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Original Paper

Preclinical Activity of 17β-[N-[N′-(2-chloroethyl)-N′-nitrosocarbamoyl]-L-alanyl]-5α-dihydrotestosterone (E91) against Tumour Colony Forming Units and Haematopoietic Progenitor Cells

P. Rank,[1] R. Peter,[1] H. Depenbrock,[1,2] G. Eisenbrand,[3] P. Schmid,[4] H. Pitzl[4] and A.-R. Hanauske[1,2]

[1]Technische Universität München, Klinikum Rechts der Isar, Ismaningerstr. 22, D-81675 München, Germany; [2]Universitair Ziekenhuis Gasthuisberg, Department of Oncology, Herestraat 49, B-3000 Leuven, Belgium; [3]Universität Kaiserslautern, Department of Chemistry, Division of Food Chemistry and Environmental Toxicology, D-67663 Kaiserslautern; and [4]Klinikum Harlaching, Sanatoriumsplatz 2, D-81545 München, Germany

E91 (17β-[N-[N′-(2-chloroethyl)-N′-nitrosocarbamoyl]-L-alanyl]-5α-dihydrotestosterone) (CNC-ala-DHT) is a newly synthesised alkylating compound consisting of N-[N′-(2-chloroethyl)-N′-nitrosocarbamoyl]-L-alanine (CNC-ala) as the alkylating moiety and of 5α-dihydrotestosterone (DHT) as a steroid carrier molecule. We studied the antitumour activity of E91 (final concentrations: 0.1, 1, 10 and 30 μmol/l) against freshly explanted human tumours, using an *in vitro* soft agar cloning system. A total of 54 tumour samples was evaluated using 1 h-exposure and 51 tumour specimens were studied using a continuous exposure for 21–28 days. In addition, the compound's activity was compared with other clinically used anticancer agents. After short-term exposure, 49 of 53 evaluable specimens (92%) had adequate colony formation, as compared with 49 of 50 (98%) after long-term exposure. After short-term exposure, E91 exhibited only marginal antitumour activity. However, in long-term exposure experiments, E91 had marked and concentration-dependent antitumour activity ($P < 0.001$). At concentrations of > 10 μmol/l, E91 was as active as the other clinically used antineoplastic agents and at 30 μmol/1, E91 was significantly more active than 5-fluorouracil ($P = 0.041$). E91 showed activitiy against a wide spectrum of tumour types. The highest activity was observed against colorectal carcinomas (3/4 tumour specimens inhibited at 30 μmol/l). Sensitivity was also high remarkable in breast cancer specimens with 3/6 specimens inhibited at 30 μmol/l. *In vitro* myelotoxicity was less than that of doxorubicin. At 30 μmol/l, E91 induced a reduction of colony forming units–granulocyte macrophage (CFU–GM) to only 53% of control and of CFU-GEMM to 20% of control. We conclude that because of broad activity and reduced myelotoxicity further clinical development of E91 appears warranted.

Key words: clonogenic growth, human tumours, steroidal alkylating agents
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INTRODUCTION

THE DISCOVERY of steroid hormone receptors and hormone responsiveness in a series of human tumours, such as breast cancer [1, 2], prostate cancer [3], ovarian- and endometrial cancers [4, 5], gastrointestinal cancers [6, 7], small cell lung cancer [8] and meningiomas [9–11] has led to the concept of a receptor-mediated chemotherapy. Various investigators have subsequently focused on the synthesis of steroid hormone-linked cytotoxic agents in order to direct the cytotoxic moiety specifically to the hormone receptor containing tumour tissue

Correspondence to A.-R. Hanauske, Centre for Haematology and Oncology, Landwehrstr. 2, D-80336 München, Germany.
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and by doing so to achieve higher antitumour efficacy and lower toxicity. These studies have indicated that the chemical link between cytotoxic moiety and hormone should allow for release of the cytotoxic moiety by hydrolysis or by enzymatic cleavage. Consequently, an ester-type link has appeared to be appropriate [12, 13]. At present, only prednimustine (prednisolone ester of chlorambucil) and estramustine phosphate (oestradiol-3-N-lost-carbamate-17-phosphate) are hormone-linked antitumour compounds that carry clinical promise [14, 15]. Eisenbrand and colleagues have synthesised and evaluated a series of 2-chloroethylnitrosoureas linked both to oestradiol and to androgens in various ester positions [16, 17]. Among the oestradiol-linked compounds, the 17-ester of N-(2-chloroethyl)-N-nitrosocarbamoyl (CNC)-α-alanine was superior with regard to its antitumour efficacy in methylnitrosourea (MNU)-induced mammary cancer of the rat. However, in animals, this oestradiol-17β-conjugate retained the cumulative toxicity to the bone marrow, characteristic for nitrosoureas, and also showed substantial toxic side-effects to ovaries and uterus, inducing marked local inflammation and pyometra. Within the androgen-linked group, E91 (Figure 1) appeared to be the most promising agent. This compound showed *in vivo* activity against the androgen-, progesterone- and oestrogen-receptor containing MNU-induced mammary cancer of the rat and against the transplantable rat leukaemia L5222 [17, 18]. *In vitro*, E91 effectively inhibited growth of a number of established malignant cell lines including K-562F, HL-60, MDA-MB-231 [19]. IC_{50}-values of 1.1 and 1.8 μmol/l have been reported for K-562F and HL-60, respectively. *In vivo* studies in female NMR-mice indicated nearly no toxicity of E91 to bone marrow [20].

E91 has considerable binding affinity to androgen receptors and less affinity to progesterone receptors, while it is virtually without affinity to oestrogen receptors. The relative binding affinity value (RBA) is 5.0 for the androgen receptor, 0.12 for the progesterone receptor and 0.01 for the oestrogen receptor [18]. The predominant mechanism of absorption of E91 in tissues still remains to be determined. Jager has reported accumulation both in steroid receptor-positive and receptor-negative normal tissues (uterus, ovary, bladder, brain, kidney) as well as in receptor-positive and receptor-negative tumours [19]. From these observations, it has been concluded that both specific binding to steroid hormone receptors and binding to other proteins, for example sex steroid hormone binding globulin (SHBG), might be of importance. The purpose of our present study was to evaluate the extent of myelotoxicity and antitumour activity of E91. Using a capillary soft agar cloning system, we studied the antitumour activity of E91 on freshly explanted clonogenic tumour cells and compared the conjugate's activity with other clinically used antineoplastic agents. The toxicity of E91 on haematopoietic stem cells was evaluated in a methylcellulose-based cloning assay and compared with toxicity of doxorubicin.

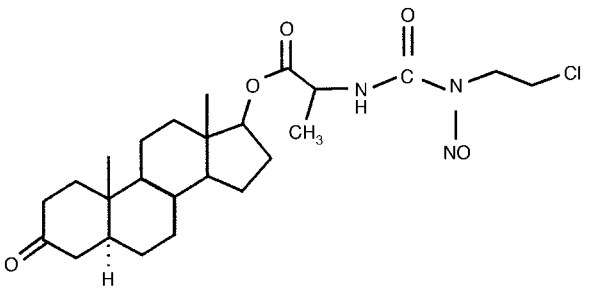

Figure 1. Structure of 17β-[N-[N′-(2-chloroethyl)-N′-nitroso-carbamoyl]-L-alanyl]-5α-dihydrotestosterone (E91), MW 496.1.

MATERIALS AND METHODS

Antitumour agents

E91 was synthesised as described previously [18]. Stock solutions and final solutions were prepared in Solutol HS15/1,2-propanediole [0.134 mg/ml]. E91 was studied at final concentations of 0.1, 1, 10 and 30 μmol/l using appropriate solvent controls. Other antitumour agents were clinical preparations and were used at final concentrations corresponding to 0.1 of their clinically observed peak plasma concentration: cisplatin [0.2 μg/ml], doxorubicin [0.04 μg/ml], mitomycin-C [0.1 μg/ml], bleomycin [0.2 μg/ml], paclitaxel [0.34 μg/ml], vinblastine [0.05 μg/ml], 5-fluorouracil [6.0 μg/ml], etoposide [3.0 μg/ml]. Stock solutions of all agents were stored at − 80°C prior to use.

Capillary soft agar cloning system

Tumour specimens from previously untreated patients and from patients who had received a variety of chemotherapy regimens were obtained by sterile standard procedures as part of routine clinical measures. Biopsies of solid tumours were stored in McCoy's 5A medium containing 5% fetal calf serum, 10 mmol/l hydroxyethylpiperazine ethanesulphonic acid (Hepes), 1 mmol/l sodium pyruvate, 90 U/ml penicillin, and 90 μg/ml streptomycin (all supplied by Gibco, Renfrewshire, U.K.) for transport to the laboratory. Preservative-free heparin (10 U/ml, Novo Nordisk, Mainz, Germany) was added immediately after collection of fluids to prevent coagulation. Solid tumour specimens were minced and repeatedly passed through metal sieves with mesh widths of 100 μm (Linker, Kassel, Germany) to obtain single cell suspensions. Effusions were centrifuged at 112 ***g*** for 7 min. If clumps of cells were detected by microscopic inspection, the suspension was passed through metal sieves with mesh widths of 50 μm (Linker) and 25 gauge needles. Cells were cryopreserved in culture medium containing 10% dimethyl sulphoxide (DMSO, Serva, Heidelberg, Germany) by freezing with a rate of − 2°C/min down to − 175°C and stored in liquid nitrogen. Prior to experiments, the cells were thawed and centrifuged at 112 ***g*** for 7 min. Then the supernatant with the DMSO was removed and the cells resuspended in McCoy's 5A medium. A capillary soft agar cloning system was used as described earlier [21–23]. The median density of seeded cells was 2.4×10^4 cells/capillary (range 1.7×10^4–5.6×10^4) in long-term exposure and 2.7×10^4 (range 2.0×10^4–6.3×10^4) in short-term exposure. Cells were seeded into 100 μl glass capillaries (Brand, Wertheim, Germany) in a mixture of 0.3% agar (Sigma, Deisenhofen, Germany) in double-enriched Connaught Medical Research Laboratories' Medium 1066 (Gibco) containing 15% horse serum (Gibco), 2% fetal calf serum (Gibco), 0.3 mmol/l vitamin C (Merck, Darmstadt, Germany), 90 U/ml penicillin, 90 μg/ml streptomycin, 10 mmol/l Hepes, 100 μg/ml asparagine (Gibco), 2 mmol/l sodium pyruvate, 0.1 mmol/l non-essential amino acids (Gibco), 4 mmol/l glutamine (Gibco), 4 ng/ml hydrocortisone (Sigma), 50 U/ml catalase (Serva, Heidelberg, Germany) and 0.1 nmol/l Epidermal Growth Factor (Flow, Meckenheim, Germany). For each data point, six capillary tubes were used.

Each experiment contained one set of controls with 0.134 mg% Solutol HS15/1.2-propanediole for E91 and one set of control with 0.9% NaCl for the clinically used antineoplastic agents and a third set with 1 mmol/l ammonium monovanadate (Merck) to assure the presence of a good single cell suspension [24]. Colony formation was evaluated with an inverted microscope after an incubation period of 21–28 days at 37°C, 5% CO_2 and 100% humidity. An experiment was considered evaluable if the solvent control had a mean $\geq$ three colonies per capillary and the vanadate control showed $<30\%$ colony formation compared with Solutol or NaCl, respectively.

Effects on clonogenic haematopoietic precursor cells

Cells from two frozen peripheral stem cell harvests were thawed and seeded at a density of 10^5 cells/plate in Petri dishes (Nunc, Naperville, Illinois, U.S.A.) in MethoCult H4431 medium (Stemcell Technologies Inc., Vancouver, B.C., Canada) containing 10% fetal calf serum and 1% glutamine. Colony forming units were evaluated with an inverted microscope after an incubation period of 10–14 days at 35°C, 5% CO_2 and 100% humidity and were classified as CFU-GEMM, CFU-GM and clusters. E91 was studied at concentrations of 0.1, 1, 10 and 30 μmol/l. For comparison, doxorubicin was investigated at concentrations of 0.0069, 0.069 and 0.69 μmol/l (corresponding to 0.01, 0.1 and 1×peak plasma concentration).

Statistical analysis

Data were calculated as means and standard deviations of at least three evaluable determinations per data point. Colony survival was calculated by expressing the average number of colony forming units from cells exposed to each antitumour agent relative to the average number of colony forming units from untreated controls. Results were evaluated statistically by the Friedman test and McNemar's test [25, 26]. P values ≤ 0.05 were interpreted as indicating significant differences. The Friedman test was used to compare the percentage survival of tumour cells exposed to different concentrations of E91. McNemar's test was used to compare the number of tumours resistant or sensitive to E91 and clinically used antitumour agents.

RESULTS

The antitumour effect of E91 was studied in a total of 51 tumours following long-term exposure and in a total of 54 tumours following short-term exposure. One specimen had to be excluded from further analysis because of bacterial contamination following both exposures. In long-term exposure experiments, 49 of the remaining 50 specimens (98%) showed sufficient tumour colony growth for evaluation. In short-term exposure experiments 49 of the 53 specimens (92%) were evaluable. Median colony formation in solvent controls was 14.8 colonies/capillary (range 3.2–97) in long-term exposure and 10.8 colonies/capillary (range 3.1–84.1) in short-term exposure. Table 1 summarises the tumour types studied and the evaluability per tumour type. The major tumour types accrued were non-small cell lung cancer, ovarian cancer, breast cancer, mesothelioma and colorectal cancer. The inhibitory activity of E91 following long-term exposure on tumour colony forming units is summarised in Table 2. *In vitro* growth of specimens was inhibited in a statistically significant concentration-dependent manner ($P<0.001$, Friedman test). E91 showed clear antitumour efficacy at concentrations >10 μmol/l, with 11/49 (22%) inhibited specimens at 10 μmol/l and 20/46 (43%) inhibited specimens at 30 μmol/l. Thus, E91 exhibited broad-spectrum antitumour efficacy rather than tumour-specific activity. After long-term exposure to 30 μmol/l, the highest activity was noted against colorectal cancer with 3/4 specimens inhibited. Marked antitumour activity was also observed against breast cancer (3/6 inhibited) and ovarian cancer (4/9 inhibited). E91 was less active against non-small cell lung cancer with 3/13 (23%) specimens inhibited, while no activity was notable against mesothelioma. Twenty per cent (10/50) of the tumours were from previous untreated patients. At concentrations ≥ 10 μmol/l these tumours were more sensitive to E91 than tumours from patients who had previous exposure to antitumour agents (at 10 μmol/l: 56% (5/9) inhibited versus 15% (6/40), at 30 μmol/l: 88% (7/8) inhibited versus 34% (13/38)). Results of short-term exposure are summarised in Table 3. E91 had only minor antitumour activity at 30 μmol/l with 3/47 (6%) specimens inhibited. In summary, antitumour efficacy of E91 was significantly higher in long-term exposure than in short-term exposure ($P<0.001$ at 30 μmol/l and $P=0.013$ at 10 μmol/l, Friedman test).

Table 1. *Types of tumours studied and evaluability per tumour type*

Tumour type	Long-term exposure No. evaluable*/ No. attempted	1 h-exposure No. evaluable*/ No. attempted
Non-small cell lung	13/13	13/13
Ovarian	10/10	11/11
Breast	6/6	5/6
Mesothelioma	4/4	5/5
Colorectal	4/4	4/5
Other†	12/13	11/13
Total	49/50 (98%)	49/53 (92%)

In the short-term (1-h) drug exposure schedule 49/50 (98%) tumour specimens were evaluable and in the long-term drug exposure schedule 49/53 (92%). *≥ 3 colonies/capillary in controls; †pancreas, unknown primary site, renal cell, liver cell, small cell lung, non-Hodgkin's lymphoma, testicular, soft tissue sarcoma, bladder, malignant melanoma.

Table 2. *Inhibitory activity of E91 against tumour colony forming units from freshly explanted human tumours* in vitro *using a long-term exposure schedule indicating concentration-dependent antitumour activity of E91*

Tumour type	No. specimens inhibited*/ no. specimens evaluable† E91 [μmol/l] 0.1	1.0	10.0	30.0
Non-small cell lung	1/13	1/13	1/13	3/13
Ovarian	1/10	0/10	0/10	4/9
Breast	0/6	2/6	2/6	3/6
Mesothelioma	0/4	0/4	0/4	0/3
Colorectal	0/4	0/4	3/4	3/4
Other‡	0/12	1/12	5/12	7/11
Total	2/49 (4%)	4/49 (8%)	11/49 (22%)	20/46 (43%)

*$\leq$ 50% survival of tumour colony forming units; †≥ 3 colonies/capillary in controls; ‡pancreas, unknown primary site, renal cell, liver cell, small cell lung, non-Hodgkin's lymphoma, testicular, soft tissue sarcoma, bladder, malignant melanoma.

Table 3. Inhibitory activity of E91 against tumour colony forming units from freshly explanted human tumours in vitro *using a short-term exposure schedule (1 h)*

Tumour type	No. specimens inhibited*/ no. specimens evaluable† E91 [μmol/l] 0.1	1.0	10.0	30.0
Non-small cell lung	0/13	0/13	0/12	1/13
Ovarian	0/11	0/11	0/10	0/10
Breast	0/5	0/5	1/5	2/5
Mesothelioma	0/4	0/4	0/5	0/4
Colorectal	0/3	0/3	0/4	0/4
Other‡	0/10	0/11	0/11	0/11
Total	0/46 (0%)	0/47 (0%)	1/47 (2%)	3/47 (6%)

In this exposure schedule E91 had only marginal activity. *≤ 50% survival of tumour colony forming units; †≥ 3 colonies/capillary in controls; ‡pancreas, unknown primary site, renal cell, liver cell, small cell lung, non-Hodgkin's lymphoma, testicular, soft tissue sarcoma, bladder, malignant melanoma.

The *in vitro* evaluation of toxicity of E91 on haematopoietic stem cells from two stem cell harvests is summarised in Figure 2a–c. Toxicity of E91 on GEMM, CFU-GM and cluster was more pronounced following long-term exposure. With long-term exposure, IC_{50}-values of E91 for GEMMs, CFU-GMs and clusters were in the range of 5–50 μmol/l. At 30 μmol/l, CFU-GM were reduced to 53% of control levels and CFU-GEMM to 20%. In short-term exposure, there was only marginal haematotoxicity with 62% survival for CFU-GMs, 80% for GEMMs and 84% for clusters at 30 μmol/l (data not shown). Haematotoxicity induced by doxorubicin at concentrations 0.0069, 0.069 and 0.69 μmol/l (corresponding to 0.01, 0.1, and 1.0× peak plasma concentration) was markedly more pronounced than haematotoxicity induced by E91. We could not observe interindividual variation in sensitivity to E91. In comparing the antitumour activity of E91 to that of clinically used antitumour agents (concentrations corresponding to 0.1× peak plasma concentration), sufficient data for statistical analysis were obtained for cisplatin, doxorubicin, mitomycin-C, bleomycin, paclitaxel, vinblastine, 5-fluorouracil and etoposide. In long-term exposure at 10 μmol/l, E91 was as active as the other antineoplastic agents (1 h exposure). At 30 μmol/l, E91 was significantly more active than 5-fluorouracil ($P = 0.041$, McNemar's test). After short-term exposure there was no significant difference in antitumour efficacy between E91 and mitomycin-C, vinblastine, cisplatin, bleomycin, doxorubicin and etoposide. However, paclitaxel was significantly more active ($P = 0.046$, McNemar's test). We also investigated whether growth inhibition of breast cancer depends on progesterone- and oestrogen-receptor status. The receptor status of 4 of 6 breast carcinomas were available. Three specimens were oestrogen- and progesterone-receptor-positive and one specimen was receptor-negative. After long-term exposure two of three receptor-positive breast cancers were inhibited by E91 at concentrations > 1 μmol/l. One receptor-positive specimen showed no sensitivity towards E91. The receptor-negative specimen was inhibited at 30 μmol/l. In short-term exposure, only two receptor-positive specimens were available: one specimen was inhibited at concentrations of > 10 μmol/l, while the other was resistant to E91. One receptor-negative specimen was inhibited at concentrations of > 10 μmol/l.

DISCUSSION

This study showed that E91 is significantly more active following long-term exposure rather than short-term exposure. Whilst showing only marginal activity following short-term exposure, E91 displayed a clear concentration-dependent activity in long-term exposure with relevant antitumour activity. At 30 μmol/l, the antitumour activity was comparable to that of clinically used anticancer agents and was higher than that of 5-fluorouracil. Our data indicate that future clinical phase I trials should preferably include prolonged or rapidly repeating dosing schedules to improve the likelihood of clinically notable antitumour activity. Also, our results support findings of earlier studies, which compared the efficacy of split dose administration (5×21 μmol/kg per week for 5 weeks) with high dose single administration of E91 (1×105 μmol/kg per week for 5 weeks) in the MNU-induced mammary carcinoma [27]. Split dose administration was found to be superior to high dose administration. After six weeks of therapy, T/C values (the median tumour volume of treated versus controls) were 9% for split dose administration and 26% for high dose administration. In an attempt to predict the extent of myelosuppression in humans, we evaluated the effects of E91 on human haematopoietic progenitor cells. At 30 μmol/l, survival of CFU-GEMMs was 20% of control, CFU-GM survival was 53% and cluster formation survival

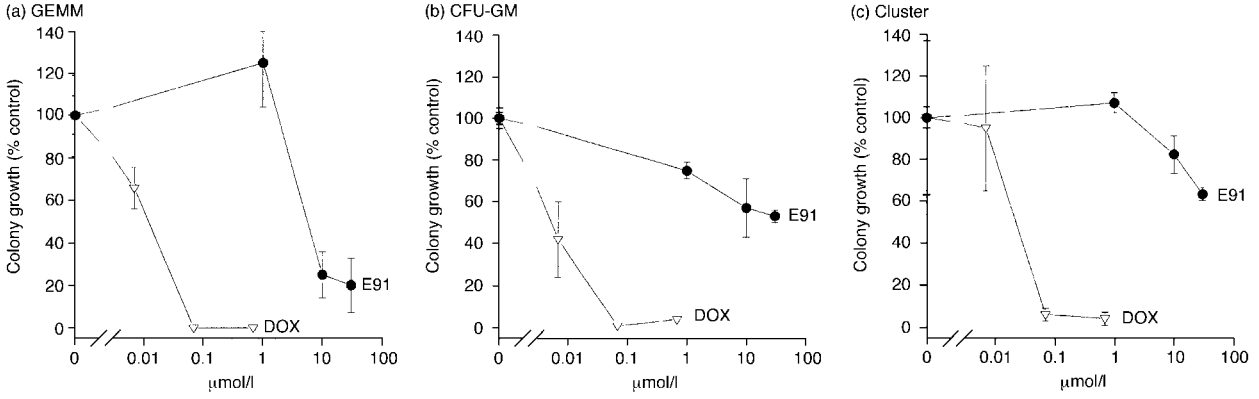

Figure 2. *In vitro* evaluation of toxicity of E91 on haematopoietic precursor cells using long-term exposure. Toxicity of E91 at 30 μmol/l was markedly lower than toxicity of doxorubicin at 0.069 μmol/l (1/10 peak plasma concentration): (a) GEMM; (b) CFU-GM; (c) cluster; DOX, doxorubicin.

was 63% of controls. The IC_{50}-value for human CFU-GMs was 30 μmol/l. Based on our results it may thus be hypothesised that clinically active concentrations of E91 might also induce moderate leucopenia. Our data are also compatible with reports from Eisenbrand and Brix indicating that myelotoxicity of CNUs might be reduced through a link to appropriate hormone carriers [17, 18]. Of note is that our results do not confirm tumour-specific efficacy of E91. Instead, E91 retained the broad antitumour spectrum known for nitrosoureas and had a marked activity against colorectal carcinoma. The activity against breast cancer was of interest, since E91 is known to be active against MNU-induced rat mammary carcinomas [16] and against both MCF-7 (receptor-positive) and MDA-MB 231 (receptor-negative) breast cancer cells. Particularly following long-term exposure, we observed a tendency for higher efficacy of E91 in receptor-positive specimens. Interestingly, Jager [19] described a significantly higher sensitivity of receptor-negative cells *in vitro* following long-term exposure. It was suggested that the presence of 3β, 17β-steroid dehydrogenase in receptor-positive cells might be responsible for decomposition of E91 to metabolites with oestrogenic activity, which potentially counteracts the activity of E91. Since the activity of E91 was not limited to receptor-containing tumour-types in our study, further studies are required to evaluate the influence of hormone receptor status on tissue-specific absorption and antitumour activity of E91. Clinical evaluation of the activity of E91 should, however, not be restricted to hormone receptor expressing tumour types. Comparative studies with unconjugated nitrosoureas also appear warranted to determine the difference in activity and toxicity between both compounds.

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