formula (I) and derivatives and pharmaceutically acceptable salts thereof, preferably water soluble ones, for the treatment of tumours that do not express p53 protein or express an inactive p53 protein (p53-null tumours)

A further object of the invention relates to the kits containing one or more emicoron compound(s) (in particular emicoron) and one or more conventional anti-tumour compound(s) which are active in differentiated and differentiating tumour cells, for example irinotecan, oxaliplatin, paclitaxel, 5-fluorouracil and similar drugs, for the combined, simultaneous, delayed or sequential administration, and to the corresponding administration schedules

Further objects will be clear from the following Detailed Description of the Invention.

#### Brief Description of the Figures

Figure 1 shows the synthetic scheme of the compound N,N'-Bis[2-(1-piperidino)-ethyl]-1-(1-piperidinyl)-6-[2-(1-piperidino)-ethyl]-benzo[ghi]perylene-3,4;9,10-tetracarboxyl-diimide (emicoron);

Figure 2. Evaluation of the emicoron telomerase activity by means of the "Telomerase Repeat Amplification Protocol" (TRAP). In Figure 2A is shown a representative TRAP assay. The quantitative analysis shown in the graph (Figure 2B) is based on the average of three independent experiments. Within the graph is shown the IC<sub>50</sub> of emicoron calculated on the basis of the curve obtained;

Figure 3. Evaluation of coron an of PPL3C compound telomerase activity. Telomerase activity was evaluated by means of the TRAP assay as shown in Figure 2. (A) PPL3C. (B) PPL3C IC<sub>50</sub> calculated on the basis of the curve obtained (average of three experiments). (C) TRAP assay for the coron compound. It is reported the percentage

inhibition of the telomere activity calculated at the 10  $\mu$ M dose.

**Figure 4. Activation of the response to the DNA damage by emicoron in the transformed cells is associated to delocalization of POT1 from telomeres.** In this Figure, untreated control cells are indicated by the symbol “-” on the ordinates. In (A) e (A’): percentage of BJ HELT o BJ TERT cells (from three independent experiments) treated with emicoron and positive for  $\gamma$ H2AX (gray bars) or positive for 53BP1 (black bars); the SD is shown. \*\* (p<0.01). In (B) and (B’): immunofluorescence images obtained with a Leica Deconvolution (100x) microscope from control or emicoron-treated BJ EHLT cells. (B) Antibodies against TRF1 and  $\gamma$ H2AX; (B’) antibodies against TRF1 and 53BP1. Enlarged details from the pictures of nuclei treated with emicoron are shown to the right of their “merge”. In (C) percentage of positive cells for telomere damage foci (TIF) and (C’) average number of TIF per nucleus in control and emicoron treated samples. Gray bars indicate cells with 4 o more TRF1/ $\gamma$ H2AX foci, black bars indicate cells with 4 o more TRF1/53BP1 foci. Average of three independent experiments. In (D) and (D’): representative immunoprecipitation experiment of chromatin (ChIP) with antibodies against TRF1, TRF2, and POT1. The antibody against  $\beta$ -actin was used as the negative control. In (D’) the gray bars show the control samples and the black bars the treated samples. Four different experiments were evaluated. SD is indicated;

**Figure 5. Activation of the DNA damage response by PPL3C compound and coron.** In this Figure the untreated control cells were labelled with the symbol (-) on the ordinates. (A) Human transformed fibroblasts (BJ EHLT) e (A’) immortalized (BJ-hTERT). Cells positive for  $\gamma$ H2AX are indicated by gray bars. Cells positive for 53BP1 are indicated by black bars. Average of three independent experiments. The standard deviation is indicated (SD). (B) percentage of positive cells for telomere damage foci (TIF). (B’) average number of TIF per nucleus in control and treated samples. Average of three independent experiments. (C) BJ EHLT and (C’) BJ-hTERT cells treated with coron. Cells positive for

$\gamma$ H2AX are indicated by gray bars. Cells positive for 53PB1 are indicated by black bars. Average of three independent experiments. The standard deviation is indicated (SD). (D) Percentage of cells positive for the telomere damage foci (TIF). (D') average number of TIF per nucleus in control and treated samples. The average of three independent experiments is shown;

Figure 6. The antiproliferative and cytotoxic effect of emicoron is specific for transformed and tumour cells. In (A) are shown the *in vitro* proliferation curves of BJ EHLT and in (B) those of BJ-hTERT cells untreated (■) and treated (□) with a 0.1  $\mu$ M dose of emicoron. Average of five experiments. The standard deviation is indicated (SD). (C) Cell proliferation percentage inhibition of BJ-HELT cells. (D) Cell proliferation percentage inhibition of BJ-hTERT treated with a 0.1  $\mu$ M dose of emicoron with respect to the correspondent untreated cells, calculated at day 2, 4, 6 and 8 of cell culture. (E) Emicoron cytotoxicity evaluated *in vitro* within the NCI Drug Screen Program in a panel of 60 human tumour cell lines, belonging to 9 different categories of tumour hystotypes;

Figure 7. PPL3C and coron antiproliferative and cytotoxic effect. In (A) is shown the *in vitro* proliferation curve of BJ EHLT and in (B) of BJ-hTERT cells untreated (■) and treated (□) with a 0.5  $\mu$ M dose of PPL3C. In (C) and in (D) are shown the *in vitro* proliferation curves of BJ EHLT e BJ-hTERT respectively, untreated (■) and treated (□) with a 0.025  $\mu$ M dose of coron. Average of five different experiments. SD is shown. In (E) coron *in vitro* cytotoxicity is showed as measured within the NCI Drug Screen Program in a panel of 60 human tumour cell lines, belonging to 9 different categories of tumour hystotypes;

Figure 8. Emicoron biological activity in a tumour cell line defective for p53. *In vitro* percentage survival of HCT116 cells with a functional p53 (A) or a delete one (B) after treatment with emicoron for 96 hours. Average of three independent experiments. The SD is indicated (SD);

Figure 9. Emicoron *in vivo* cytotoxicity. Emicoron *in vivo* biological activity evaluated in HT29 cell inoculated i.m. in nude mice at a density of  $3 \times 10^6$  cells/animal. The treatment was started at day 4 from the inoculation (tumour weight about 500 mg). Mice were treated as follows: emicoron at a 20 mg/kg i.v. dose (days 4, 7, 10, 13); irinotecan at a 15 mg/Kg i.p. dose (days 4-8); irinotecan (days 4 and 8) and emicoron (days 9, 12, 15, 18);

Figure 10. Emicoron antiproliferative effect in ALT tumour lines. In (A) are shown the *in vitro* proliferation curves for the human osteosarcoma cell line U2OS treated with the G-quadruplex ligand RHPS4. Concentrations: ■ = 0.5  $\mu$ M; ▲ = 1  $\mu$ M; ♦ = Untreated control. In (B) are shown the *in vitro* proliferation curves for the same cell line with emicoron. Concentrations: ■ = 0.25  $\mu$ M; ▲ = 0.5  $\mu$ M; ♦ = Untreated control. In (C) are shown the *in vitro* proliferation curves for the human osteosarcoma cell line SaoS2 treated with the G-quadruplex ligand RHPS4. In (D) are shown the *in vitro* proliferation curves for the same cell line treated with emicoron. The concentrations used for the two compounds were the same as mentioned before. Average of five different experiments. The SD is shown;

Figure 11. Colon cancer stem cells are sensitive to treatment with emicoron. Colon cancer stem cells AG2, R511 and colon carcinoma cells HCT116 were treated with emicoron, 5-fluorouracil [5FU] and oxaliplatin [OXA]. In (A) emicoron was administered for 96 hours. In (B) 5FU was administered for 72 hours. In (C) OXA was administered for 72 hours. Average of three independent experiments The standard deviation (SD) is shown.

Figure 12. Emicoron potentiates the effect of paclitaxel *in vitro*. Colon carcinoma HT29 cells were treated with emicoron for 96 hours and with paclitaxel for 24 hours at the indicated concentrations. In (A), histograms show the results obtained by evaluating colony formation after 10 days in culture. In (B) the combination index (CI) is shown as calculated according to Chou-Talalay. Data show the 50% (white

squares), 75% (light gray squares), 90% (dark gray squares) and 95% CI of the killed cells. Average of three independent experiments. The standard deviation (SD) is shown;

Figure 13. Emicoron potentiates oxaliplatin effect *in vitro*. HT29 colon carcinoma cells were treated with emicoron for 96 hours and with oxaliplatin for 24 hours at the indicated concentrations. In (A) histograms show the results obtained evaluating colony formation after 10 days in culture. In (B) the combination index (CI) calculated according to Chou-Talalay is shown. Data show the 50% (white squares), 75% (light gray squares), 90% (dark gray squares) and 95% CI of the killed cells. Average of three independent experiments. The standard deviation (SD) is shown;

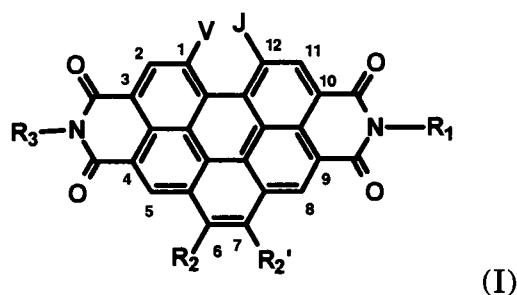
Figure 14. Emicoron potentiates 5-fluorouracil effect *in vitro*. HT29 colon carcinoma cells were treated with emicoron for 96 hours and with fluorouracil for 24 hours at the indicated concentrations. In (A) histograms show the results obtained evaluating colony formation after 10 days in culture. In (B) the combination index (CI) calculated according to Chou-Talalay is shown. Data show the 50% (white squares), 75% (light gray squares), 90% (dark gray squares) and 95% CI of the killed cells. Average of three independent experiments. The standard deviation (SD) is shown;

Figure 15. Emicoron effect on oncogene c-myc promoter. Measures were taken in the presence of increasing concentrations of emicoron (0-50  $\mu$ M) by means of polymerase stop assay in the G-quadruplex containing region of c-myc promoter. In (A) the accumulation of truncated products indicates the presence of G-quadruplex structures which hinder the DNA-polymerase passage. In (B) is shown the sequence of c-myc promoter that forms G-quadruplex (77-mer) and the one of the primer used for the extension reaction (18-mer). The underlined part of the 77-mer is complementary to the 18-mer. Data are representative of three different experiments with similar results;

**Figure 16. Emicoron effect on c-myc transcription.** In (A) RT-PCR of c-myc in human melanoma M14 cells treated with 1  $\mu$ M emicoron for 16, 20, 24 and 28 hours. In (B) western blot analysis in human melanoma M14 cells treated with 0.5 and 1  $\mu$ M emicoron for 24 and 48 hours. Data shown are representative of three different experiments with similar results. In (C) the sequence of the forward and reverse primers used for the RT-PCR assay is shown.

### Detailed Description of the Invention

The present invention relates to benzo[ghi]perylene derivatives (emicorons), in particular the derivatives corresponding to the following general formula (I):



wherein, R2' is null or has the same meaning as R1, R3 and R2 described below, V and J represent, independently from each other, but without forming a ring, H, Cl, Br, I or C, N, O, NH or NH-R with R having the same meaning as R1, R3 and R2 described below, or having one of the structures defined with A and B as follows, R1, R2, and R3 each represent, independently from each other, a group having the general formula:



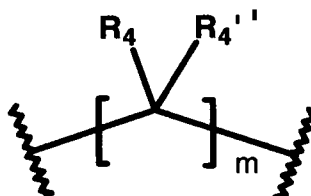
wherein:

n = 0, 1, 2 or 3

X = NH, O, S, NMe or NCOMe

(wherein Me represents a methyl group, -CH<sub>3</sub>)

L<sub>1</sub> and L<sub>2</sub>, independently from each other, represent a bridging group according to the formula:



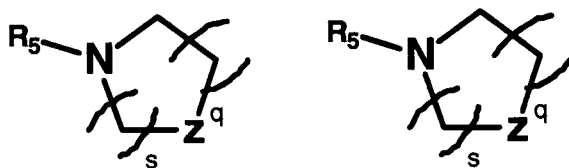
wherein:

$m = 0, 1, 2, 3$  or  $4$

$R_4$  and  $R_4' =$  independently from each other, H, OH, Me, OMe, halogen

Y is chosen among H,  $NH_2$ , OH, OCOMe, NHCOME,  $NMe_2$ ,  $N^+Me_3$ , or has one of the following structures

A) pyrrolidinyl/piperidinyl/morpholinyl/piperazinyl



wherein:

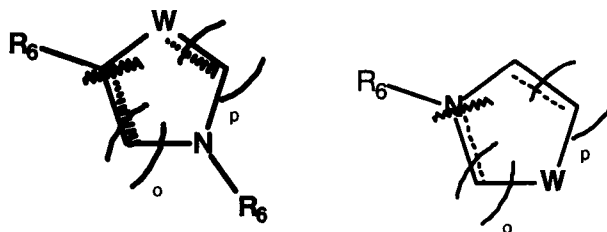
$s = 0, 1$  or  $2$

$q = 0, 1$  or  $2$

$Z = CH_2, O, NH, NMe, NEt, N^+Me_2, NCOMe$

$R_5 = H, Me$

B) piridinyl/pyrrolyl



wherein:

$o = 0$  or  $1$

$p = 1, 2$  or  $3$

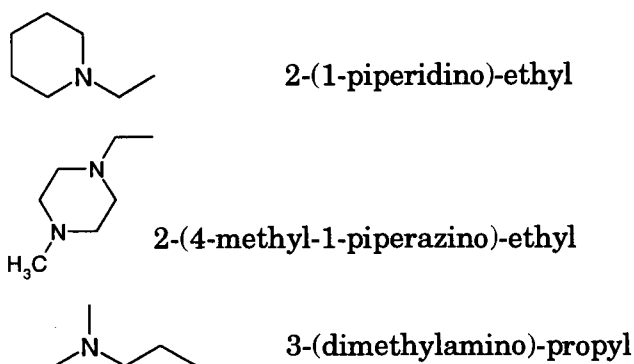
$R_6 = H, Me$  or null

$W = CH, O, S, N, NH$

with the proviso that at least two among R1, R2, R2', R3, are different from null and from H.

Preferably, in general formula (I) R1 and R3 are different from H and are the same, while R2 or R2' are different from H.

According to some preferred embodiments of the present invention, in general formula (I), R1, R3, R2', are different from hydrogen and can preferably be chosen, both the same or in couples, from the group consisting of:



The emicoron derivatives of formula (I) of the invention can be prepared starting from a perylenetetracarboxyl dianhydride, for example the one indicated with 1 in the scheme of Figure 1, easily available on the market, by means of the synthetic procedure comprising the following steps:

(A) the starting compound (perylenetetracarboxyl dianhydride 1) is dissolved in an acidic solvent, e.g., sulphuric acid, it is heated to 80°C or higher (typically 80-100°C) and a halide is added, usually bromine, under agitation; the mixture is left to react, is left to cool down and is filtered and the solid thus obtained, usually a mixture of the two 1,6 and 1,7 isomers, is purified; in the scheme of Figure 1 is shown the formation of the 1,7-dibromoperylene-3,4:9,10-tetracarboxyl dianhydride [a mixture of (2a) e (2b)];

(B) the mixture of the two 1,6 and 1,7 isomers is dissolved in a polar aprotic solvent, for example dioxane, with the addition of other solvents, for example dimethylacetamide; this is heated to a temperature in between 80°C and 120°C in an atmosphere of inert gas and is left to react with a primary amine carrying suitable functional

groups at the opposite end for a minimum of 30 minutes to a maximum of 8 hours; then water is added to the reaction mixture and the resulting solid (**23**) is filtered and purified and brought to dryness; the diamide derivative of dibromoperylene is thus obtained (in the Figure 1 scheme the formation of the N,N'-Bis[2-(1-piperidino)-ethyl]-1,7-dibromoperylene-3,4:9,10-tetracarboxyl diimide (PIPER-Br) (**23**) is shown;

(C) a functionalized alkynyl derivative is prepared in parallel by standard procedures starting from an alkyne with a terminal alcohol function (alkynolol), a commercially available product, (in Figure 1 scheme the formation of 3-butynyl-metansulfonate is shown by means of the reaction with methanesulfonyl chloride); the reaction is aimed at obtaining the formation of a better leaving group with respect to OH;

(D) thus the product obtained in step (C) is reacted by standard procedures with an amine, typically a secondary amine; (in the Figure 1 scheme the formation of 1-(3-butynyl)-piperidine by means of a nucleophilic substitution reaction between the compound of step (C) and piperidine is shown);

(E) the diamide obtained in step (B) is reacted with hydroquinone in a polar solvents mixture of which one is a secondary amine, for example anhydrous piperidine and dioxane at a temperature between 80°C and 120°C for a minimum of 30 minutes up to a maximum of 2 hours; after cooling water is added and an extraction is performed in a solvent, for example chloroform, thus obtaining a mixture of compounds wherein the halogens initially present have been substituted in different ways by the secondary amine used; the mixture of compounds is shown in Figure 1 with (27, 28 and 29);

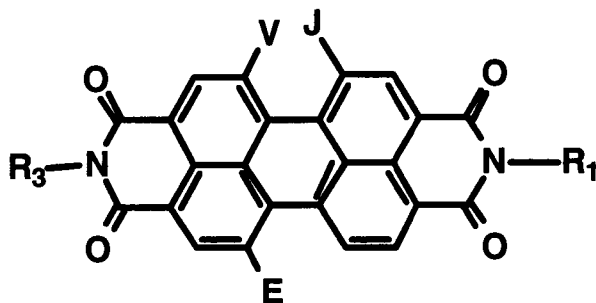
(F) the mixture of compounds obtained in the previous step, dissolved in an anhydrous solvent, for example tetrahydrofuran, is reacted with the alkyne-derivative obtained in step (D) by means of the Sonogashira reaction (Kenkichi Sonogashira, Yasuo Tohda, Nobue Hagihara *Tetrahedron Letters* Vol. 16, Issue 50, 1975, Pages 4467-4470) a cross-coupling catalyzed by Pd(PPh<sub>3</sub>)<sub>4</sub> and CuI; after cooling down, the organic mixture is purified and brought to dryness, obtaining an intermediate of the general formula (III) which in Figure

1 is indicated with (30) [synthetic intermediate N,N'-Bis[2-(1-piperidino)-ethyl]-1-(1-piperidinyl)-7-[3-(1-piperidino)-butynyl]-perylene-3,4;9,10-tetracarboxyl diimide]

(G) the intermediate obtained in step (F) is let to cyclize in re-fluxed organic solvent together with a strong non-nucleophilic base and under an inert atmosphere, for example toluene and DBU (diazabicycloundecene) with the formation of the expected emicoron derivative of the formula (I), in Figure 1 the N,N'-Bis[2-(1-piperidino)-ethyl]-1-(1-piperidinyl)-6-[2-(1-piperidino)-ethyl]-benzo[ghi]perylene-3,4;9,10-tetracarboxyl diimide).

The details of the synthetic conditions are described below in the experimental section according to the scheme shown in Figure 1 (Example 1).

According to the Inventors, the reaction intermediates of step (F) are new and can be rationalized by means of the following general formula (III):



(III)

wherein R<sub>1</sub>, R<sub>3</sub>, V and J have the same meaning as described in the general formula (I) but are not Cl, Br, I, F, while E is selected from Cl, Br, I, F, CR<sub>6</sub>=CR<sub>6'</sub>R<sub>6''</sub>, CR<sub>6</sub>=NR<sub>6'</sub>, N=CR<sub>6</sub>R<sub>6'</sub>, C≡CR<sub>6</sub>, wherein R<sub>6</sub>, R<sub>6'</sub> and R<sub>6''</sub>, have, independently from each other, the same meaning as R<sub>1</sub> and R<sub>3</sub> as described above.

A particularly preferred intermediate is the compound indicated with 30 in Figure 1 (N,N'-Bis[2-(1-piperidino)-ethyl]-1-(1-piperidinyl)-7-[3-(1-piperidino)-butynyl]-perylene-3,4;9,10-tetracarboxyl diimide).

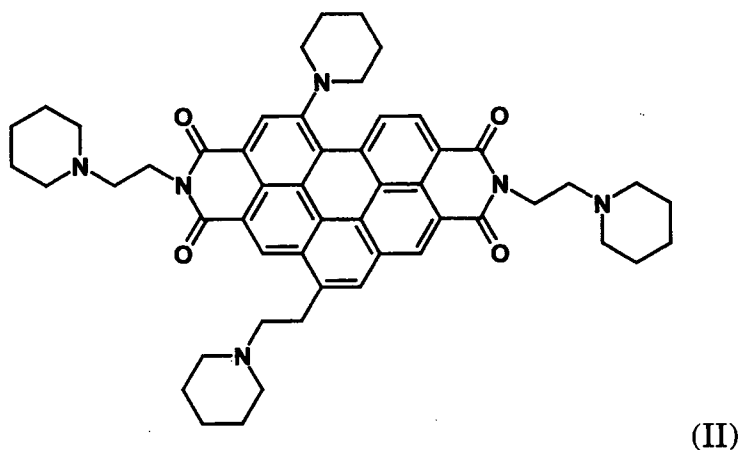
As already said in the Summary of the Invention, it has been unexpectedly found that the emicoron compounds of the invention are endowed with a mechanism of action that is completely different from coron's one, which do not act on telomerase, but directly on telomers, independently from telomerase and on DNA portions rich in guanine by damaging them. Such an effect was experimentally demonstrated (see also Figure 4) and makes emicorons anti-tumour drugs that can act on tumours which express excess oncogenes, such as, for example, MYC, BCL-2, KIT, MYB, KRAS, VEGF, HIF1A and are well known to the expert in the field as potential pharmacological targets. The promoters of these genes form G-quadruplex structures (Balasubramanian *et al.* (2011) *Nature Rev*, **10**, 261-275) and have an essential role in the genesis and aggressiveness of most part of tumours such as melanoma (see Kraehn GM *et al.* (2001); Ramsden A.J. *et al.* (2007); *supra*), breast cancer, lymphomas and colon carcinoma. A significant effect was also found in cancer stem cells (see Figure 11) and it can be significant for metastases and tumours that maintain telomer by means of mechanism different from telomerase, such as glioblastoma multiforme, osteosarcoma, some soft tissues sarcomas and tumours that do not express telomerase, such as sarcomas, astrocytomas, liposarcomas (see *supra*).

This new mechanism of action is responsible for the effectiveness and selectivity for tumour and transformed cells shown by emicorons of the formula (I).

Emicoron shows such features as, for example, selectivity for tumour cells and activity in cancer stem cells, which make them particularly useful for the use in combination with known anti-tumour drugs for the prevention of recurrences and of metastases formation. These features, in addition, allow obtaining a better effect in tumour cells while keeping low the dosage of the known anti-tumour agent. This allows obtaining a higher therapy efficacy without increasing the toxicity linked to an increase in the dose of the anti-tumour agent.

In a preferred embodiment the emicoron compound is N,N'-Bis[2-(1-piperidino)-ethyl]-1-(1-piperidinyl)-6-[2-(1-piperidino)-ethyl]-

benzo[ghi]perylene-3,4;9,10-tetracarboxyl diimide corresponding to the following formula (II)



herein conventionally called “emicoron”.

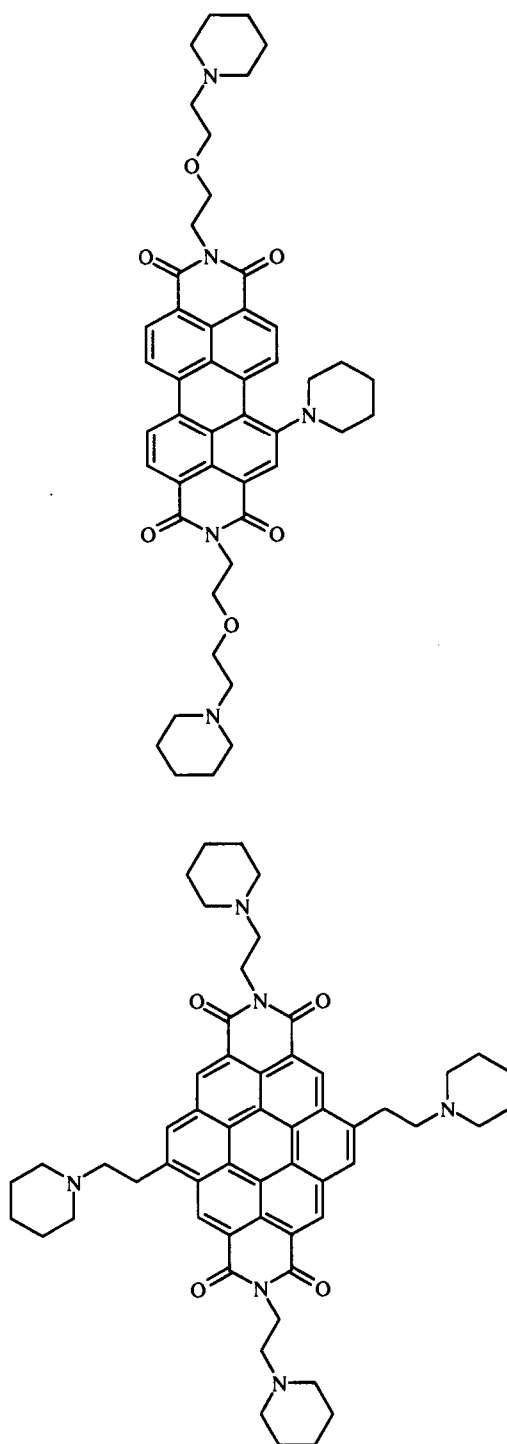
In vitro study of affinity to DNA quadruplex and duplex forms

The bounding activities peculiar for the emicoron compound synthesized was carried out by measuring linking stoichiometry and bounding affinity of the complexes between oligonucleotides and the compounds of the invention.

To this end, the technique ESI-MS (ionizing mass electrospray-spectrometry) (Rosu, De Pauw *et al.* (2008) *Biochimie*, **90**, 1074-1087) has been used, which allows preserving non-covalent interactions responsible for the stability of complexes between oligonucleotides and small molecules (in this case the assayed compounds compared were emicoron of formula (II) and the perylene PPL3C and coron of the following formulas, respectively:

**PPL3C** = Molecular Weight = 867.11; Exact Mass = 866; Molecular Formula =  $C_{52}H_{62}N_6O_6$

18



**CORON** = Molecular Weight = 883.16; Exact Mass = 882; Molecular Formula = C<sub>56</sub>H<sub>62</sub>N<sub>6</sub>O<sub>4</sub>

For the ESI-MS assays the single strand oligonucleotides (SEQ ID NO. 2) 5'-GGGTTAGGGTTAGGGTTAGGTT-3' (21TT) e (SEQ ID NO. 1) 5'-CGTAAATTTACG-3' (DK66) (Eurofins MWG Operon; Ebersberg, Germany) were used, each able to form G-quadruplex DNA structures (being an *in vitro* model of telomere sequence) and duplex DNA in appropriate conditions (for the experimental details see section "Examples" herein below).

The formation of complexes having 1:1 and 2:1 stoichiometry (compounds to be assayed:DNA) which are the most present in solution in all the experiments, can be represented by two different equilibria, which are described by the following equations:

$$K_1 = [1:1] / ([DNA] [\text{compound to be assayed}]) \text{ and}$$

$$K_2 = [2:1] / ([1:1] [\text{compound to be assayed}])$$

Wherein ([DNA] [compound to be assayed]), [1:1], and [2:1] each represent the concentration of the different species in solution.

The association constants  $K_1$  and  $K_2$  are calculated directly from the relative intensity of the different peaks obtained in the mass spectra as described in Rosu, De Pauw *et al.*, *supra*; Mazzitelli *et al.* (2006) *J. Am. Soc. Mass Spectrom.*, **17**, 593-604; Rosu *et al.* (2002) *Rapid Commun. Mass Spectrom.*, **16**, 1729-1736 and Casagrande *et al.* (2009) *J. Mass Spectrom.*, **44**, 530-540.

In Table 1 are shown the association constants  $K_1$  e  $K_2$  for the complexes having a ([compound to be assayed]:[DNA]) stoichiometry equal to 1:1 and 2:1 respectively, and their standard deviations.

Table 1.

|                 | 21-TT (G-quadruplex) |           |             | DK66 (duplex) |           |             |
|-----------------|----------------------|-----------|-------------|---------------|-----------|-------------|
|                 | Log $K_1$            | Log $K_2$ | % DNA bound | Log $K_1$     | Log $K_2$ | % DNA bound |
| <b>PPL3C</b>    | 5.8±0,2              | 5.0±0,1   | 56±7        | 3.9±0,2       | 4.2±0,1   | <5          |
| <b>CORON</b>    | 6.6±0,3              | 4.2±0,1   | 78±5        | 6.0±0,3       | 5.2±0,2   | 59±5        |
| <b>EMICORON</b> | 6.5±0,2              | 6.3±0,2   | 73±2        | 5.4±0,1       | 4.9±0,1   | 38±1        |

The  $K_1$  and  $K_2$  values and % bound DNA were calculated at a ratio compound to be assayed:DNA of 1:1. Results from at least three independent experiments.

These data show that emicoron is a stronger G-quadruplex DNA ligand with respect to coron ( $K_2 = 6.3 \pm 0.2$  vs.  $4.2 \pm 0.1$ ) at essentially the same percentage of DNA bound in solution ( $73 \pm 2$  vs.  $78 \pm 5$ ). With respect to the precursor PPL3C, emicoron is better a G-quadruplex ligand both in terms of  $K_1$  and  $K_2$  and of percentage of bound DNA ( $K_2 = 6.3 \pm 0.2$  vs.  $5.0 \pm 0.1$ ).

In contrast with PPL3C, its selectivity for G-quadruplex is anyway less marked, as it shows a  $38 \pm 1\%$  bond to duplex DNA vs  $5\%$  of PPL3C. This value does, anyway, point to a remarkably higher selectivity of emicoron towards DNA quadruplex with respect to coron, which shows a  $59 \pm 5\%$  binding also to duplex DNA.

Emicoron was also assayed in competition experiments (for details see the “Examples” section herein below) between the 21-TT (G-quadruplex) oligonucleotide and calf thymus DNA (CT), where a fixed quantity of G-quadruplex DNA and of a test molecule are used, and variable quantities of calf thymus DNA (“natural” duplex DNA). For emicoron a molecular ratio 0.5:1 was used, while the ratio used with the other molecules was 1:1.

The results obtained are shown in Table 2. N% represents the normalized quadruplex DNA percentage that was bound (see the “Examples” section herein below).

Table 2

|                 | DNA 21-TT/CT Ratio |            |      |            |      |
|-----------------|--------------------|------------|------|------------|------|
|                 | CT = 0             | 1:1        | N%   | 1:5        | N%   |
| <b>PPL3C</b>    | $56 \pm 7$         | $47 \pm 3$ | 0.84 | $39 \pm 1$ | 0.70 |
| <b>CORON</b>    | $78 \pm 5$         | $63 \pm 5$ | 0.81 | $42 \pm 5$ | 0.54 |
| <b>EMICORON</b> | $38 \pm 1$         | $33 \pm 1$ | 0.87 | $22 \pm 1$ | 0.58 |

Taking into consideration the fact that emicoron was assayed at a molar ration of 0.5:1 with respect to the DNA present in the assay, the data reported in Table 2 clearly show that not only emicoron selectively binds to quadruplex DNA, but mainly that it has a higher binding affinity for telomere DNA with respect both to PPL3C and coron.

This series of assays brought us to hypothesize a selectivity for tumour cells as compared to healthy cells, a selectivity that was confirmed by *in vitro* studies and that is particularly promising for obtaining a selective drug with less side effects in healthy tissues with respect to the drugs currently used in clinical practice.

This behaviour of emicoron is unexpected and can not be directly inferred a priori from the behaviour and features of PPL3C and of coron in the same assay.

### Emicoron's Biological Activity

Regarding the study of emicoron's biological activity, the following assays were carried out:

- TRAP assay (inhibition of cellular telomerase activity);
- *in vitro* telomere protein displacement assay;
- *in vitro* assay of the telomere DNA induced damage;
- *in vitro* cytotoxicity;
- cell proliferation and activity on tumour cell survival, both transformed and non-transformed;
  - effect on p53 +/+ and p53 -/- (p53-null) tumour cell colonies formation *in vitro* (proliferation);
  - combined *in vivo* and *in vitro* effect with known anti-tumour agents;
  - inhibitory action on c-myc expression.

### TRAP assay, emicorons mechanism of action and their application field

The assays were carried out using a TRAP kit based on the use of PCR technique (Chemicon International, Ma, USA), as described in Biroccio *et al.*, (2002) *Oncogene*, **21**, 3011-3019, followed by electrophoretic analysis of the products obtained, as explained in the "Examples" section herein below.

In case of a presence of telomerase activity in the samples tested, in the gel autoradiography a series of bands can be seen, that can be attributed to fragments that differ from each other for a telomere repetition (six nucleotides). On the contrary, in the presence of a telomerase inhibitor, the synthesis of telomere repetitions on the

oligonucleotide is reduced or absent and the TRAP assay shows a decrease in the number and/or of the intensity of the electrophoretic bands due to the different fragments in the solutions wherein the test molecule is present with respect to a solution where it is absent. The internal standard (IS) shows the possible inhibition of Taq polymerase by the chemical species present in the reaction environment.

As shown in Figure 2B, emicoron, although showing a dose-response effect in the TRAP assay, was a weak inhibitor of telomerase activity, with an  $IC_{50}$  about 30  $\mu$ M.

In the same assay, PPL3C showed an  $IC_{50}$  about 15  $\mu$ M, while at 10  $\mu$ M (Figure 3C) coron already inhibited by about 40% the telomerase activity. Figure 3C shows the TRAP assay for coron as reported in the paper by Franceschin *et al.*, *Bioorg Med Chem.* (2007).

The data obtained show that emicoron, although showing selectivity and a great affinity for the DNA quadruplex structure, does not interfere in a significant way with the telomerase activity *in vitro*, at variance with its precursor molecule PPL3C and from the chemically similar molecule of coron. Actually, in Figure 2 it can be seen that telomerase activity inhibition is observed at rather high doses which do not justify a biological effect on the inhibition of tumour proliferation.

Such a high decrease of the telomerase interference activity could not be foreseen based on the behaviour of the two molecules PPL3C and coron with respect to the activity of this enzyme.

The fact that emicoron is not so effective in inhibiting the telomerase enzymatic activity, although it has a very good ability to bind the quadruplex and of inducing a damage to telomeres in a specific way in transformed cells, indicates that the binding specificity of emicoron is independent from the presence or absence of telomerase on the telomere. Thus, emicoron and emicorons can exert a pharmacological action also on tumours which do not express the telomerase, such as, for example, sarcomas, astrocytomas, liposarcomas, (*supra*).

This aspect was confirmed by the experimental studies shown in Figures 4 and 10. They demonstrate that the main mechanism of action of emicoron is the damage produced at the telomere level

(Figure 4) and that emicoron is effective in tumour cells of the ALT type, i.e., that maintain telomeres by means of mechanisms other than the telomerase, such as U2OS and Saos2 cells (Figure 10).

In addition, experimental data clearly show that emicoron is more active in tumour cells negative for the telomerase (ALT) with respect to those positive for telomerase, as a matter of fact a cell proliferation decrease of more than 50% is observed very early, already after two days of treatment, as shown by Figure 10.

The peculiar emicoron's and emicorons' mechanism of action makes them very interesting agents for the anti-tumour therapy of glioblastoma multiforme, osteosarcoma and some soft-tissues sarcomas.

Further to this, the experiments shown in Figure 11 demonstrate that emicoron works with great efficiency in cancer stem cells (CSC), i.e., on cells that are highly tumorigenic and resistant to conventional chemotherapy treatments that are present inside tumours and are responsible for the origin of metastases, as already explained above. To have the possibility of eradicating CSC in a tumour means to remarkably reduce the chances of recurrences and of metastases appearance.

In vitro assays for telomere damage and telomere proteins displacement (uncapping). Selectivity of emicoron's action

The assay for detecting telomere damage is based on the analysis, by means of immunofluorescence in cultured cells, of the presence in the cell nucleus of phosphorylated  $\gamma$ H2AX or of 53BP1 protein co-localized with the telomere protein TRF1. These proteins are indicators known to the expert in the field of double strand DNA damage in cells (see Yu Xu e Price B.D., *Cell Cycle* (2011) 10, 261-267; Noon AT e Goodarzi A.A. (2011) *DNA Repair*, 10, 1071-6). It is also a known convention the fact that only cells that have at least four or more positive foci localized to the telomere are defined as positive, for a damage to the telomeric DNA as obtained by superimposing the immunofluorescence images for  $\gamma$ H2AX/TRF1 (gray bars) or 53BP1/TRF1 (black bars). Telomere damage foci are called TIF from Telomere-dysfunction Induced Foci (Takai H, et al. *Curr Biol.* (2003)

13:1549-56) and cells positive for at least 4 or more TIF in their nuclei are considered TIF positive.

The experiments, carried out in human immortalized BJ fibroblasts (BJ-h TERT) and in human immortalized BJ fibroblasts transformed with early region SV40 (BJ EHLT), are described further below in the “Examples” section.

In Figures 4 and 5 are shown the results obtained with this assay, using the compounds emicoron, PPL3C and coron.

Emicoron was shown to be a stronger inducer of selective telomere damage in transformed cells than PPL3C. As a matter of fact, PPL3C and emicoron selectively act on transformed cells (BJ EHLT), but the generation of telomere damage foci containing phosphorylated  $\gamma$ H2AX and 53PB1 by emicoron is seen at very lower concentrations with respect to PPL3C. Actually, as shown in Figure 4C, the telomere damage can already be measured for emicoron at the 0.1  $\mu$ M concentration (more than 40% treated cells are positive) while about 50% of the cells showed TIF presence at the 0.5  $\mu$ M concentration. PPL3C produced measurable telomere damage at concentrations at least equal to 0.25  $\mu$ M (Figure 5B). In the experiment shown in Figure 4C', emicoron induced a significant number of TIF foci, with an average of about 7 foci per positive cell. Therefore, the treatment with emicoron increased in a significant way the percentage of cells that showed more than 4 co-localization of  $\gamma$ H2AX e TRF1.

TIF foci formation was seen in association with the displacement of POT1 protein from the telomere DNA (Figure 4D, for the ChIP assay and uncapping, see “Examples” section), while proteins TRF1 and TRF2 continued to be associated to telomeres following treatment with emicoron. In Figure 4D' the results of the experiment of Figure 4D are shown in a graph as telomere DNA percentage associated with TRF1, TRF2 and POT1 proteins in control cells (gray bars) and in those treated with emicoron (black bars).

The results that refers to the TIF foci formation by PPL3C (Figure 5B and 5B'), again show a lower potency of PPL3C with respect to emicoron (compare the TIF % per cell) and the ability to form a smaller average number of TIF foci per cell at comparable

concentrations. For example, the average number of TIF foci in a positive cell is not higher than 4 at the 0.25  $\mu$ M PPL3C dose.

Coron, on the contrary, is a potent inducer of telomere damage which, anyway, is not selective for the transformed BJ EHLT cells (see Figure 5C) but is also induced in immortalized BJ-hTERT cells (Figure 5C'), presenting itself as an anti-tumour agent whose use is not advisable *in vivo*.

Also in this case emicoron behaviour, which has remarkable structural similarities with coron molecule although it has PPL3C as the synthetic precursor, could not be foreseen based only on the chemical structure of the molecule. Actually, it would have been more reasonable to expect a less selective behaviour with respect to transformed cells, according to the structural similarity between coron and emicoron. To the contrary, emicoron experimentally demonstrated to have an unexpected ability to create a very selective telomere damage in transformed cells only.

#### Cell proliferation and effect on tumour cell survival. Effect on p53-null tumour cells

PPL3C, coron and emicoron were tested for their ability to interfere with non-transformed, transformed and tumour cells growth. The results are shown in Figure 6 and 7. For the experimental procedures used, see section "Example" herein below.

As expected, based on the results already obtained for the experiments on telomere damage, emicoron at the 0.1  $\mu$ M dose showed a selective toxicity on transformed BJ EHLT cells growth (Figure 6A and 6C). BJ-hTERT cells were not influenced by the same treatment (Figure 6B and 6D).

In Figure 7A and 7B are shown the growth curves that are obtained using the same cells in the presence of 0.5  $\mu$ M PPL3C, while in Figure 7C and 7D are shown the growth curves in the presence of 0.025  $\mu$ M coron. As can be seen from the proliferation curves, the compound PPL3C is less effective, as it is necessary to use a higher dose with respect to the one used for emicoron to obtain a comparable

inhibition of the transformed cells, while coron, although being more effective than emicoron in transformed cells, is also toxic to normal cells and, thus, is not selective.

The *in vitro* cytotoxicity experiments were carried out at the National Cancer Institute del National Institutes of Health (Bethesda, U.S.A.), on a panel of 60 tumour cell lines, belonging to 9 different tumour hystotype categories (Figure 6E; Monks, A. *et al.* (1991) *J. Natl. Cancer Inst.*, **83**, 757-766).

The cells used in this assay comprised the following tumour subgroups: leukemia, non-small cell lung cancer, colon tumour, central nervous system tumour, ovarian carcinoma, kidney tumour, prostate carcinoma and breast carcinoma. From the results obtained with this assay it is possible to extrapolate the following data:

- GI<sub>50</sub> (concentration that inhibits 50% of the net cell growth);
- TGI (concentration that causes inhibition of the total cell growth); and
- LC<sub>50</sub> (concentration that causes 50% of cell death).

The GI<sub>50</sub> results coming from this assay with reference to emicoron are shown in Figure 6D, while the same results referring to coron are shown in Figure 7E. Coron was more potent (GI<sub>50</sub> about 0.1 µM) with respect to emicoron (GI<sub>50</sub> about 1 µM) as an inhibitor of the tumour cells growth studied.

A further unexpected data was obtained treating colorectal cancer cells HCT116 containing or not containing the protein p53. As a matter of fact, as shown in Figure 8, emicoron showed a particularly potent inhibitory effect on the *in vitro* growth of cells HCT116 p53<sup>-/-</sup>, an effect that was clearly higher than the one observed in the same cells wherein the p53 activity was still present. HCT116 p53<sup>-/-</sup> cells are more sensible to emicoron treatment with respect to the cell line that expresses the wild-type protein. This data is particularly significant in view of an anti-tumour therapy, considering the fact that many tumours do not have p53 or express a non-functional p53 variant (Kmat *et al.* (2003) *Cancer*, 97, 389-404; Soussi & Wiman (2007) *Cancer Cell*, 12, 303-12; Goh *et al.* (2011) *J Pathol*, 223, 116-26).

*In vitro* and *in vivo* combined effect with known anti-tumour agents: irinotecan

Table 3

| TREATMENT *                         | TWI (%) <sup>#</sup> | TRD (days) <sup>&amp;</sup> | ILS (%) <sup>§</sup> |
|-------------------------------------|----------------------|-----------------------------|----------------------|
| a) EMICORON <sup>°</sup>            | 27                   | 2                           | 5                    |
| b) IRINOTECAN                       | 57                   | 7                           | 62                   |
| c) IRINOTECAN-EMICORON <sup>°</sup> | 74                   | 14                          | 76                   |

# Inhibition of tumour weight (TWI), calculated at nadir of the effect by comparing the treated groups with the non-treated ones.

& Time of tumour recurrence (TRD), determined as the average time, expressed in days, of tumour recurrence after treatment.

§ Increase of survival time in treated mice (ILS), evaluated by comparing their average survival time with that of non-treated mice (about 21 days)

The data of Table 3 show a potentiating effect, i.e., an unexpected synergic effect, of irinotecan activity. Such an effect can be generalized to other topoisomerase I inhibitors, the pharmacological class to which irinotecan belongs and to other anti-tumour drugs having a different mechanism of action (such as, for example, taxol, adriamycin, cisplatin, 5-fluorouracil).

This potentiating effect of the classical anti-tumour drug shows itself as a significant extension of the development time of tumours, and, thus, in a significant extension of treated subjects survival. In addition, cooperation between the action of emicoron compounds and that of topoisomerase inhibitors can contribute to the use of the latter at lower doses than those currently used in the clinical practice, with a subsequent lowering of their toxic effects.

It has also been possible to experimentally verify the administration sequence for the two drugs that allows obtaining the synergic effect observed. In particular, emicoron is preferably administered after chemotherapy (at least 24 hours after, the doubling time for tumour cells), to prevent DNA damage repairing after the first drug administration. It is likely that, during the *in vivo* treatments, multiple courses of alternate administration of the two drugs will significantly increase their anti-tumour efficacy.

In particular, in Figures 12, 13, and 14 are shown the effects of emicoron in combination with paclitaxel, oxaliplatin and 5-fluorouracil. In all cases, emicoron action is of the synergic type, as shown by the graphs obtained according to the Chou-Talalay method. In particular, in the case of paclitaxel, also known as taxol and mostly used in breast cancer therapy (Seidman A.D. (1995) *Clinical Cancer Research* 1, 247-256) but also in the case of oxaliplatin, data clearly show that the use of low emicoron concentrations in combination with the currently used anti-tumour molecule allows obtaining a significantly better cell survival while keeping the anti-tumour drug concentrations low.

#### Effect on c-myc transcription

The data obtained and shown in figures 15 and 16 demonstrate that emicoron interacts with G-quadruplex structures that are in the promoter of the gene encoding c-myc causing the reduction of its transcription, as shown by the results of the polymerase stop assay and the decrease of its expression, as measured in the human melanoma cell line M14 for c-myc and by means of RT-PCR and western blot analysis.

#### Pharmaceutical formulation and administration routes for the compounds of the invention

Within the scope of the invention, emicoron compounds of the formula (I) can be transformed into the corresponding derivatives and salts, by means of known techniques. Particularly advantageous are the derivatives obtained by irreversibly turning into a quaternary nitrogen at least one of the nitrogen atoms of the molecule, thus increasing its solubility in water.

The emicoron compounds of the invention and derivatives thereof can advantageously be formulated as active principles in compositions containing excipients, carriers and/or adjuvants suitable for the preparation of pharmaceutical forms known to the expert in the field for the topical, oral, spray or parenteral administration.

Particularly advantageous are the intravenous and mainly *per os* administrations, allowing treatment also outside a hospital environment.

Herein are defined as excipients, diluents, carriers and/or adjuvants those substances that used alone are not able to exert a pharmacological effect in a subject which takes said composition and that support stability, preservation, administration and absorption of active principles in pharmaceutical preparations

All the excipients, diluents, carriers and/or adjuvants necessary for the preparation of the composition of the invention in solid or liquid form are those known to the expert in the field and can be chosen, for example, among substances such as water, saline, glycerol, ethanol, polyethylene glycols, polyoxyethylenes, carbowax, glycols, PEG, non-polar diluents (oils), buffer substances, emulsifiers, diluents, preservatives, sweeteners, thickeners, stabilizers, flavourings and similar agents.

Dosages are chosen according to the type of disease to be treated, of the administration routes and of the treated subject's response (either human or animal) and the physician will be able to find the more appropriate routes and administration doses.

The emicoron compounds of the invention, conveniently formulated and prepared in useful pharmaceutical forms, as explained above, and conveniently packed, can be used together with other therapeutic agents, in particular other anti-tumour agents, also conveniently formulated, prepared and packed, for the manufacture of kits for the combined, simultaneous or separate, sequential or delayed administration. Said kits will also contain the description of the correct administration route and of the correct administration schedule for the drugs therein contained.

In the whole, the experimental results herein shown indicate that emicorons, in particular emicoron, act in a selective way in tumour or transformed cells, a feature that makes them completely different from the already known coron and derivatives thereof (coronens), as shown by the results reported in Figure 6A and 6B and in Figure 7C and 7D. In addition, as shown in Figure 2A and 3C, this

different selectivity is in agreement with a different mode of action of the two molecules: coron is a very effective inhibitor of cellular telomerase while emicoron is not in a significant biological way.

Regarding the compound of the prior art PPL3C, emicoronens, in particular emicoron, were shown to be more effective in their selectivity toward tumour and transformed cells. Actually, as shown in Figure 6A and 6B and in Figure 7A and 7B, emicoron is effective already at a 0,1  $\mu\text{M}$  concentration, while a comparable effectiveness is obtained only with a 0,5  $\mu\text{M}$  concentration of PPL3C, i.e., a five-fold higher concentration. Moreover, always with respect to PPL3C, emicoron has a lower activity on telomerase which is mirrored in a greater  $\text{IC}_{50}$  value (see Figure 2B and 3B) and is able to induce a greater damage in telomeres at a lower concentration (see Figures 4A and 5A, 4C' and 5B').

As already evidenced above, this emicoron behaviour is unexpected and can not directly *a priori* be inferred from PPL3C and coron structural features nor from their *in vitro* behaviour.

The experimental results described above also clearly demonstrate that emicoron presents such features as selectivity for tumour cells and an activity in cancer stem cells which make particularly useful its use in combination with known anti-tumour drugs which, as is known to the expert in field, show a not particularly selective cell toxicity, do not have an action in cancer stem cells but do show an activity in differentiating and differentiated tumour cells and are, therefore, little or at all effective against recurrence and metastases appearance. The features of differentiating and differentiated tumour cells and of cancer stem cells are well known to the expert in the field.

The following examples are given for the sake of illustration of the invention and are not to be considered as limiting its scope.

### Examples

Example 1: Synthesis of N,N'-Bis[2-(1-piperidino)-ethyl]-1-(1-piperidinyl)-6-[2-(1-piperidino)-ethyl]-benzo[ghi]perylene-3,4:9,10-tetracarboxyl diimide (emicoron)

For the synthetic scheme of emicoron, see Figure 1.

A): Synthesis of 1,7-dibromoperylene-3,4:9,10-tetra-carboxyl dianhydride

The commercially available 3,4:9,10-perylenetetracarboxyl dianhydride (**1**) (5 g) was initially dissolved in 96% sulfuric acid (100 ml) and stirred for 2 hours at room temperature. 113 g of elemental iodine were then added and the mixture was heated. When a temperature of 80°C was attained, elemental bromine was added dropwise (1.65 ml). The reaction mixture was left stirring for 4-6 hours at 100°C. At the end of cooling, it was added dropwise to ice and was then filtered and washed with a 5% sodium metabisulfite solution to give a red solid (6 g), which was brought to dryness and characterized (yield 87%). In addition to the more important isomer (1,7) (**2a**), a small quantity of the 1,6-dibromoperylene-3,4:9,10-tetracarboxyl dianhydride (**2b**) was obtained. The two isomers could not be separated, thus the mixture was used in the next steps without further purification.

<sup>1</sup>H NMR (200MHz, D<sub>2</sub>SO<sub>4</sub>): δ 10.71 (d, J = 8Hz, 2H, H aromatic), 10.04 (s, 2H, H aromatic), 9.82 (d, J = 8Hz, 2H, H aromatic) ppm. The signals from the lesser isomer (1,6) are mostly superimposed to the ones for (**2a**). Elemental analysis: C<sub>24</sub>H<sub>6</sub>O<sub>6</sub>Br<sub>2</sub>: calc C 52.4 %, H 1.1 %; found C 51.6 %, H 1.1 %.

(B): Synthesis of N,N'-Bis[2-(1-piperidino)-ethyl]-1,7-dibromoperylene-3,4:9,10-tetracarboxyl (PIPER-Br) diimide (**23**).

The dibromo anhydride obtained as described above, a mixture of the two possible isomers (6.0 g) was dissolved in N,N-diethylacetamide (60 ml) and 1,4-dioxane (60 ml). Commercially available 1-(2-aminoethyl) piperidine (3.6 ml) was added and the reaction mixture was left to stir at 120°C for 6 hours in an argon atmosphere. Following water addition a red solid was obtained that was repeatedly washed with water, separated by filtration and brought to dryness, to give the

desired product **23** (6 g, yield 71%; see the synthetic scheme for the structural formula of this compound).

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  9.44 (d,  $J=8\text{Hz}$ , 2H, H aromatic), 8.89 (s, 2H, H aromatic), 8.67 (d,  $J=8\text{ Hz}$ , 2H, H aromatic), 4.41 (t,  $J=7\text{ Hz}$ , 4H,  $\text{N}_{\text{imidic}}\text{-CH}_2$ ), 2.8-2.6 (broad, 12H,  $\text{N}_{\text{piperidine}}\text{-CH}_2$ ), 1.8-1.4 (br, 12H,  $\text{CH}_2\text{piperidine}$ ) ppm.  $^{13}\text{C}$  NMR APT (200 MHz,  $\text{CDCl}_3$ ):  $\delta$  162.14 (C=O), 161.64 (C=O), 137.31 ( $\text{CH}_{\text{ar}}$ ), 132.17 ( $\text{C}_{\text{ar}}$ ), 132.02 ( $\text{C}_{\text{ar}}$ ), 129.29 ( $\text{CH}_{\text{ar}}$ ), 128.50 ( $\text{C}_{\text{ar}}$ ), 127.78 ( $\text{CH}_{\text{ar}}$ ), 126.28 ( $\text{C}_{\text{ar}}$ ), 122.55 ( $\text{C}_{\text{ar}}$ ), 122.12 ( $\text{C}_{\text{ar}}$ ), 120.18 ( $\text{C}_{\text{ar}}$ ), 55.74, 54.24, 37.41, 25.48, 23.81 ppm. MS (ESI)  $m/z$ : 769.1005 [(M+H) $^+$ ] (calc for  $\text{C}_{38}\text{H}_{35}\text{N}_4\text{O}_4\text{Br}_2$ : 769.1025).

(C): Synthesis of the functionalized alkyne 3-Butynyl-1-Mesylate

The 3-butyne-1-ol was converted into its mesylate by reacting with methansulfonyl chloride. The reagent (4.3 ml) was added dropwise at  $0^\circ\text{C}$  to a mixture of anhydrous dichloromethane (80 ml), triethylamine (11 ml) and the alcohol (4 ml). The reaction mixture was then stirred for 2 hours at room temperature. Later, the organic layer was washed with water, HCL 0.5 M, saturated with a saturated solution of  $\text{NaHCO}_3$  and a saturated solution of NaCl. After treatment with anhydrous  $\text{Na}_2\text{SO}_4$  most of the solvents was evaporated under vacuum and the remainders were removed by bubbling  $\text{N}_2$  in the liquid. 7.2 g of a pale yellow liquid were obtained (94% yield).

$^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$ ):  $\delta$  4.25 (t,  $J=7\text{ Hz}$ , 2H,  $\text{SO-CH}_2$ ), 3.01 (s, 3H,  $\text{S-CH}_3$ ), 2.62 (td,  $J'=7\text{ Hz}$ ,  $J''=3\text{ Hz}$ , 2H,  $\text{CH}\equiv\text{C-CH}_2\text{-CH}_2\text{-OS}$ ), 2.06 (t,  $J=3\text{ Hz}$ , 1H,  $\text{CH}\equiv\text{C}$ ) ppm.

D): Synthesis of 1-(3-butynyl)-piperidine

4.5 g of 3-butynyl-mesilate were left to stir with piperidine (6 ml) under reflux with acetonitrile (50 ml) overnight. After cooling, dicloromethane was added and the organic layer was repeatedly washed with water. After treating with anhydrous  $\text{Na}_2\text{SO}_4$  most of the solvent was evaporated under vacuum and the remainders were removed by bubbling  $\text{N}_2$  in the liquid. 2.4 g of the product were obtained, with a 58% yield.

$^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$ ):  $\delta$  2.51 (m, 2H,  $\equiv\text{C-CH}_2$ ), 2.4–2.2 (br, 6H,  $\text{N-CH}_2$ ), 1.91 (t,  $J=3\text{Hz}$ , 1H,  $\text{C}\equiv\text{C-H}$ ), 1.52 (m, 4H,  $\text{CH}_2\text{piperidine}$ ), 1.38 (m, 2H,  $\text{CH}_2\text{piperidine}$ ) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  82.9 ( $\equiv\text{C}$ ), 68.7 ( $\equiv\text{CH}$ ),

57.7, 54.1, 25.8, 24.2, 16.5 ppm.

*E): Synthesis of N,N'-Bis[2-(1-piperidino)-ethyl]-1-(1-piperidinyl)-7-bromoperylene-3,4:9,10-tetracarboxyl diimide (PP3CBr) (29)*

50 mg of PIPER-Br (**23**; see the synthetic scheme in Figure 1), a mixture of the two possible isomers, and 25 mg of hydroquinone were stirred in piperidine (2 ml) and anhydrous dioxane at 100°C in an argon atmosphere for 40 minutes. After cooling, water was added and the raw product was extracted with chloroform. The organic layer was extracted with water to neutrality of the water phase. After drying onto Na<sub>2</sub>SO<sub>4</sub> and evaporation under vacuum, the raw product was purified by silica gel column chromatography (CHCl<sub>3</sub>:MeOH 98:2). It was not possible to proceed with the separation of the different PP3CBr products (**29**; see synthetic scheme), PIP-PIPER(1,7) (**27**; see synthetic scheme) and PIP-PIPER(1,6) (**28**; see synthetic scheme). The fractions obtained by chromatography which showed a suitable aromatic profile in the <sup>1</sup>H-NMR spectra were collected and the mixture was used in the following step.

<sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>): δ 9.27 (d, J = 8Hz, 1H, aromatic H), 9.22 (d, J = 8Hz, 1H, H aromatic), 8.66 (s, 1H, H aromatic), 8.36 (s, 1H, H aromatic), 8.52 (d, J = 8Hz, 1H, H aromatic), 8.40 (d, J = 8Hz, 1H, H aromatic), 4.59 (m, 4H, N<sub>imidic</sub>-CH<sub>2</sub>), 3.37 (m, 2H, C<sub>ar</sub>-N<sub>piperidine</sub>-CH<sub>2</sub>), 2.95 (m, 2H, C<sub>ar</sub>-N<sub>piperidine</sub>-CH<sub>2</sub>), 3.11 (broad, 12H, N<sub>piperidine</sub>-CH<sub>2</sub>), 1.93 (broad, 8H, N<sub>piperidine</sub>-CH<sub>2</sub>-CH<sub>2</sub>), 1.73 (m, 4H, C<sub>ar</sub>-N<sub>piperidine</sub>-CH<sub>2</sub>-CH<sub>2</sub>), 1.62 (broad, 4H, N<sub>piperidine</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>), 1.48 (broad, 2H, C<sub>ar</sub>-N<sub>piperidine</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>) ppm.

*F): Synthesis of the intermediate compound N,N'-Bis[2-(1-piperidino)-ethyl]-1-(1-piperidinyl)-7-[3-(1-piperidino)-butynyl]-perylene-3,4:9,10-tetracarboxyl diimide (30)*

The starting compound PP3CBr (**29**; see the synthetic scheme in Figure 1) (2.5 g), which was a mixture of perylene mono- and di-substituted derivatives, was dissolved in anhydrous THF (40 ml) and triethylamine (40 ml), following, CuI (61 mg) and Pd(PPh<sub>3</sub>)<sub>4</sub> (367 mg) were added. After bubbling with argon, the reaction mixture was heated to 80°C under agitation and 1-(3-butynyl)-piperidine (829 mg) was added dropwise. The mixture was left to stir at 80°C overnight in

an argon atmosphere. After cooling, dilute HCl was added and the product was extracted with dichloromethane after neutralizing with NaOH 2 M. The organic layer was washed with water to neutrality. After treating with anhydrous Na<sub>2</sub>SO<sub>4</sub>, the solvents were evaporated under vacuum. Since at this point a partial cycle formation could take place, the complete separation of the different products and a full characterization were not possible. The raw product (3 g) was then used in the next cyclization step with no further purification.

G): Synthesis of N,N'-Bis[2-(1-piperidino)-ethyl]-1-(1-piperidinyl)-6-[2-(1-piperidino)-ethyl]-benzo[ghi]perylene-3,4,9,10-tetracarboxyl diimide (Emicoron)

2.7 g of intermediate compound (**30**, see the synthetic scheme in Figure 1), were added to 100 ml of toluene and 1.66 ml of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were then added. The reaction mixture was stirred under a reflux in an argon atmosphere for 20 hours. After cooling, dichloromethane was added and the organic layer was extracted with water until neutralization of the water phase. The raw product was purified by means of silica gel column chromatography (CHCl<sub>3</sub>:MeOH 100:0, 98:2, 95:5, 90:10, 80:20, 70:30) to give 1.5 g (55% yield) of the desired compound.

The product was then crystallized by dissolving it in a mixture of MeOH and 37% of a water solution of HCl 95:5 and by precipitating the correspondent hydrochloride with diethyl ether. 350 mg of hydrochloride were obtained from 420 mg of starting compound (71% yield).

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 10.30 (d, J=8.7 Hz, 1H, H aromatic), 9.11 (s, 1H, H aromatic), 8.91 (s, 1H, H aromatic), 8.60 (d, J=8.7 Hz, 1H, H aromatic), 8.55 (s, 1H, H aromatic), 8.26 (s, 1H, H aromatic), 4.51 (m, 4H, N<sub>imidic</sub>-CH<sub>2</sub>), 3.70 (broad, 2H, C<sub>ar</sub>-CH<sub>2</sub>), 3.34 (broad, 2H, C<sub>ar</sub>-N<sub>piperidine</sub>-CH<sub>2</sub>), 2.94 (broad, 2H, C<sub>ar</sub>-N<sub>piperidine</sub>-CH<sub>2</sub>), 2.84 (broad, 18H, N<sub>piperidine</sub>-CH), 1.69 (broad, 24H, CH<sub>piperidine</sub>). <sup>13</sup>C NMR (300 MHz, CDCl<sub>3</sub>): δ 163.8 (C=O), 163.7 (C=O), 163.6 (C=O), 163.5 (C=O), 152.8 (ar.), 138.7 (ar.), 133.1 (ar.), 132.4 (ar.), 129.0 (ar.), 128.9 (ar.), 128.4 (ar.), 127.2 (ar.), 126.7 (ar.), 126.1 (ar.), 125.6 (ar.), 125.0 (ar.), 124.9 (ar.), 123.3 (ar.), 123.0 (ar.), 122.9 (ar.), 121.9 (ar.), 121.7 (ar.), 121.6

(ar.), 121.2 (ar.), 120.2 (ar.), 60.3, 56.3, 54.8, 54.7, 54.6, 53.4, 37.9, 37.7, 30.8, 26.0, 25.9, 24.4, 24.3, 23.8 ppm. MS (ESI)  $m/z$ : 831.4580 [(M+H)+] (calc. for  $C_{52}H_{59}N_6O_4$  M = 831.4598).

## Example 2: ESI-MS study of the emicoron bounding affinity and selectivity

### 2.1: Sample preparation

For this study, the compounds were assayed at three different concentrations comprised between 0.5 and 1 mM.

Samples of three different DNA types were used, as follows:

- calf thymus DNA (CT) as an example of B-DNA used for the competition experiments (Sigma-Aldrich; average molecular weight equal to 13000 base couples). To obtain fragments of lower molecular weight, 10 mg of DNA were dissolved in 10 ml of ultrapure water and treated with ultrasound in a sonicator (SONIPREP 150) at 10W for 8 min. The average molecular weight of the fragments, about 500 base couples, thus obtained was obtained by measuring the electrophoretic mobility in an agarose gel:
- An oligonucleotide auto-complementary sequence (SEQ ID NO. 1), called DK66 (5'-CGTAAATTTACG-3') that is able to give human double strand structures (Eurofins MWG Operon, Ebersberg, Germania);
- The oligonucleotide F21TTT (SEQ ID NO. 2) made of 23 nucleotides with 4 guanine blocks (5'-GGGTTAGGGTTAGGGTTAGGTT-3') which reproduce the human telomere sequence (Eurofins MWG Operon, Ebersberg, Germania).

The oligonucleotide coupling (annealing) was carried out in ultrapure water in the presence of ammonium buffer 150 mM. The solution was heated to 90°C for 10 minutes and left to slowly cool down at room temperature. The final concentration of the nucleotide stock was 50  $\mu$ M both for the duplex DNA and for the quadruplex one.

The samples were prepared by mixing the suitable volumes of ammonium acetate buffer, of a 50  $\mu$ M oligonucleotide solution, of a solution of the test compound and methanol. The final DNA concentration in each sample was 5  $\mu$ M in a final volume of 50  $\mu$ l. The

reference DNA sample contained only 5  $\mu$ M DNA without the addition of a test compound.

The test compounds were added at different ratios with respect to the oligonucleotides according to the results obtained. Methanol was added to the mixture before the injection (15% w:v) after the equilibrium for the formation of complexes in ammonium acetate was complete.

Samples for the competition tests were prepared according to the procedure described above by adding the suitable CT volume. The final oligonucleotide concentration was 5  $\mu$ M and the CT was added in two different ratios 1:1 and 5:1 duplex/quadruplex (calculated based on the phosphate groups concentration). Each experiment was repeated at least three times and the results were mediated to minimize casual errors. Data were processed by means of the SIGMA-PLOT software.

This study was carried out with a Q-TOF MICRO spectrophotometer (Micromass, now Waters, Manchester, UK) that is made up of an ESI source in a negative ionization mode.

The flux speed for the sample in the mass spectrometer was set at 3  $\mu$ l/min and the capillary voltage at -2.6kV. The source temperature was set at 70°C and the source pressure at 1.30 mbar. The cone voltage was set at 30V and the collision energy at 5V. The complete scan of the mass spectre was recorded in the m/z range comprised between 800 and 2500 with 100 data captures per spectre. Data were analyzed using the MassLynx software developed by Waters.

## 2.2: Data processing

Complex formation with a stoichiometry test compound: DNA of 1:1 and 2:1, which are the most represented in solution in all the experiments, is represented by the two following equation:

$$K_1 = [1:1] / ([DNA] [test compound]) \text{ and}$$

$$K_2 = [2:1] / ([1:1] [test compound])$$

wherein ([DNA], [test compound], [1:1] and [2:1] each represent the concentration of the different species in solution.

The association constants  $K_1$  and  $K_2$  can be calculated directly from the relative intensity of the corresponding peaks evidenced in the mass spectre assuming that the response factors of the oligonucleotides

alone and those that refer to the complex test compound-DNA are the same. In this way the relative intensities of the spectra can be assumed as proportional to the relative concentrations of the injected solutions (see Rosu, De Pauw *et al.*, *supra*; Rosu, De Pauw *et al.*, *supra*; Mazzitelli *et al.* (2006) *J. Am. Soc. Mass Spectrom.*, **17**, 593-604; Rosu *et al.* (2002) *Rapid Commun. Mass Spectrom.*, **16**, 1729-1736 e Casagrande *et al.* (2009) *J. Mass Spectrom.*, **44**, 530-540). Mazzitelli *et al.* (2006) *J. Am. Soc. Mass Spectrom.*, **17**, 593-604; Rosu *et al.* (2002) *Rapid Commun. Mass Spectrom.*, **16**, 1729-1736 and Casagrande *et al.* (2009) *J. Mass Spectrom.*, **44**, 530-540).

The bound DNA percentage was calculated according to an equation developed by Mazzitelli *et al.* (2006) (*supra*), that represents the DNA-bound ligand %:  $\text{DNA bound} = 100 \frac{([1:1] + [2:1])}{([DNA] + [1:1] + [2:1])}$ . To process the data obtained in the competition experiments, this percentage was normalized with respect to the same percentage obtained in the presence and in the absence of calf thymus DNA according to the formula:  $\text{N\%} = \frac{\% \text{ bound quadruplex in the presence of CT}}{\% \text{ quadruplex bound in the absence of CT}}$ .

#### Example 3: TRAP (Telomeric Repeat Amplification Protocol) Assay

The telomerase enzymatic activity was evaluated by means of TRAP (Telomeric Repeat Amplification Protocol) assay. The assay was carried out using the kit commercialized by Chemicon International, MA, USA, as previously described (Biroccio *et al.* (2002) *supra*).

Before proceeding with the PCR assay the lengthening products and the bound ligand were extracted with fenol/chloroform/isoamyl alcohol (50:49:1) and the DNA was precipitated in ethanol at -20°C overnight to remove all the unwanted products. The reaction products were amplified in the presence of 36 bp ITAS sequences as the internal control of the PCR reaction. Each TRAP assay group also included a control without the extract (negative control).

The samples were then separated in 12% PAGE and visualized by labelling with the DNA intercalator SYBR Green (Sigma Aldrich). Each gel image was processed with a scanner. The data about the intensity of the bands were measured by denaturing gel scanning and the integration of the total signal of each PCR product on the gel.

The samples that contained the test compounds were corrected for the background value by subtracting the fluorescence readings from the negative controls.

The dose (IC<sub>50</sub>) that inhibits by 50% the enzymatic activity of telomerase was determined based on the dose-response effect according to methods known to the expert in the field.

#### Example 4: In vitro experiments in cell cultures

##### 4.1: Cell cultures

BJ fibroblast expressing hTERT or hTERT and the SV early region (BJ EHLT) were obtained as already described in Salvati *et al.* (2007) *J. Clin Invest.*, **117**, 3236-3247.

HCT116 e HCT116 p53/- cells were a gift from Prof. Bert Vogelstein (Johns Hopkins Medical Institutions, Baltimore, MD, USA).

U2OS, Saos2, AG2, R511 e HCT116 cells were purchased from ATCC. All the cells were cultured in D-MEM (Invitrogen, Carlsbad, CA, USA) with the addition of 10% calf fetal serum, 2 mM glutamine and antibiotics, in an environment of 5% CO<sub>2</sub>, 95% humidity at 37°C.

##### 4.2: Immunofluorescence and telomere damage study

In the following description the following abbreviations are used: mAb = monoclonal antibody(ies); pAb = polyclonal antibody(ies).

Immunofluorescence was carried out as previously described (Salvati *et al.* (2007) *supra*). Cells were fixed with 2% formaldeide and permeabilized with 0.25% Triton X100 in PBS for 5 minutes at room temperature. For the immunofluorescence experiments the primary antibodies used were the following: pAb and mAb anti-TRF1 (Abcam Ltd, Cambridge, UK); mAb anti-γH2AX (Upstate, Lake Placid, NY); pAb anti-53BP1 (Novus Biologicals Inc., Littleton, CO). The secondary antibodies used were a goat anti-rabbit pAb conjugated with TRITC and a goat anti-mouse pAb conjugated with FITC (Jackson Lab).

Human transformed (BJ EHLT) and immortalized (BJ hTERT) fibroblasts were treated for 24 hours with different concentrations for the test compounds (doses are shown in Figures 4A-A', 5A-A', 5C-C'), fixed and processed for immunofluorescence (IF) using the antibodies against γH2AX and 53PB1.

Fluorescent signals were collected by means of a Leica DMIRE2 microscope equipped with a Leica DFC 350FX camera and were processed by means of the deconvolution software Leica FW4000 (Leica, Solms, Germania).

4.3: Chromatin immunoprecipitation (ChIP) and displacement study of the telomere proteins (uncapping)

BJ-HELT fibroblasts were treated for 72 hours with 0,5  $\mu$ M. emicoron.

The ChIP assays were carried out as previously described in Salvati *et al.* (2007) *supra*, using the following antibodies: pAb anti-TRF1 (Santa Cruz Biotechnology, Santa Cruz, Ca, USA); mAb anti-TRF2 (Imgenex, San Diego, CA, USA) and pAb anti-POT1 (Abcam Ltd, Cambridge, UK). For the negative controls the anti- $\beta$ -actin mAb purchased from Sigma Chemicals, Milan, Italy was used.

At the end of the immunoprecipitation and chromatin purification procedure, each precipitated chromatin sample was transferred by "dot-blot" onto a Hybond-N (Amersham International, Buckinghamshire, UK) membrane, and the presence of telomere DNA was detected by hybridization of the membrane with a telomere probe previously labeled with  $\alpha$ -P<sup>32</sup>-CTP. As the non-specific probe a probe that recognizes the ALU repetitive sequences interspersed in the genome DNA was used. To normalize the chromatin amount for each sample, samples representing 10% and 1% of the immunoprecipitated chromatin were included (input). The membrane was exposed to a phosphorimager screen to visualize the radioactivity and the signal was quantified by using the ImageQuant software.

4.4: In vitro proliferation assays

The growth curves shown in Figures 6A, 6B, 7A, 7B, 7C e 7D were carried out by plating  $5 \times 10^4$  cells in 60-mm petri capsules (Nunc, MasciaBrunelli, Milan, Italy). Emicoron was added to the culture medium 24 hours after seeding at the final concentration of 0.1  $\mu$ M. The number of cells and the cell viability of treated and control samples, evaluated by trypan blue staining, were daily detected from day 1 to day 8 in culture.

The ability of HCT116 cells to form colonies as shown in Figures 8A and 8B was evaluated by plating  $2 \times 10^5$  cells in 60-mm petri dishes. After 24 hours from seeding emicoron was added to the cells for 96 hours at the doses indicated in the Figure. At the end of treatment, cells were collected and plated again at such a density to allow colony formation in 60-mm dishes and after 10-12 days in culture. Colonies were stained with methylene blue in 95% ethanol and counted (considering exclusively the colonies formed by at least 50 cells).

For the experiments in U2OS e Saos2 cells, cells were seeded at the concentrations shown in Figure 10 graphs and cultured for 10 days in the medium indicated above in the presence of RHPS4 0.5  $\mu\text{M}$  ( $\square$ ) and 1  $\mu\text{M}$  ( $\square$ ), or emicoron 0.25  $\mu\text{M}$  ( $\square$ ) and 0.5  $\mu\text{M}$  ( $\square$ ). With the symbol ( $\square$ ) are indicated the control cells.

The results shown in Figure 11 were obtained by means of the standard colorimetric MTT assay (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide). The cell mitochondrial enzymes reduce MTT producing a blue/violet coloured substance in vital cells only. This reaction is measured by a spectrophometric reading of the sample at 570 nm wavelength. The cell fraction that survived the treatment was calculated as the ratio between the absolute survival of the treated samples/absolute survival of the control samples. 5FU (Fluorouracil Teva, Teva Pharma, Italy) was added for 72 hours at 5, 10, 20 and 30  $\mu\text{M}$  doses. OXA (Oxaliplatin Teva, Teva Pharma, Italy) was added for 72 hours at 0.35, 0.7, 1.5, 3, 6, 9, 12 e 15  $\mu\text{M}$  doses.

#### 4.5: *In vitro combined cytotoxicity*

HT29 human colon carcinoma cells were treated with emicoron for 96 hours, with oxaliplatin and paclitaxel for 24 hours and with 5-fluorouracil for 48 hours, at the concentrations shown in Figures 12, 13 and 14 or in combination. At the end of the incubation cells were trypsinized, suspended in complete culture medium and seeded in 60 mm petri capsules at a density of 500 and 2000 cells. After 10 days cells were coloured with 2% methylene blue in 95% ethanol and counted.

The surviving cell fractions were calculated as the ratio of absolute survival of the treated sample/absolute survival of control

sample. The combination index (CI) was calculated according to the Chou-Talalay method (Chou T.C. and Talalay P. (1984) *Advances in Enzyme Regulation* **22**, 27-55) and shown as graphs in Figures 12, 13 and 14 respectively.

#### Statistical analysis

Experiments were repeated from 3 to 5 times and the results obtained are shown as the average  $\pm$  SD (standard deviation). The statistical significance was analyzed by the Student test and differences with  $p < 0.05$  values were considered as significant.

#### 4.6: In vitro cytotoxicity in 60 human tumour cell lines (National Cancer Institute)

Briefly, the tumour cell lines were cultured in 1640 RPMI culture medium containing 5% fetal calf serum and 2 mM L-glutamine.

Typically, the assay is replicated 5 times as follows:

- cells are seeded in 96-well microtiter plates in 100  $\mu$ l of medium at a density of 5,000 to 40,000 cells/well, according to the doubling time of the cell type used;
- plates are then left to incubate at 37°C in 5% CO<sub>2</sub>, 95% air and 100% relative humidity for 24 hours;
- after 24 hours, two plates for each cell line are fixed *in situ* with TCA (control cell population (Tz) before adding the test compound;
- the test compounds are first diluted in dimethylsulfoxide and subsequently in complete culture medium containing 50  $\mu$ g/ml gentamicin so that the desired final concentration is present in each well;
- the plates are incubated for further 48 hours at 37°C in 5% CO<sub>2</sub>, 95% air and 100% relative humidity;
- the cells are then fixed with a cold 50% TCA solution (final TCA concentration, 10%) incubating for 60 minutes at 4°C;
- the supernatant is discarded, the cells are washed five times with running water and left to dry in the air
- the sulforhodamine B assay is carried out by adding 100  $\mu$ l of a 0.4% solution in 1% acetic acid to each well and incubating the plates for 10 minutes at room temperature. After accurate washes with 1% acetic acid the dye is solubilized in the wells by adding 10 mM Trizma

base. The absorbance is read at 515 nm.

The percentage growth is calculated as follows for each concentration of test compound assayed:

$[(Ti-Tz)/(C-Tz)] \times 100$  for concentrations where  $Ti \geq Tz$

$10 [(Ti-Tz)/Tz] \times 100$  for concentrations where  $Ti < Tz$ .

From the data obtained with this assay it is also possible to extrapolate the following data:

- GI50 (concentration that inhibits 50% of the net cell growth);
- TGI (concentration that causes the inhibition of overall cell growth); and
- LC50 (concentration that causes 50% of cell death)

#### Example 5: The c-myc gene promoter contains G-quadruplex structures

The presence of a region that forms G-quadruplex structures in the c-myc promoter was verified by means of a polymerase stop assay as shown in Figure 15. The assay was carried out using the following primer:

5'-TAA TAC GAC TCA CTA TAG-3' (18-mer) (SEQ ID NO. 3).

This primer is complementary to the underlined part of the 77-mer sequence that forms G-quadruplex structures in the c-myc promoter given herein below (SEQ ID NO. 4):

5'-TCC ACC TAT GTA TAC TGG GGA GGG TGG GGA GGG TGG  
GGA AGG TTA GCG GCA CGC AAT TGC TAT AGT GAG TCG TAT  
TA-3' (77-mer).

#### Example 6: Elicoron inhibits c-myc transcription

C-myc gene transcription was measured by means of RT-PCR. Herein below the sequences of the primers used are given:

Forward: 5'-CCA TGA GGA GAC ACC GCC-3' (SEQ ID NO. 5)

Reverse: 5'- TCT TGT TCC TCC TCA GAG TCG C-3' (SEQ ID NO. 6).

M14 human melanoma cells were treated with elicoron 1  $\mu$ M for 16, 20, 24 and 28 hours. The results are shown in Figure 16A.

The data shown in Figure 16B were obtained by western blot analysis as already described. The analyzed samples were from M14 cells treated with emicoron 0.5 and 1  $\mu$ M for 24 and 48 hours.

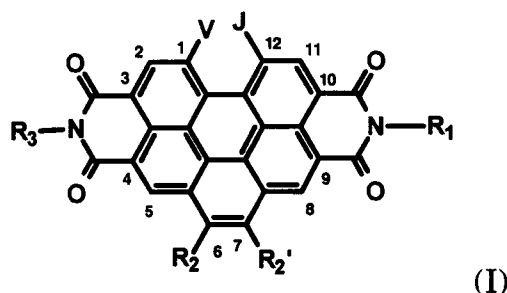
Example 7: Combined *in vivo* cytotoxicity

The *in vivo* cytotoxicity of emicoron was tested in colon adenocarcinoma HT29 injected i.m. in nude mice at a density of  $3 \times 10^6$  cells/animal. The treatment was started at day 4 from the injection (when the tumour weight is about 500 mg). Mice were treated as follows: a) emicoron administered i.v. at a 20 mg/kg dose (days 4, 7, 10, 13); b) irinotecan administered i.p. at a 15 mg/Kg dose (days 4-8); c) irinotecan (days 4-8) and emicoron (days 9, 12, 15, 18). Emicoron was administered 4 times. The parameters that were analyzed for each experiment were:

- Inhibition of tumour weight (TWI), calculated at the nadir of the effect comparing the treated vs. the untreated groups.
- Time of tumour recurrence (TRD), calculated as the average time, in days, of tumour recurrence after treatment.
- Increase of survival in treated mice (ILS), calculated as the ratio between their average survival time and the one of untreated mice (equal to about 21 days).

## CLAIMS

1. Emicoron compounds and pharmaceutically acceptable derivatives and salts thereof for the treatment of tumours that contains Cancer Stem Cells, of tumours that express in excess at least one oncogene selected from: MYC, BCL-2, KIT, MYB, KRAS, VEGF, HIF1A, of the tumours that maintain telomeres by means of mechanisms that are different from telomerase, and for the preventions of recurrences and of metastases formation, said emicoron compounds having the following general formula (I):



wherein R2' is null or has the same meaning as R1, R3 and R2 as below, V and J represent, independently from each other, but without forming a ring, H, Cl, Br, I or C, N, O, NH or NH-R with R having the same meaning as R1, R3 and R2 described below, or having one of the structures defined with A and B as follows,

R1, R2, and R3 each represent, independently from each other, a group having the general formula:



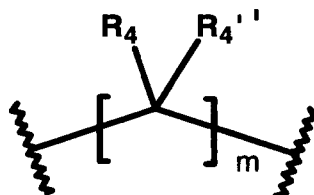
wherein:

n = 0, 1, 2 or 3

X = NH, O, S, NMe or NCOMe

(wherein Me represents a methyl group, CH<sub>3</sub>)

L<sub>1</sub> and L<sub>2</sub>, independently from each other, represent a bridging group according to the formula:

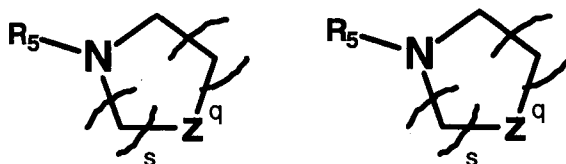


wherein:

$m = 0, 1, 2, 3$  or  $4$

$R_4$  and  $R_4'$  = independently from each other, H, OH, Me, OMe, halogen  
 $Y$  is chosen among H,  $NH_2$ , OH, OCOMe, NHCOMe,  $NMe_2$ ,  $N^+Me_3$ , or  
 has one of the following structures

A) pyrrolidiny/piperidiny/morpholinyl/piperazinyl



wherein:

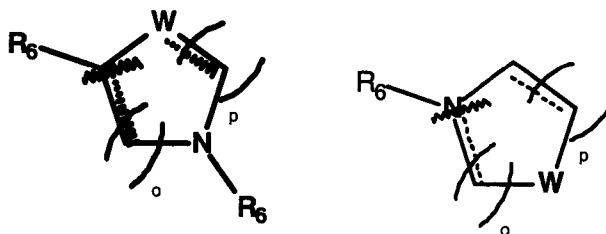
$s = 0, 1$  or  $2$

$q = 0, 1$  or  $2$

$Z = CH_2, O, NH, NMe, NEt, N^+Me_2, NCOMe$

$R_5 = H, Me$

B) piridinyl/pyrrolyl



wherein:

$o = 0$  or  $1$

$p = 1, 2$  or  $3$

$R_6 = H, Me$  or null

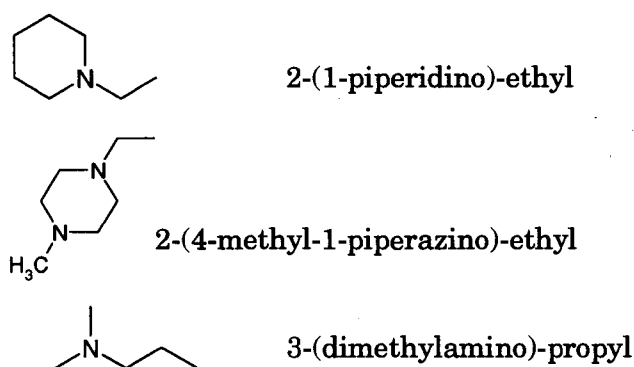
$W = CH, O, S, N, NH$

with the proviso that at least two among  $R_1, R_2, R_2', R_3$ , are different

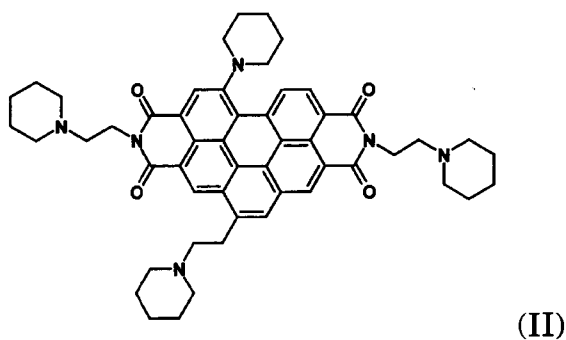
from null and from H.

2. The compounds according to claim 1, wherein in the emicoron compound of the general formula (I), R1 and R3 are different from H and are the same, while R2 or R2' and R4 or R4' are different from H.

3. The compounds according to any one of claims 1-2, wherein in the emicoron compound of the general formula (I), R1, R3, R2, are different from hydrogen and are chosen, both the same or in couples, from the group consisting of:



4. The compounds according to any one of claims 1-3, wherein the emicoron compound of the general formula (I) is the N,N'-Bis[2-(1-piperidino)-ethyl]-1-(1-piperidinyl)-6-[2-(1-piperidino)-ethyl]-benzo[ghi]perylene-3,4;9,10-tetracarboxyl-diimide of the formula (II)



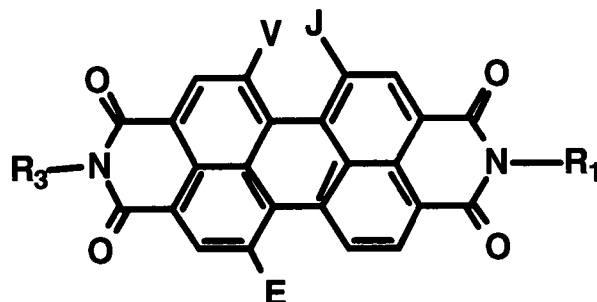
5. The compounds according to any one of claims 1-4, wherein the tumour is selected from: melanoma, prostate tumours, breast tumours, brain tumours such as glioblastoma, ovarian tumours, colon tumours, colorectal carcinomas, leukemias, non-small cell lung tumours, central nervous system tumours, ovarian carcinomas, kidney tumours, prostate carcinoma and breast carcinoma, pancreatic tumours, sarcomas such as liposarcomas, astrosarcomas, glioblastoma multiforme, osteosarcomas and soft tissues sarcomas.
6. The emicoron compounds according to any one of claims 1-5, used in combination with anti-tumour drugs which are active in differentiated and differentiating tumour cells.
7. The emicoron compounds according to claim 6, wherein the anti-tumour drugs which are active in differentiated and differentiating tumour cells are selected among topoisomerase I inhibitors.
8. The compounds according to claim 6, wherein the anti-tumour drugs which are active in differentiated and differentiating tumour cells are one or more drugs selected from: taxol, adriamycin, cisplatin, oxaliplatin, irinotecan, 5-fluorouracil and mixtures thereof.
9. The compounds of any one of claims 1-8, wherein the emicoron compounds are used in the therapy of tumours and the tumours are selected from those that do not express p53 protein or express an inactive p53 protein (p53-null tumours).
10. A kit comprising one or more emicoron compounds of the general formula (I) according to claim 1, in particular emicoron, and one or more anti-tumour drugs which are active in differentiated and differentiating tumour cells, for a combined, simultaneous, delayed or sequential administration.

11. The kit according to claim 10, wherein the anti-tumour drug is selected from: topoisomerase I inhibitors, taxol, adriamycin, cisplatin, oxaliplatin, 5-fluorouracil, irinotecan and mixtures thereof.

12. A method for the preparation of the compounds of the general formula (I) according to claims 1 starting from a perylenetetracarboxyl dianhydride, which comprises the following steps:

- (A) reacting the starting compound with a halide, stirring in the presence of an acidic solvent;
- (B) solubilizing the mixture obtained in step (A) in a polar aprotic solvent, adding an acetamide derivative; react with a primary amine by heating in an inert gas atmosphere and then purifying the diimide solid thus obtained;
- (C) simultaneously preparing a functionalized alkyne derivative starting from the corresponding alkynolol, preferably 3-butyne-1-ol;
- (D) thus reacting the product obtained in step (C) with an amine;
- (E) reacting the diamide obtained in step (B) with a hydroquinone in a polar aprotic solvent containing a secondary amine;
- (F) solubilizing in an anhydrous solvent the mixture of compounds obtained in previous step (E), reacting with the alkyne derivative obtained in step (D) by means of a cross-coupling reaction catalyzed by  $\text{Pd}(\text{PPh}_3)_4$  and  $\text{CuI}$ ; after cooling and purification an intermediate compound of the general formula (II) is obtained;
- (G) cyclization of the intermediate compound (II) by contacting a strong nucleophilic base, thus forming the expected emicoron derivative of the formula (I)

13. A reaction intermediate of the general formula (III):



(III)

wherein R1, R3, V and J have the same meaning as described in the general formula (I) of claim 1, but are not Cl, Br, I, F, while E is selected from Cl, Br, I, F, CR<sub>6</sub>=CR<sub>6'</sub>R<sub>6''</sub>, CR<sub>6</sub>=NR<sub>6'</sub>, N=CR<sub>6</sub>R<sub>6'</sub>, C≡CR<sub>6</sub>, wherein R<sub>6</sub>, R<sub>6'</sub> and R<sub>6''</sub>, have, independently from each other, the same meaning as R1 and R3 as described above.

14. The reaction intermediate according to claim 13 which is the compound N,N'-Bis[2-(1-piperidino)-ethyl]-1-(1-piperidinyl)-7-[3-(1-piperidino)-butinyl]-perylene-3,4,9,10-tetracarboxyl diimide.

15. Use of the compounds according to claims 13 and 14 for the preparation of the compounds of the formula (I).

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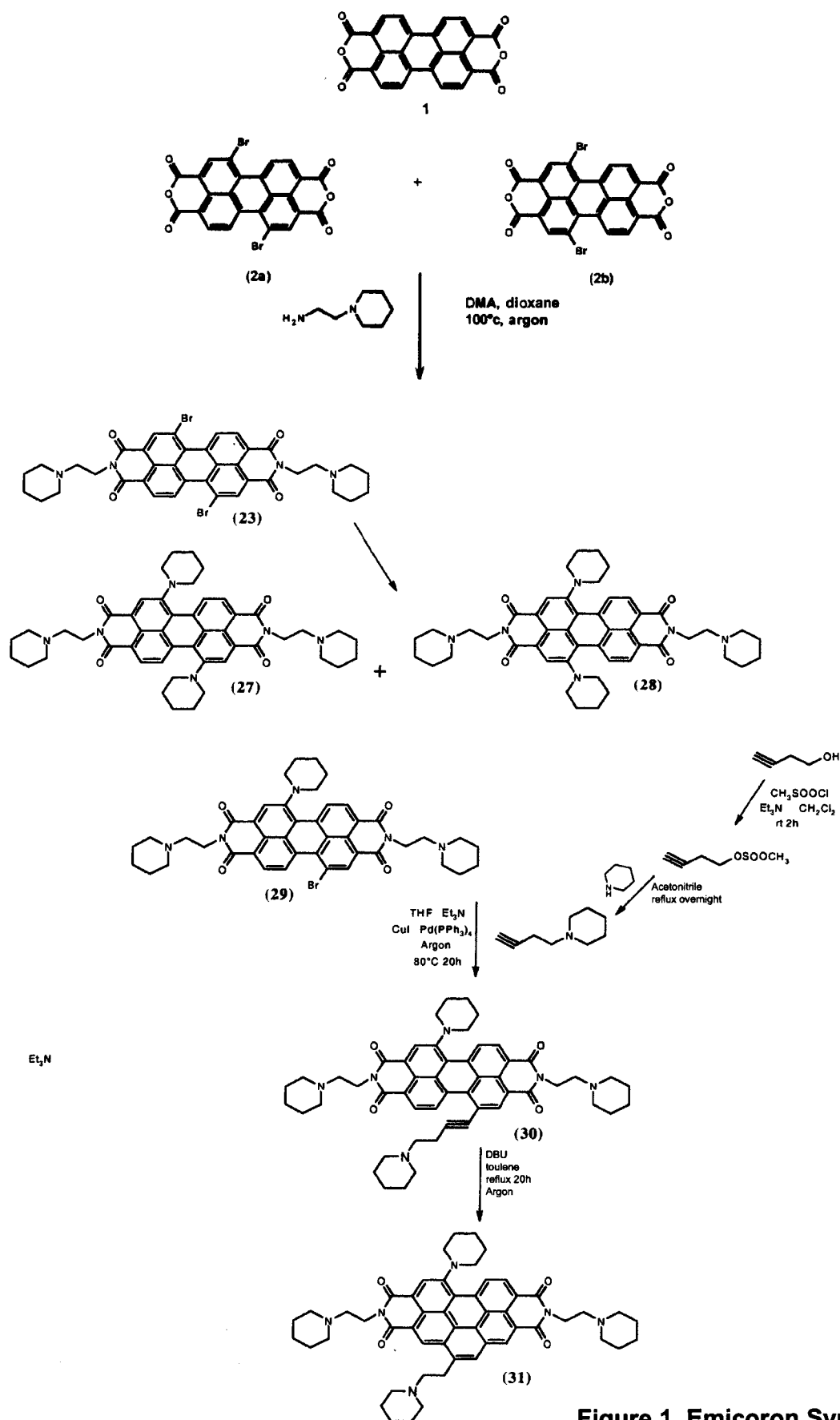


Figure 1. Emicoron Synthetic Scheme

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Figure 2

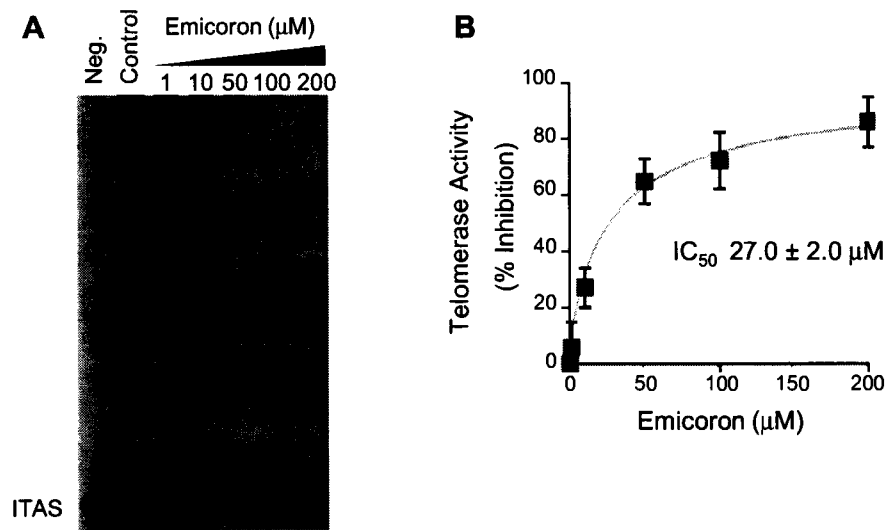


Figure 3

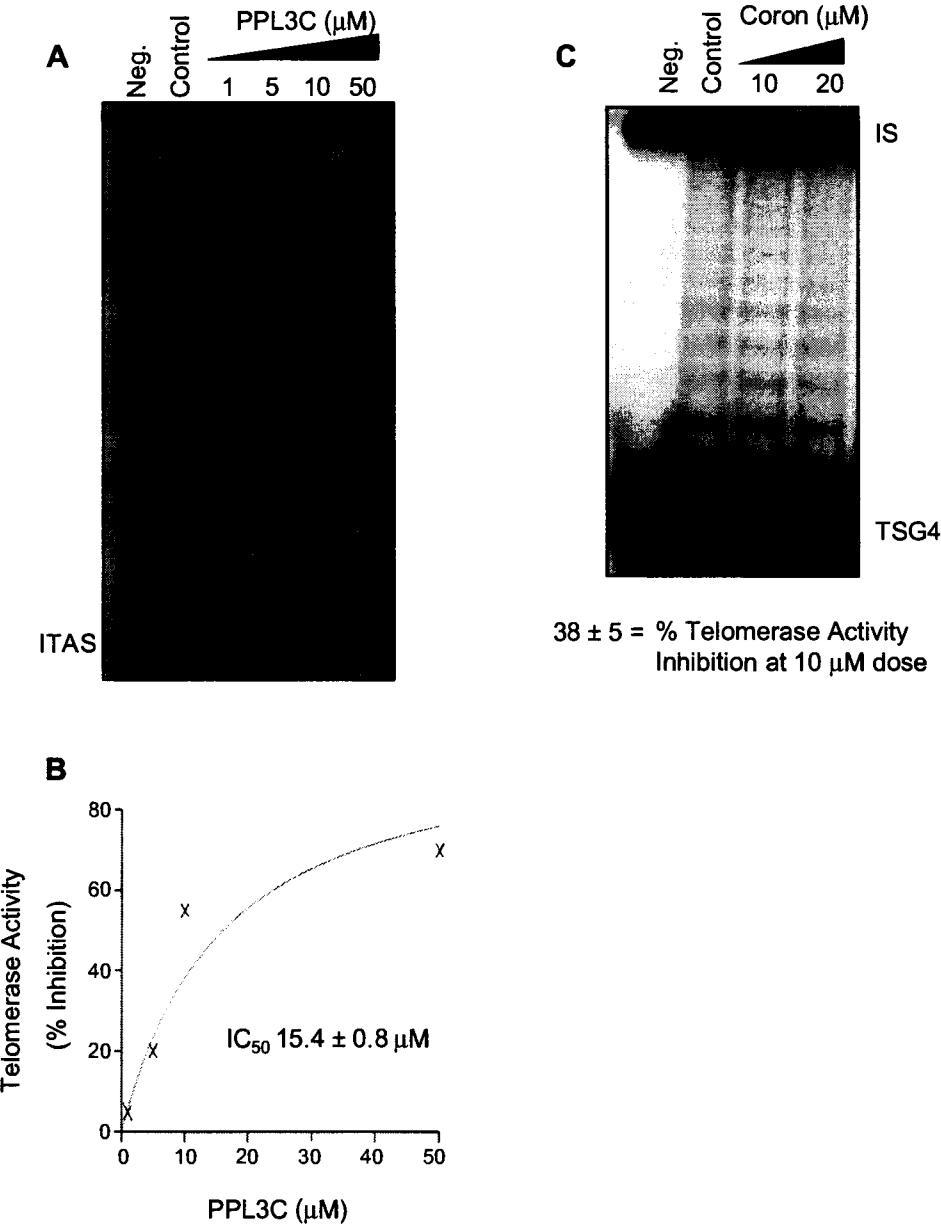
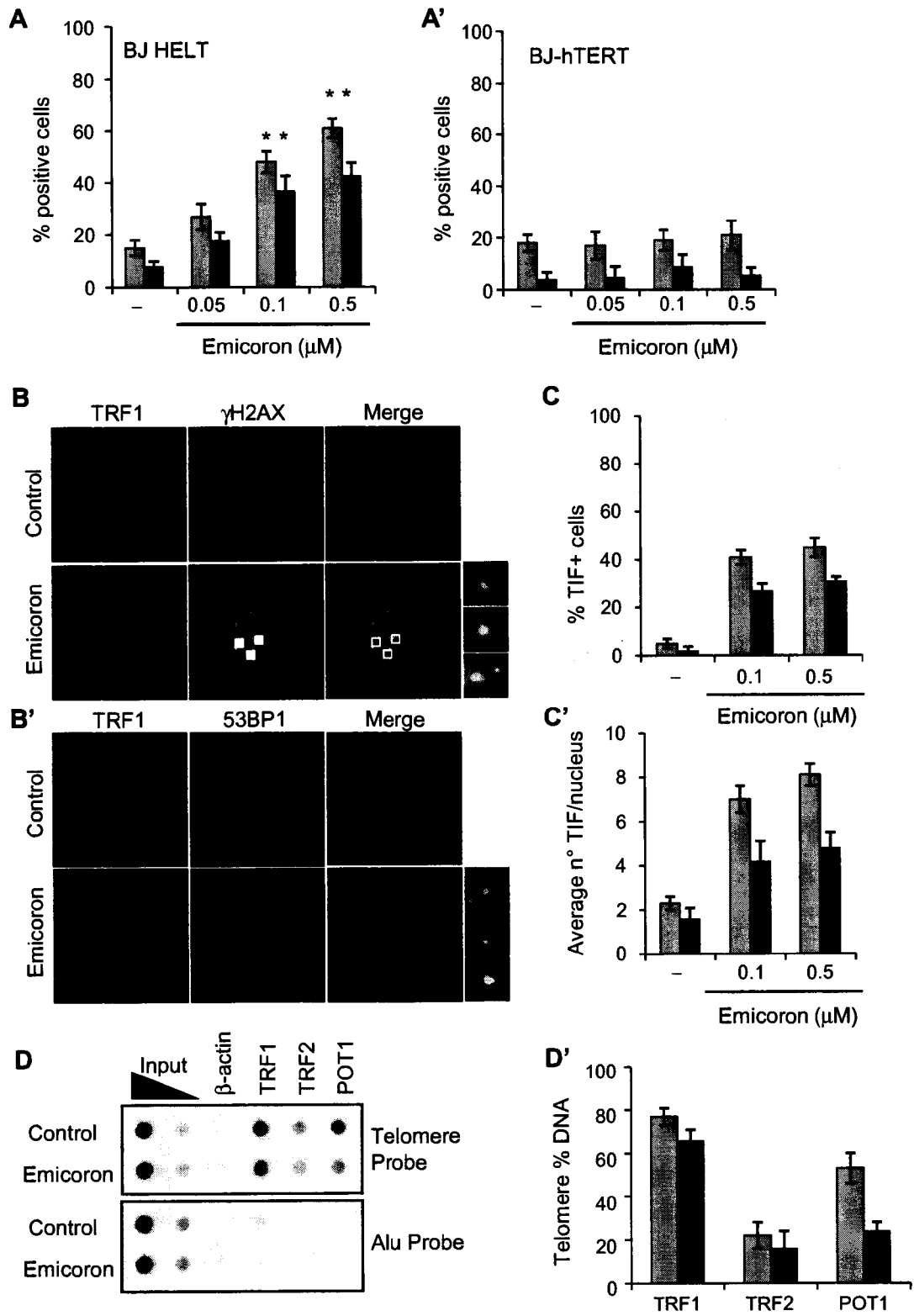
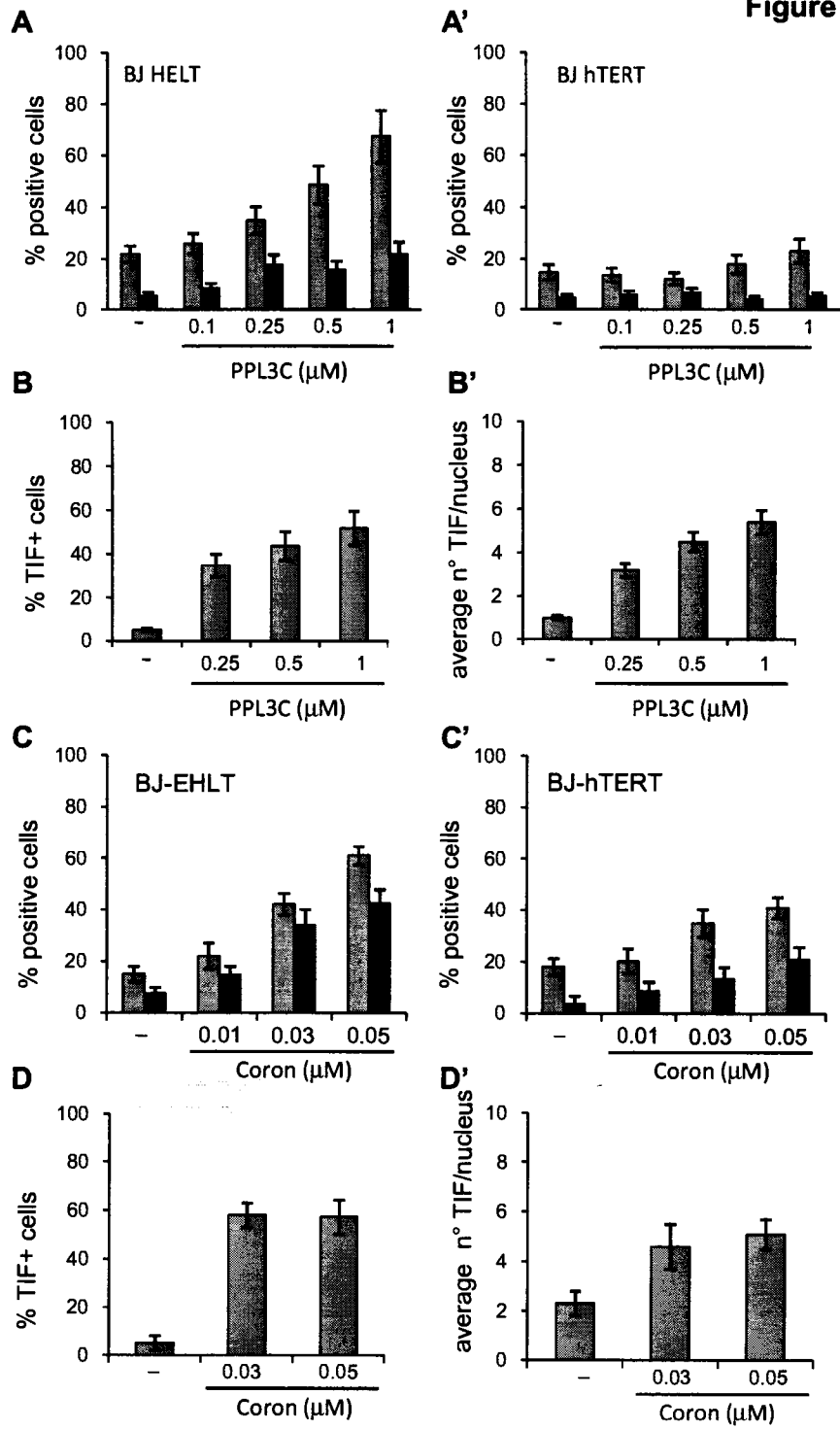


Figure 4



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Figure 5



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Figure 6

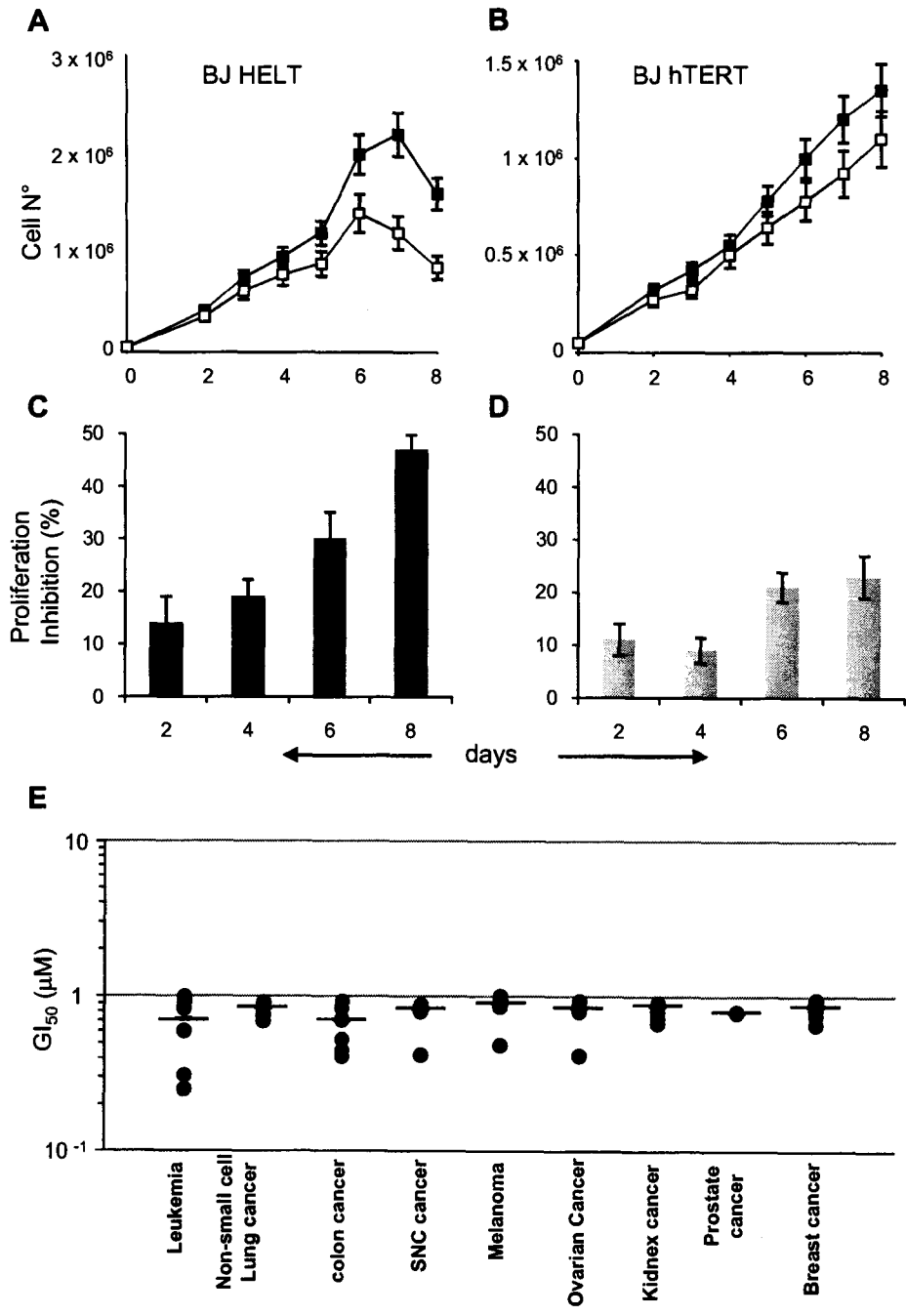
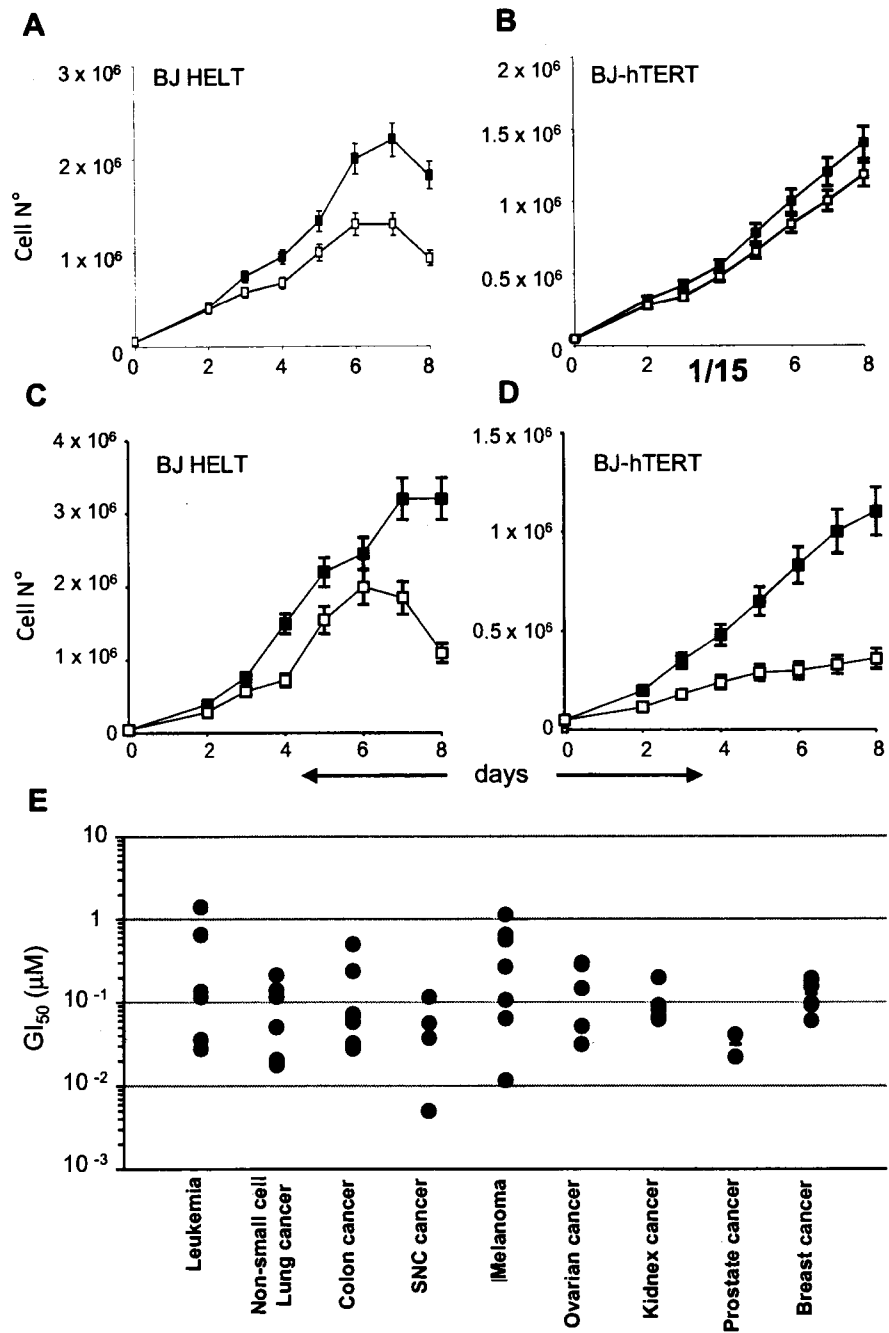


Figure 7



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Figure 8

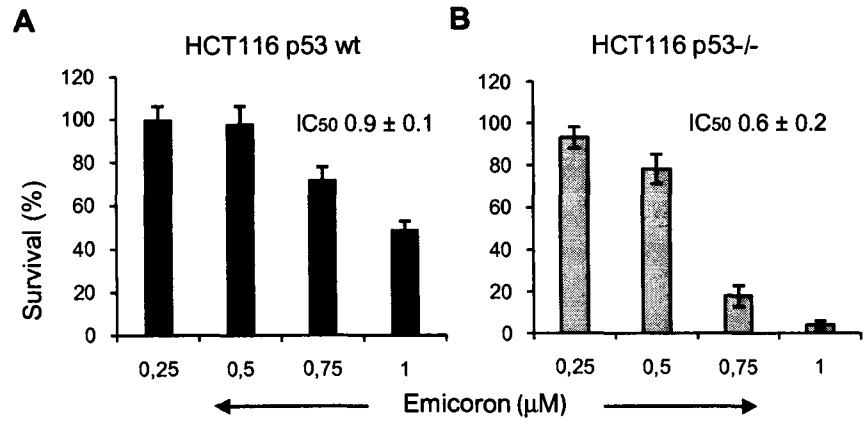
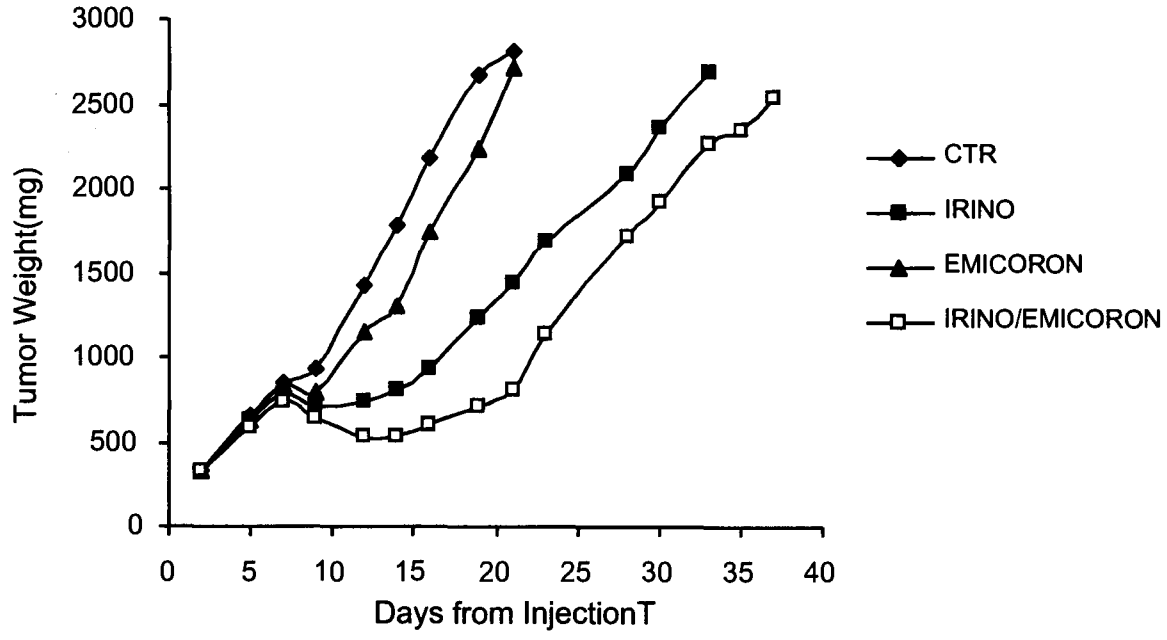


Figura 9



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Figure 10

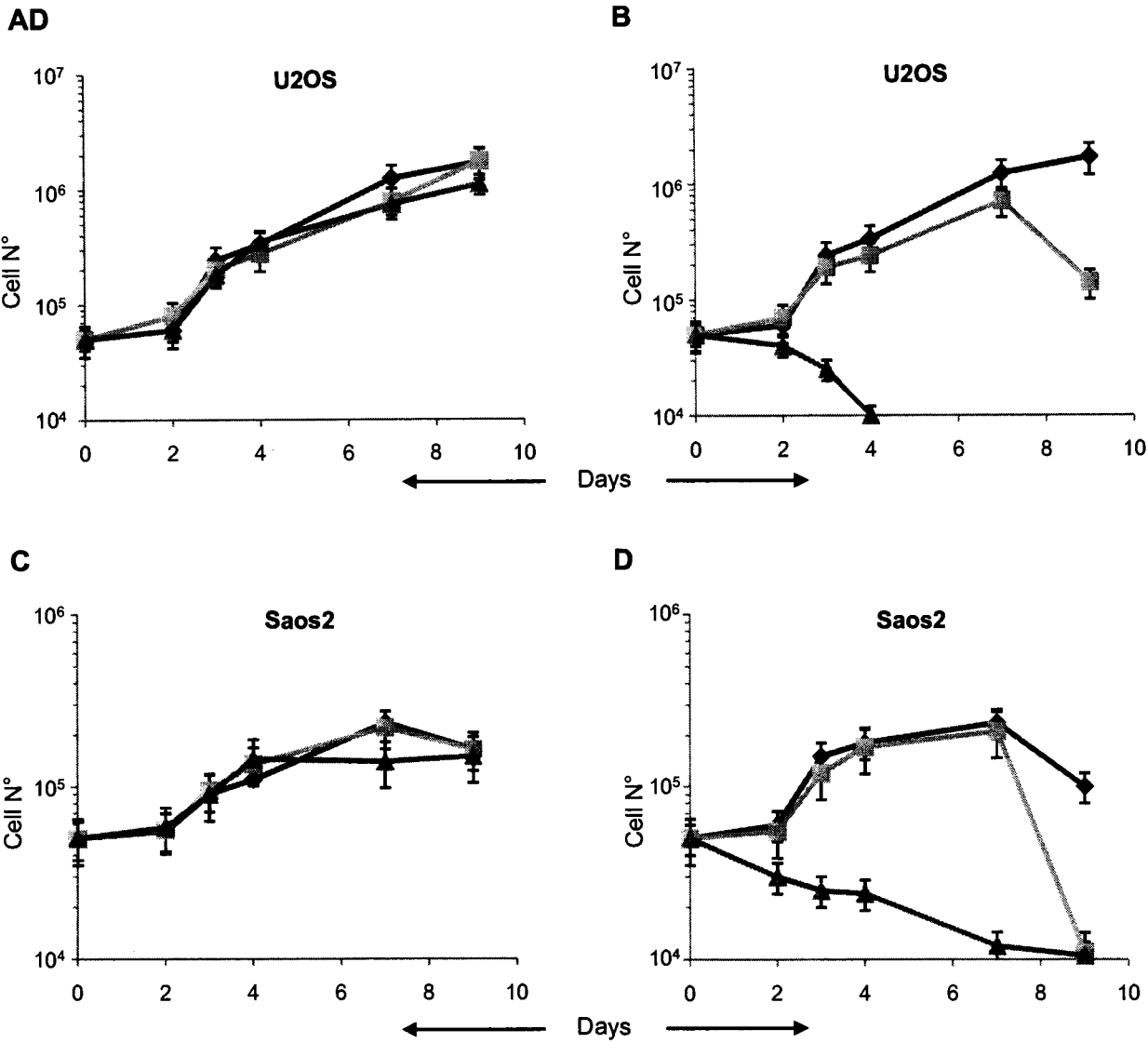
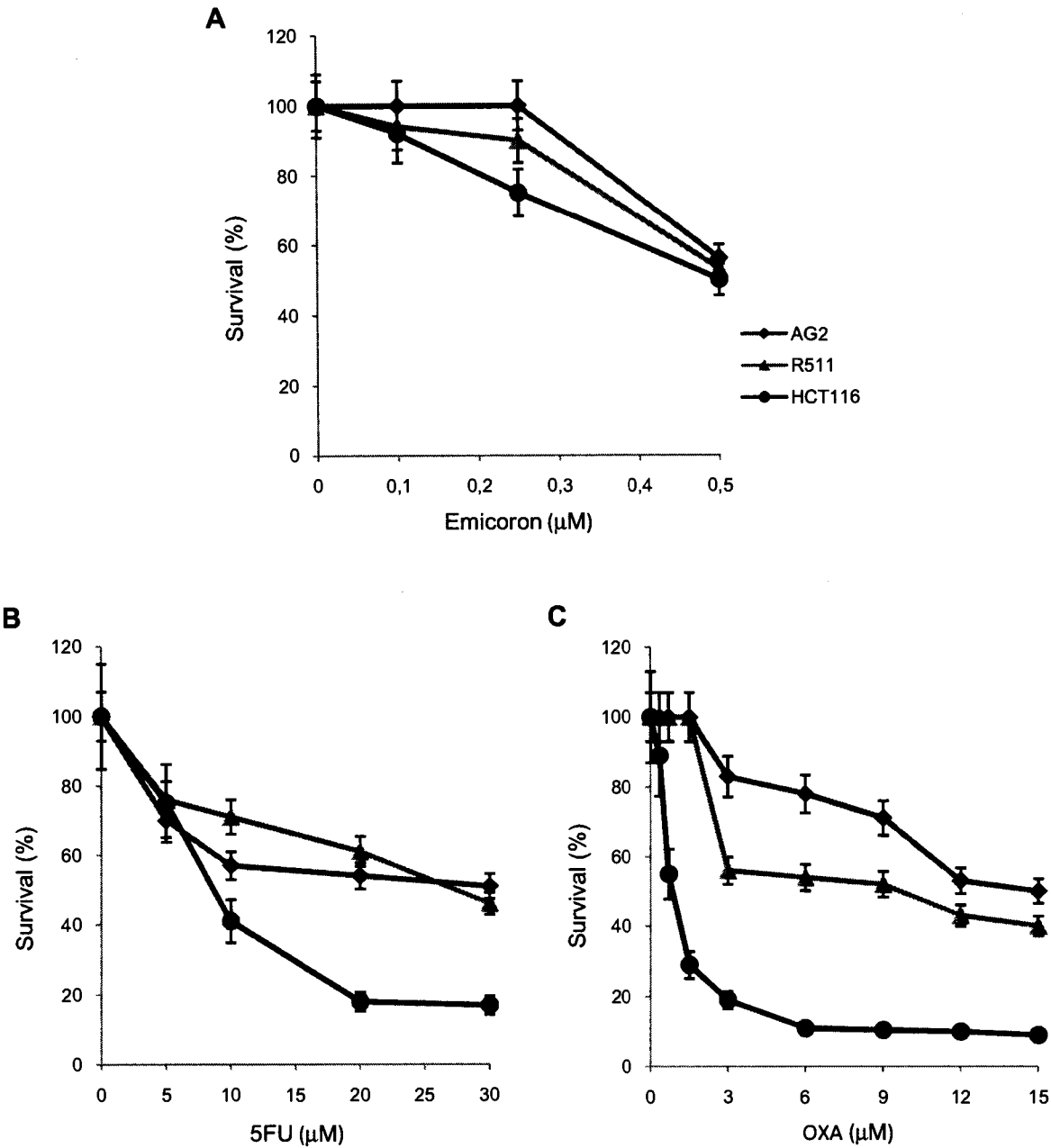


Figure 11

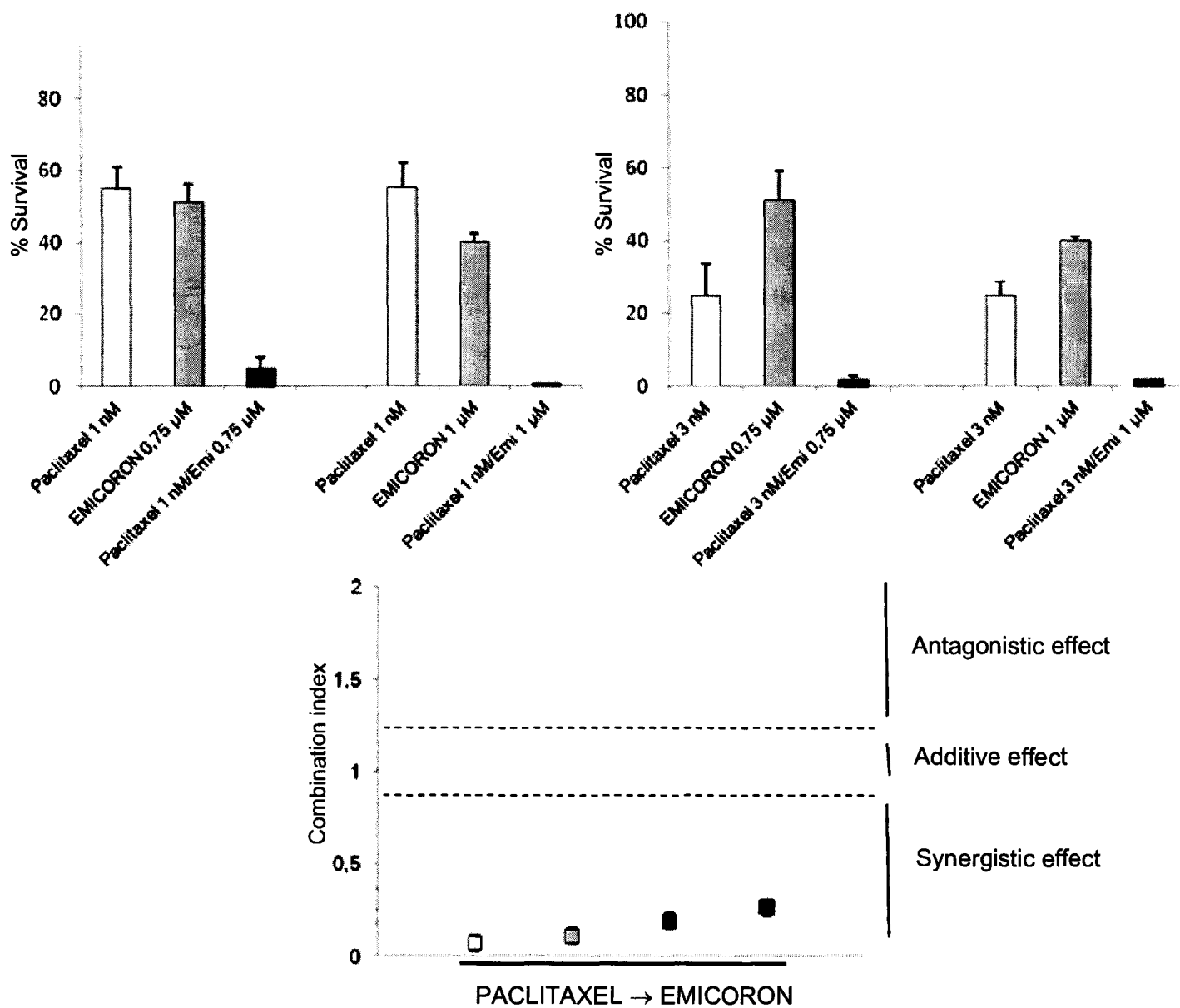
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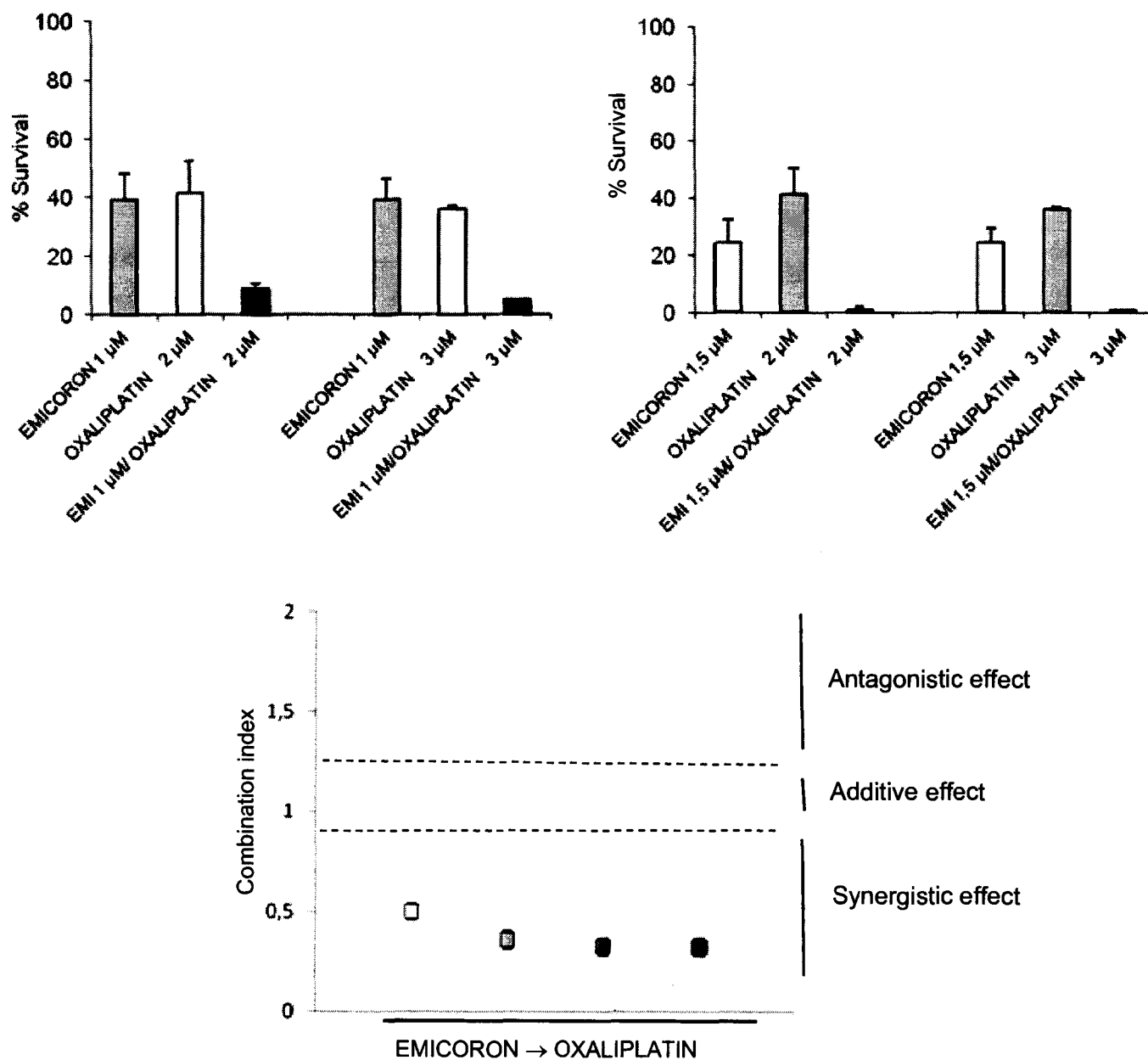
Figure 12

PACLITAXEL → EMICORON



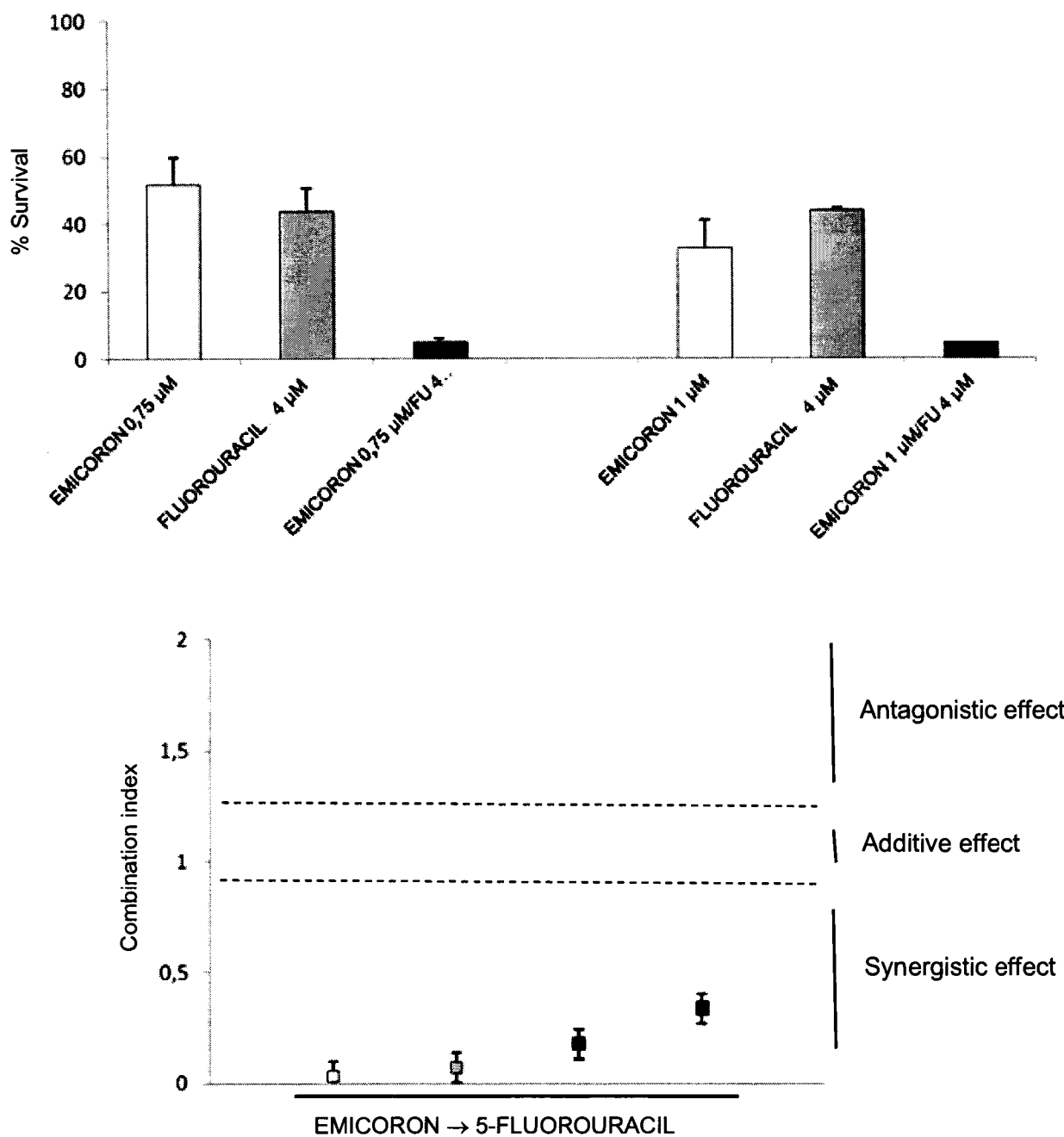
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Figure 13

**EMICORON → OXALIPLATIN**

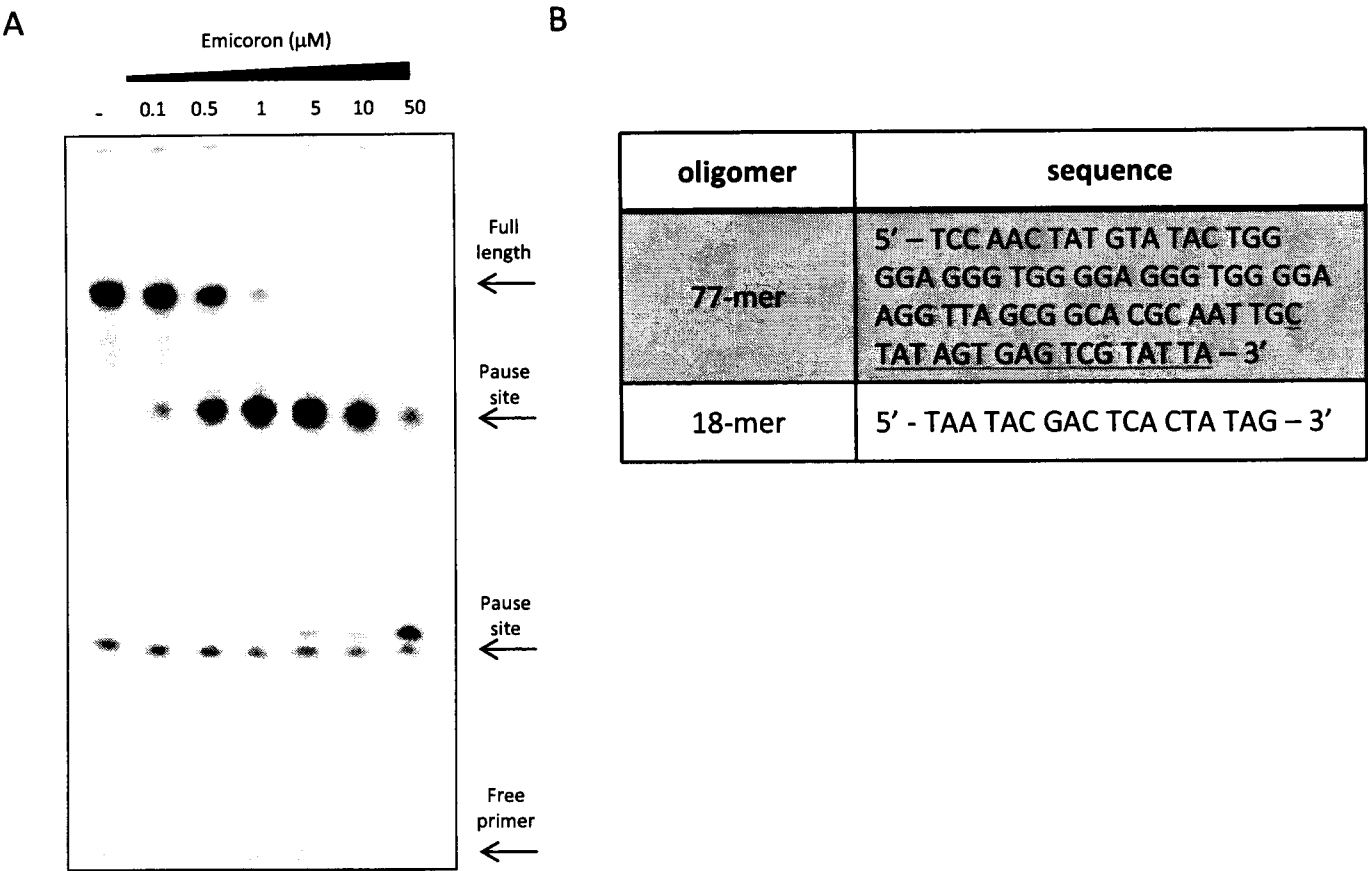
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Figure 14

**EMICORON → 5-FLUOROURACIL**

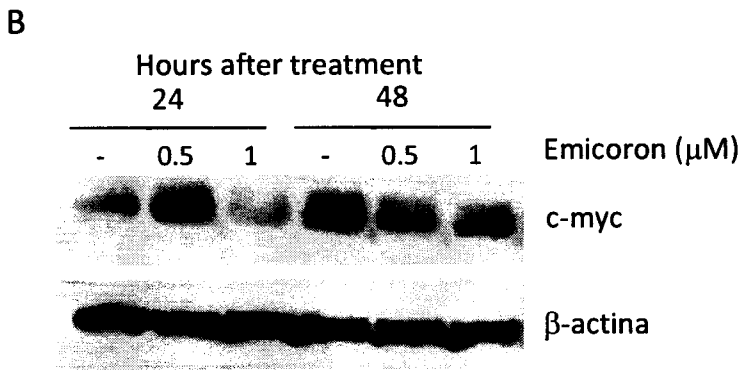
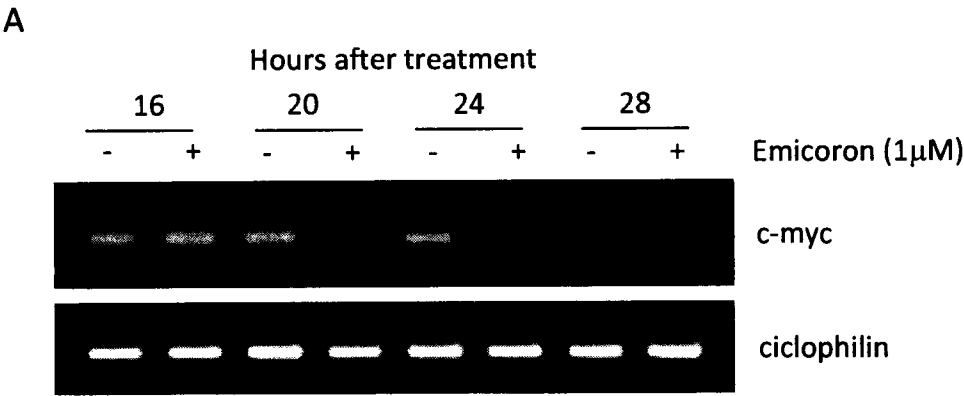
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Figure 15



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Figure 16



**C**

| oligomer | sequence                                |
|----------|-----------------------------------------|
| forward  | 5' – CCA TGA GGA GAC ACC GCC – 3'       |
| reverse  | 5' – TCT TGT TCC TCC TCA GAG TCG C – 3' |

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/IT2013/000281

A. CLASSIFICATION OF SUBJECT MATTER  
INV. A61K31/4545 A61P35/00  
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                     | Relevant to claim No. |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | <p>WO 2008/126123 A2 (UNIV ROMA [IT]; BIANCO ARMANDODORIANO [IT]; FRANCESCHIN MARCO [IT]; AL) 23 October 2008 (2008-10-23) cited in the application abstract<br/>page 3 - page 4<br/>page 7 - page 8<br/>page 10; compound VI<br/>claims 1,2,9-15</p> <p style="text-align: center;">-----<br/>-/-</p> | 1-4                   |

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

\* Special categories of cited documents :

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"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search

5 February 2014

Date of mailing of the international search report

17/02/2014

Name and mailing address of the ISA/

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Authorized officer

Damiani, Federica

## INTERNATIONAL SEARCH REPORT

International application No

PCT/IT2013/000281

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                                                                                  |                       |
|------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                               | Relevant to claim No. |
| Y                                                    | <p>VALENTINA CASAGRANDE ET AL: "N -Cyclic Bay-Substituted Perylene G-Quadruplex Ligands Have Selective Antiproliferative Effects on Cancer Cells and Induce Telomere Damage",<br/>JOURNAL OF MEDICINAL CHEMISTRY,<br/>vol. 54, no. 5, 10 March 2011 (2011-03-10)<br/>, pages 1140-1156, XP055059669,<br/>ISSN: 0022-2623, DOI: 10.1021/jm1013665<br/>the whole document</p>                                                      | 1-15                  |
| Y                                                    | <p>ERICA SALVATI ET AL: "Telomere damage induced by the G-quadruplex ligand RHPS4 has an antitumor effect",<br/>JOURNAL OF CLINICAL INVESTIGATION,<br/>vol. 117, no. 11,<br/>1 November 2007 (2007-11-01), pages<br/>3236-3247, XP055060207,<br/>ISSN: 0021-9738, DOI: 10.1172/JCI32461<br/>the whole document</p>                                                                                                               | 1-15                  |
| X,P                                                  | <p>MARCO FRANCESCHIN ET AL: "Aromatic Core Extension in the Series of N-Cyclic Bay-Substituted Perylene G-Quadruplex Ligands: Increased Telomere Damage, Antitumor Activity, and Strong Selectivity for Neoplastic over Healthy Cells",<br/>CHEMMEDCHEM,<br/>vol. 7, no. 12,<br/>24 October 2012 (2012-10-24), pages<br/>2144-2154, XP055100318,<br/>ISSN: 1860-7179, DOI:<br/>10.1002/cmdc.201200348<br/>the whole document</p> | 1-15                  |

## INTERNATIONAL SEARCH REPORT

### Information on patent family members

International application No

PCT/IT2013/000281

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|-------------------------------------------|---------------------|----------------------------|---------------------|
| WO 2008126123                             | A2                  | 23-10-2008                 | NONE                |
| -----                                     |                     |                            |                     |