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EO9 (Apaziquone): From the clinic to the laboratory and back again.

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Running title: Preclinical and clinical history of EO9

Abstract

EO9 (Apaziquone) is a bioreductive drug that has a chequered history. It underwent clinical trial but failed to show activity in phase II clinical trials when administered intravenously. Poor drug delivery to tumours caused by a combination of rapid pharmacokinetic elimination and poor penetration through avascular tissue are the major causative factors responsible for EO9's poor efficacy. Based upon an understanding of why EO9 failed, a further clinical trial against patients with superficial transitional cell carcinoma of the bladder was conducted. The rationale for this was that intravesical administration directly into the bladder would circumvents the drug delivery problem and any drug reaching the blood supply would be rapidly cleared thereby reducing the risk of systemic exposure. EO9 was well tolerated and clinical activity against marker lesions was recorded in both phase I and II clinical trials. This article charts the pharmacological history of EO9 and discusses the potential implications that 'the EO9 story' has for the development of other loco-regional therapies.

Abbreviations: AUC: Area under the curve; EORTC: European Organisation for the Research and Treatment of Cancer; ICL: Interstrand Cross Link; NQO1: NAD(P)H:Quinone oxidoreductase-1; PD: Pharmacodynamics; PK: Pharmacokinetics; NSCLC: Non Small Cell Lung Cancer; TCC: Transitional Cell Carcinoma of the bladder; TUR: Trans-Urethral Resection.

Introduction

EO9 (3-hydroxy-5-aziridinyl-1-methyl-2 (1H-indole-4,7-dione)prop-β-en-α-ol) is a prodrug that belongs to a class of anti-cancer agents known as bioreductive drugs. Various chemical classes of bioreductive drugs have been developed (Denny, 2004; Hay *et al.*, 2008; Hay *et al.*, 2007a; Hay *et al.*, 2007b; Milbank *et al.*, 2009; Tercel *et al.*, 2009) and all require enzymatic reduction by various oxidoreductases in order to generate cytotoxic metabolites. This activation process is reversed in the presence of oxygen and these agents have preferential activity against hypoxic tumour cells (McKeown *et al.*, 2007; Stratford *et al.*, 1998). Selectivity is determined by the presence of elevated levels of reductases in tumours and the absence of oxygen (figure 1). At the time EO9 was developed, these compounds represented a from the classical way anticancer drugs were developed of identifying active compounds first and then identifying mechanisms of action towards targeted therapeutic agents that selectively exploit aspects of tumour biochemistry and physiology.

EO9 was originally developed at the University of Amsterdam in the mid 1980's (Oostveen *et al.*, 1987). The project was initially sponsored by the Dutch Cancer Society and a series of 90 indolequinone (given the name EO) derivatives of Mitomycin C were developed, the ninth of which was EO9 (figure 1). Preclinical and clinical evaluation was co-ordinated by the New Drug Development Office of the European Organisation for the Research and Treatment of Cancer (EORTC) and a number of laboratories belonging to the Screening and Pharmacology (SPG) and Pharmacology and Molecular Mechanism (PAMM) groups across Europe played key roles in the pharmacological evaluation of these compounds. Clinical evaluation of EO9 was halted by lack of efficacy in phase II trials (Dirix *et al.*, 1996; Pavlidis *et al.*, 1996). Based upon an understanding of why EO9 failed, a further phase I/II clinical trial against superficial bladder cancer using intravesical administration was commissioned in 2001 and sponsored by Spectrum Pharmaceuticals (Irvine, California). Significant anti-tumour activity was reported in the phase I/II study (Puri *et al.*, 2006) and this was subsequently confirmed in phase II

studies (van der Heijden *et al.*, 2006). Phase III trials are currently underway and the results are expected in the first quarter of 2012. The purpose of this article is to review the pharmacology of EO9, its preclinical and clinical history and to discuss the potential implications that this story has for the development of other loco-regional therapies.

Pharmacology of EO9.

EO9 is activated by several enzymes, the most widely studied of these is NAD(P)H:Quinone oxidoreductase 1 (NQO1 or DT-diaphorase). NQO1 is a cytosolic flavoprotein that catalyses the two electron reduction of a wide range of substrates (Ernster, 1987) and its physiological function is detoxification of quinones (Cadenas, 1995). The chemistry of the side chains attached to the quinone nucleus dictates the reactivity of the reduced form (Cadenas, 1995) and in the case of EO9, it is reduced to a DNA damaging species. In cell free systems, reduction of EO9 by NQO1 results in the generation of DNA damage in the form of single strand breaks (Walton *et al.*, 1991). Catalase inhibits this process (Phillips *et al.*, 1999) suggesting that hydrogen peroxide is formed during the redox cycling of the EO9 hydroquinone in oxygen (Bailey *et al.*, 1998; Butler *et al.*, 1996). Alkylation of DNA is possible via the release of hydroxyl groups at C2 and C3 as well as protonation and opening of the aziridine ring (Hargreaves *et al.*, 2000). DNA inter-strand cross links (ICL) following the reduction of EO9 by purified rat NQO1 under hypoxic conditions have been reported (Maliepaard *et al.*, 1995) although other groups have not observed ICLs following reduction by purified human NQO1 under aerobic conditions (Phillips, 1996). Limited information exists about the formation of mono-adducts. Other purified enzymes have been shown to reduce EO9 and induce either single strand breaks or DNA cross links including xanthine oxidase (Maliepaard *et al.*, 1995) and NADPH cytochrome P450 reductase (Bailey *et al.*, 2001).

In cell based assays, EO9 does not behave as a classical hypoxia targeted bioreductive drug as it also has activity against aerobic cells (Collard *et al.*, 1995; Hendriks *et al.*, 1993; Phillips *et al.*, 1992; Plumb *et al.*, 1994a; Roed *et al.*, 1989; Smitskamp-Wilms *et al.*, 1994). Activation of EO9 still conforms to

the concept of 'enzyme directed bioreductive therapy' (Workman *et al.*, 1990) as therapy could still be targeted at tumours that expressed high levels of NQO1. The role of NQO1 in activating EO9 to DNA damaging species in cell free assays is clear but its role in determining cellular response is more complex. Under aerobic conditions, good correlations between NQO1 activity and chemosensitivity *in vitro* have been reported (Collard *et al.*, 1995; Fitzsimmons *et al.*, 1996; Plumb *et al.*, 1994b; Plumb *et al.*, 1994c; Robertson *et al.*, 1994; Robertson *et al.*, 1992; Smitskamp-Wilms *et al.*, 1994). In hypoxia however, significant potentiation of EO9's activity was only seen in cell lines that lack NQO1 activity (Plumb *et al.*, 1994b; Plumb *et al.*, 1994c; Robertson *et al.*, 1994). In cell lines where NQO1 was high, EO9 was as effective against aerobic and hypoxic cells. Mechanistically, it is likely that one electron reductases play a prominent role in the hypoxia selectivity whereas reduction of EO9 by NQO1 is an oxygen independent process (Workman, 1994). EO9 can therefore be used to target the hypoxic regions of NQO1 deficient tumours whereas in NQO1 rich tumours, EO9 will target both the aerobic and hypoxic fraction. This feature of EO9's pharmacology was seen as a unique and attractive feature as it suggested that EO9 has the capacity to exhibit single agent activity against solid tumours (Hendriks *et al.*, 1993). These preclinical studies also suggested that EO9 might find its optimal use in combination with radiation or other drugs.

In animal tumour models, EO9 was inactive against the P388 murine leukaemia but exhibited anti-tumour activity against human tumour xenografts and the generally chemo-resistant murine adenocarcinomas of the colon (MAC) tumours (Collard *et al.*, 1995; Hendriks *et al.*, 1993; Roed *et al.*, 1989). Initial evidence that *in vivo* response correlated with NQO1 activity (Walton *et al.*, 1992) was not substantiated in subsequent studies where poor relationships between NQO1 activity and *in vivo* activity were reported (Collard *et al.*, 1995; Cummings *et al.*, 1998). EO9 was selected for clinical evaluation based upon its novel mechanism of action (which was distinct from MMC), its preferential activity against cells derived from solid tumours *in vitro* and *in vivo*, its ability to target both aerobic and hypoxic cells and the lack of myelosuppression in mice and rats (Hendriks *et al.*, 1993).

Clinical evaluation: Two phase I trials started in 1992 under the auspices of the EORTC. In the first study, the maximum tolerated dose following a three week schedule (q3wk) of 5 min intravenous infusion was 27 mg/m^2 (Schellens *et al.*, 1994). Bone marrow suppression was not observed and the dose limiting toxicity was reversible proteinuria. In a second phase I trial using a weekly bolus intravenous schedule (q1wk), a maximum tolerated dose of 14 mg/m^2 was reported and the dose limiting toxicity was again reversible proteinuria (Aamdal *et al.*, 2000; McLeod *et al.*, 1996). Damage to glomeruli was observed in the clinical trial and this correlated with high levels of NQO1 in the kidney (Segura-Aguilar *et al.*, 1994; Zappa *et al.*, 2003). A total of three partial responses were recorded in the phase I studies; two in patients with adenocarcinoma of unknown primary site and one in a patient with bile duct cancer. Phase II clinical trials commenced in the summer of 1994 and two studies were conducted. In the first, a total of 92 patients with advanced breast, gastric, pancreatic and colorectal cancer were treated with a 5 min intravenous infusion of EO9 at a weekly dose of 12 mg/m^2 (Dirix *et al.*, 1996). No anti-tumour activity was seen. A second study involved the treatment of 38 chemotherapy naïve patients with advanced non-small cell lung cancer (NSCLC). Two treatment schedules were evaluated; a single bolus intravenous injection at 12 mg/m^2 administered weekly and intravenous bolus injection at 22 mg/m^2 administered every three weeks. Dose limiting toxicity was reversible proteinuria and whilst stable disease was reported in thirteen patients, these studies concluded that EO9 at these doses and schedules had no clinical activity against NSCLC (Dirix *et al.*, 1996; Pavlidis *et al.*, 1996).

Reasons for EO9's failure: In a critique of the design of the clinical studies conducted, Connors (Connors, 1996) highlighted certain key deficiencies in clinical trial design. These included the fact that NQO1and/or hypoxia were not measured in patient tumour samples, a fact that can be partially explained by an incomplete understanding of EO9s mechanism of action in the 1990's when the trials were designed. Both these parameters are key determinants of EO9's activity and it is conceivable that tumours lacked the appropriate biochemistry required for drug activation. This is however unlikely as high

NQO1 expression and hypoxia is typically found in many solid tumours (Siegel *et al.*, 1998; Siegel *et al.*, 2000; Vaupel *et al.*, 2001; Vaupel *et al.*, 2007). On the other hand, EO9 should have been evaluated in hypoxic tumours that lack NQO1 in combination with other modalities (such as radiotherapy) that target the aerobic fraction. Post irradiation treatment of tumours *in vivo* with EO9 indicated that radiosensitization was obtained in various preclinical tumour models (Adams *et al.*, 1992; Burd *et al.*, 2005) but no clinical trials of this nature were conducted. In any future trials of Apaziquone or other bioreductive drugs, it is therefore advised that levels of hypoxia and enzymology (particularly NQO1 in the case of apaziquone) should be measured so that they can be used as potential biomarkers to further stratify patient outcomes.

In addition to concerns about the design of the clinical trials, attention also focused on whether or not drug delivery to tumours was impaired. The factors that determine how much drug is delivered to tumours can be broadly grouped into supply (extent of tumour vasculature and pharmacokinetics), flux (the drugs ability to penetrate through multiple layers of cells) and metabolism/sequestration of drugs within cells or the extracellular matrix (Minchinton *et al.*, 2006). In mice, the half life was 1.9 ± 0.1 min and the AUC was 4.8 µg.min/ml following the iv administration of EO9 at 12 mg/kg and in male Sprague-Dawley rats, the half life was 3.0 ± 0.2 min with an AUC of 6.2 µg.min/ml following an iv dose of 3 mg/kg (Workman *et al.*, 1992). The rapid clearance and extremely short half life of EO9 in rodents was replicated in man with half lives ranging from 0.8 to 19 min at the maximum tolerated dose of 27 mg/m^2 administered intravenously (Schellens *et al.*, 1994). Similar preclinical and clinical data were reported by other groups (Bibby *et al.*, 1993b; McLeod *et al.*, 1996).

Given this data, it was clear that the supply of EO9 to tumours is likely to be impaired by its poor PK properties. To some extent, poor PK can be offset if the 'flux' of drugs through avascular tissue is good but early studies using 3

dimensional multilayered post-confluent cultures and multicell spheroids suggested that drug penetration barriers may exist (Bibby *et al.*, 1993a; Pizao *et al.*, 1993). Resistance in these models could be due to a multitude of reasons including low cell proliferation rates, reduced extracellular pH, reduced nutrient status etc. EO9 is however preferentially active against cells in acidic extracellular pH (Phillips *et al.*, 1992), is able to kill confluent monolayer cultures (Phillips *et al.*, 1997) and is active against hypoxic cells. In 1996, Cowan et al (Cowan *et al.*, 1996) described an assay that could quantify the rate at which drugs crossed multicell layers *in vitro*. EO9 is able to cross DLD-1 human colorectal cancer multicell layers but in comparison to tirapazamine (a nitroimidazole based bioreductive drug), its penetration rate is slow (Phillips *et al.*, 1998). This study concluded that when EO9's rapid pharmacokinetic elimination is taken into consideration, EO9 would only penetrate a few microns from a blood vessel within its pharmacokinetic lifespan and this is the probable reason for its failure to demonstrate efficacy in the clinic (Phillips *et al.*, 1998).

Whilst inadequate drug delivery to tumours appears a plausible explanation, the question remains as to why EO9 is active against preclinical tumour models (Hendriks *et al.*, 1993) but inactive in clinical trials despite PK properties being similar in rodents and humans? A critical review of the preclinical studies however reveals that the magnitude of anti-tumour response observed is low with specific growth delays of only a few days reported. This level of activity would be acceptable if EO9 functioned purely as a hypoxia targeted agent as cytotoxic effects against hypoxic cells would be masked by the continued growth of the aerobic fraction of cells. In NQO1 rich tumours however, EO9 would target the aerobic fraction of cells and in this case, a much greater level of activity would be expected. EO9 does induce some responses in preclinical tumours and partial responses and stable disease was seen in phase I and II studies (Pavlidis *et al.*, 1996; Schellens *et al.*, 1994) so some EO9 is reaching the tumour. Direct intra-tumoural injection of EO9 results in improved anti-tumour activity (Loadman *et al.*, 2002) supporting the fact that sub-optimal concentrations of EO9 reach tumours following systemic administration.

Clinical evaluation of EO9 against superficial bladder cancer: Based upon this understanding of why EO9 failed, investigators were presented with two options; to develop analogues of EO9 that retained its good PD properties but had improved PK properties (Loadman *et al.*, 2002; Phillips, 1996; Phillips *et al.*, 2004; Phillips *et al.*, 1999) or utilise EO9's bad properties to our advantage. In this latter case, loco-regional administration of drug would circumvent the problem of drug delivery and if the drug could be retained at this site for long periods, improved penetration into the tumour would occur. Furthermore, any drug that reached the systemic circulation would be rapidly cleared thereby reducing the risk of systemic toxicity. Superficial transitional cell carcinoma (TCC) of the bladder provided a suitable clinical model to test this hypothesis as intravesical administration of chemotherapy or immunotherapy following transurethral resection (TUR) is an established mode of treatment (Hall *et al.*, 2007). Following studies which demonstrated that TCC of the bladder expressed the key biochemical machinery required to activate EO9 (figure 2) (Basu *et al.*, 2004; Choudry *et al.*, 2001), a phase I/II pilot study commenced in 2004.

The purpose of this trial was to establish the dose of EO9 (now renamed EOquin by the sponsor Spectrum Pharmaceuticals which was later changed to Apaziquone) that could be safely administered intravesically and obtain evidence of efficacy against a marker lesion left *in situ* at TUR (Gofrit *et al.*, 2010; Puri *et al.*, 2006). Six patients with multifocal superficial TCC of the bladder received escalating doses of EO9 (0.5 – 16 mg/40ml) administered intravesically which was retained within the bladder for 1 hour. EO9 was well tolerated at doses up to 4 mg/40ml and grade 2 and 3 dysuria and haematuria was observed at doses at or above 8 mg/40ml. No EO9 could be detected in plasma (Puri *et al.*, 2006). A further 6 patients received EO9 at 4 mg/40ml once a week for 6 weeks which was well tolerated in all cases. Analysis of EO9 in the urine at the end of the instillation demonstrated that the concentration increased linearly with dose and therapeutically effective concentrations were being achieved (Puri *et al.*, 2006). At 4 mg/40ml (100 µg/ml), the concentration of EO9 in the urine at the end of the1 hour

instillation was 72. 2 ± 11.8 µM (20.79 µg/ml) which represents a significant increase in drug exposure parameters compared to those reported following intravenous administration (table 1). A total of 8 out of 12 patients had complete remission as defined by complete loss of the marker lesion, negative cytology and histology at the site of the lesion (Puri *et al.*, 2006). Representative images of the marker lesion *in situ* before and after EO9 are presented in figure 2.

Phase II studies were conducted using an identical study design to the phase I/II pilot study and each patient received 6 weekly intravesical instillations of EO9 at 4 mg/40ml with the first instillation starting two weeks after TUR (van der Heijden *et al.*, 2006). EO9 was well tolerated with no systemic side effects and grades 1 to 3 dysuria and haematuria being the most common local side effects. Of a total of 45 patients with superficial TCC of the bladder, 30 (67%) patients had complete response as defined by complete macro- and microscopic elimination of a marker lesion (van der Heijden *et al.*, 2006). Recurrence free rates were good in comparison to the results of other ablative studies (Hendricksen *et al.*, 2009; Jain *et al.*, 2009). EO9 was reformulated in 2007 (van der Schoot *et al.*, 2007a; van der Schoot *et al.*, 2007b; van der Schoot *et al.*, 2008) and it is currently undergoing phase III clinical evaluation in multiple centres across North America and Europe. Additionally, studies where EO9 was administered within 24 hours of TUR which is the standard recommended treatment for superficial TCC of the bladder (Sylvester *et al.*, 2004) demonstrated that EO9 was well tolerated and has a good safety profile (Hendricksen *et al.*, 2008).

Conclusions and future prospects:

EO9 has had a chequered history but by understanding the reasons why it failed, EO9 has been transformed from a clinically inactive drug to one that has efficacy against superficial bladder cancer. The outcome of the phase III clinical trials is eagerly anticipated but even so, the 'EO9 story' demonstrates that compounds with poor systemic PK could be effectively used in a loco-regional setting. In the case of EO9, direct intravesical administration circumvented the drug delivery problem encountered following intravenous

administration and resulted in high concentrations of drug that are confined within the bladder. In this setting, EO9's poor PK properties were advantageous as any drug that reached the blood stream was rapidly cleared. Whilst this review has focused specifically on EO9, our experience with EO9 has potentially significant implications for the development of loco-regional therapies in general. We suggest that compounds with good PD properties but poor systemic PK could be valuable therapeutic agents for treating cancers in a loco-regional setting.

Loco-regional chemotherapy is emerging as an important adjunct to surgery and systemic chemotherapy in selected patients with certain types of cancer (Ceelen et al., 2010; Lu et al., 2010). In addition, as early detection strategies for cancer become more effective, there is a case for using loco-regional chemotherapy as an adjunct to surgical excision. Therapeutic agents used for loco-regional therapies are typically conventional chemotherapeutic that are widely used to treat systemic disease. These can be effective but the success of this approach has been restricted by local toxicity, inadequate drug penetration and systemic toxicity caused by drugs leaching out from the site of administration into the blood stream (Lu et al., 2010; Masters et al., 1996; Masters et al., 1990). In terms of selecting drugs for use in a loco-regional setting, experience with EO9 has shown that compounds with poor systemic PK can be locally efficacious without the systemic toxic side effects observed when administered intravenously. In the context of the majority of drug discovery programs, compounds with poor systemic PK are typically rejected during the process of lead compound optimisation and currently reside on the medicinal chemists shelves. Based upon our experience with EO9, we suggest that many of these compounds should be revisited and re-evaluated as potential loco-regional therapies.

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Table 1. Summary of pharmacokinetic parameters following the intravenous administration of EO9 (5 min infusion) and intravesical administration (one hour instillation) at maximum tolerated doses. The values quoted following intravenous administration refer to plasma levels whereas following intravesical administration, values quoted are in the urine.

Route of administration	Dose	Cmax (µg/ml)	Cmax (µM)	AUC (µM.h)	T½ (min)
Intravenous*	27 mg/m^2	1.418 ± 0.911	4.92 ± 3.16	0.468	7.8 ± 5.6
Intravesical[#]	4mg/40ml	100.00	347.2	204.8	36.6

* Data obtained from Schellens et al (Schellens *et al.*, 1994)
# Data obtained from Puri et al (Puri *et al.*, 2006)

Figure 1. Chemical structure of EO9 (A) and scheme for possible activation of EO9 leading to DNA damage (B). 2e and 1e in panel B represent 2 electron reduction (via enzymes such as NQO1) and 1 electron reduction (via enzymes such as cytochrome P450 reductase) respectively. Q, SQ and HQ denote Quinone (parent compound), Semi-Quinone (1 electron reduction product) and Hydroquinone (2 electron reduction product) respectively.
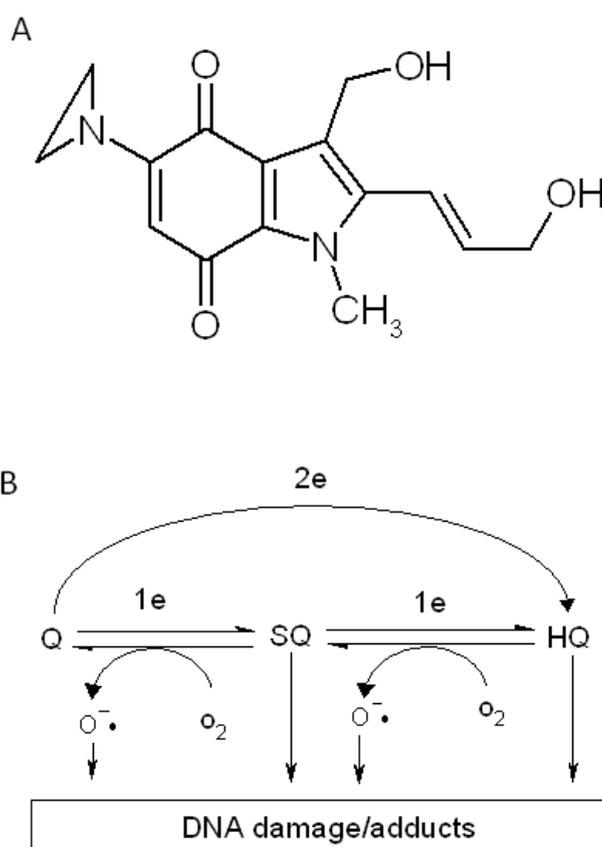

Figure 1

Figure 2. Representative immunohistochemical analysis of superficial human TCC of bladder expressing high levels of NQO1 (A), Glucose transporter 1 (Glut-1) (B) and pimonidazole treated tumour (C). Glut-1 and pimonidazole have been used as endogenous and exogenous markers of hypoxia respectively (Rademakers *et al.*, 2011). The brown staining in panels A to C represents areas of positive staining. Appearance of marker lesion (ML) *in situ* before (D) and after (E) a 6 week course of EO9 administered intravesically once a week at a dose of 4 mg/40ml.