

Tumour-Selective *Salmonella*-Based Cancer Therapy

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Introduction

Cancer therapies fail for several primary reasons; lack of drug effect on the cancerous tissue, lack of selectivity for the cancerous tissue, and/or inadequate delivery to the target tissue. Drug effect and selectivity can be improved by increased understanding of molecular and cellular differences between cancer and normal tissues, thus enabling the design of drugs that potently affect cancer-specific molecular targets associated with malignant behaviour. Another approach is to improve the selective delivery of anti-cancer agents to tumours. One approach is to use carriers that bind to cancer-specific targets, such as antibodies (Hall, 1995). However, most targeting approaches, even if selective, tend not to deliver sufficiently high concentrations of the agent to the tumour to induce significant therapeutic effects. Recent findings suggest that the pathogenic bacterium *Salmonella*, when genetically modified, can be used to selectively deliver therapeutic agents to solid tumours at high concentrations (Pawelek *et al.*, 1997; Low *et al.*, 1999a). These attenuated bacteria are administered either systemically or locally, whereupon they typically replicate 1000 times greater in the tumour than in other tissue. The basis for preferential colonization and accumulation of *Salmonella* in tumours appears to include some of the same characteristics of tumours that provide resistance to drug and immune-based therapies (Bermudes *et al.*, 2000a,b).

Why tumours are susceptible to *Salmonella* is not well understood and probably includes a variety of factors. Poor penetration of components of the immune system, including antibodies, complement, CD8+ T-cells, granulocytes and macrophages

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Abbreviations: CD, cytosine deaminase; cfu, colony forming units; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; IC₅₀, 50% inhibitory concentration; J, joule; mpk, milligrams per kilogram; PCR, polymerase chain reaction; UV, ultraviolet.

which limits an immunological response to tumours may be a major factor contributing to susceptibility of tumours to *Salmonella* (Low *et al.*, 1999a). The faster rate of growth of tumours is now recognized to result in aberrant angiogenesis (Jain, 1994) and can result in hypoxia, anoxia and necrosis as well as contributing to a positive pressure within the tumour. As facultative anaerobes, *Salmonella* are readily capable of sustained metabolism under hypoxic and anoxic conditions as well as fully aerobic respiration in well-oxygenated environments. Further, their flagellar motility (approx. 10 $\mu\text{m}/\text{sec}$) allows them to resist positive pressures in order to fully penetrate into tumours and remain there. The initial studies of genetically engineered tumour-specific *Salmonella* (Pawelek *et al.*, 1997) have demonstrated that auxotrophy for amino acids, pyrimidines, and purines, inhibited the growth of *Salmonella* in liver tissue, one of the primary sites of *Salmonella* pathogenesis, but retained the high-level growth in tumour tissue. Earlier studies by Bacon *et al.* (1950a,b, 1951), Hoiseth and Stocker (1981) and O'Callaghan *et al.* (1988) had shown that purine and amino acid mutations were highly attenuating for *Salmonella*. Introduction of free purines intravenously (Bacon *et al.*, 1951) restored virulence. Therefore, these attenuating mutations work through limiting availability of an essential requirement, possibly within macrophages where *Salmonella* virulence factors are essential for their survival (Fields *et al.*, 1986, 1989). However, these factors appear not to be limiting within the tumour as evidenced by the same high level of intra-tumoural growth by auxotrophic mutants. The auxotrophic mutations resulted in a 30-fold enhancement in specificity for tumours, increasing from a tumour to liver ratio of 300:1 for wild-type *Salmonella* to 9000:1 for a purine mutant at the two day time point (Pawelek *et al.*, 1997). This increased ratio for the auxotrophic mutant is primarily due to a decrease of the bacteria within the liver, with similarly high intra-tumour levels (1×10^9 cfu/g tissue) occurring for both strains. The apparent abundance of intra-tumoural nutrients may be a consequence of the uncontrolled tumour growth, with either the increased metabolism of the cells, and/or the associated necrosis from irregular blood supply contributing to the overall availability.

Other general features of *Salmonella* also probably contribute to their ability to reach tumours and exploit the tumour environment (Bermudes *et al.*, 2000a,b). *Salmonella* are serum resistant (Joiner, 1988), and thus readily pass through the vasculature. Inside the tumour the bacteria are both extracellular (interstitial and necrotic spaces) and intracellular (Pawelek *et al.*, 1997), thus providing for delivery in both subcellular locations.

Genetic methods for generation of tumour-specific strains

Our continuing development of tumour-specific strains has sought to include the features of antibiotic sensitivity as well as stable deletion mutations in key attenuating genes (*purI* and *msbB*) resulting in the strain VNP20009 (Low *et al.*, 1999b) which is currently in human clinical development (Clairmont *et al.*, 2000; King *et al.*, 2000; Lee *et al.*, 2000; Sznol *et al.*, 2000). Here, we briefly describe some of the genetics and selection of these strains and their anti-tumour activity. Following this, we also describe an example of the use of these bacteria in combination with X-irradiation (Platt *et al.*, 2000) and to deliver proteins such as cytosine deaminase (King *et al.*, 1998) with anti-tumour activity directly to the tumour.

MUTATION TO AUXOTROPHY

In order to introduce auxotrophic mutations (e.g. *pur*⁻) so as to attenuate various *Salmonella* strains, three mutagenic procedures have been used:

- (1) Nitrosoguanidine: The procedure of Miller (1992) was used, except that prior to a 20 minute nitrosoguanidine treatment the cells were grown in modified minimal medium 56 (Low, 1973) with 0.5% glycerol as a carbon source. The slow growth rate in this medium reduces the number of multiple DNA replication forks and reduces the number of multiple site mutations caused by the nitrosoguanidine treatment.
- (2) Ultraviolet light (UV): A dose of 50 J/m² of UV light (wavelength, $\lambda = 254$ nm) was used to treat cells prepared as for the nitrosoguanidine treatment.
- (3) Transposon pools: Tn10 donor plasmid pNK2883 (Kleckner *et al.*, 1991) was used to pseudorandomly mutagenize *Salmonella* strains when gene disruptions were desired.

For all the above approaches, the percentage of auxotrophs among survivors can be increased by enrichment by growth in penicillin (Eisenstadt *et al.*, 1994). In addition, for all the above approaches, the auxotrophic populations were sampled for randomness by identifying auxotrophs by replica plating colonies onto rich and minimal media (Low, 1973), and then by identifying the requirements of individual auxotrophs using nutrient pools (Davis *et al.*, 1980).

An additional method of introducing auxotrophic mutations (for example, the introduction of *purI*⁻) made use of existing published Tn mutations (Altman *et al.*, 1996), which were introduced into the desired strains by the use of P22-mediated transduction (see below).

Specific knock-out mutations

In order to introduce reversionless mutations for complete stability, three approaches were used:

- (1) Bochner selection from Tn10 insertions mutants: With most genetic backgrounds it is possible to derive tetracycline (Tet) sensitive derivatives of a Tet-R strain (for example, one which carries a Tn10 insertion) by selection on media containing fusaric acid (Bochner *et al.*, 1980; Davis *et al.*, 1980). In many cases the resulting Tet-S derivative arises by a deletion of the Tet-R determinant, including neighbouring DNA. Thus some of the Tet-S derivatives are deleted for one or more of the genes bordering the original Tn10 insertion. Such mutants are absolutely stable to reversion, except for any possible extragenic suppressors. In this way we derived the stable *purI* deletion mutation in strain YS1646 (= VNP20009).
- (2) Transformation of selectable engineered knock-out: If a cloned gene is available on a plasmid, it can be engineered to carry a selectable antibiotic resistance determinant as a substitution for part or all of the gene, while leaving some of the flanking chromosomal DNA (approximately 500 base pairs or, preferably, more) intact. After linearizing such a plasmid, it can be transformed into a *recD*⁻ recipient in order to select for integration of the substitution mutation and loss of

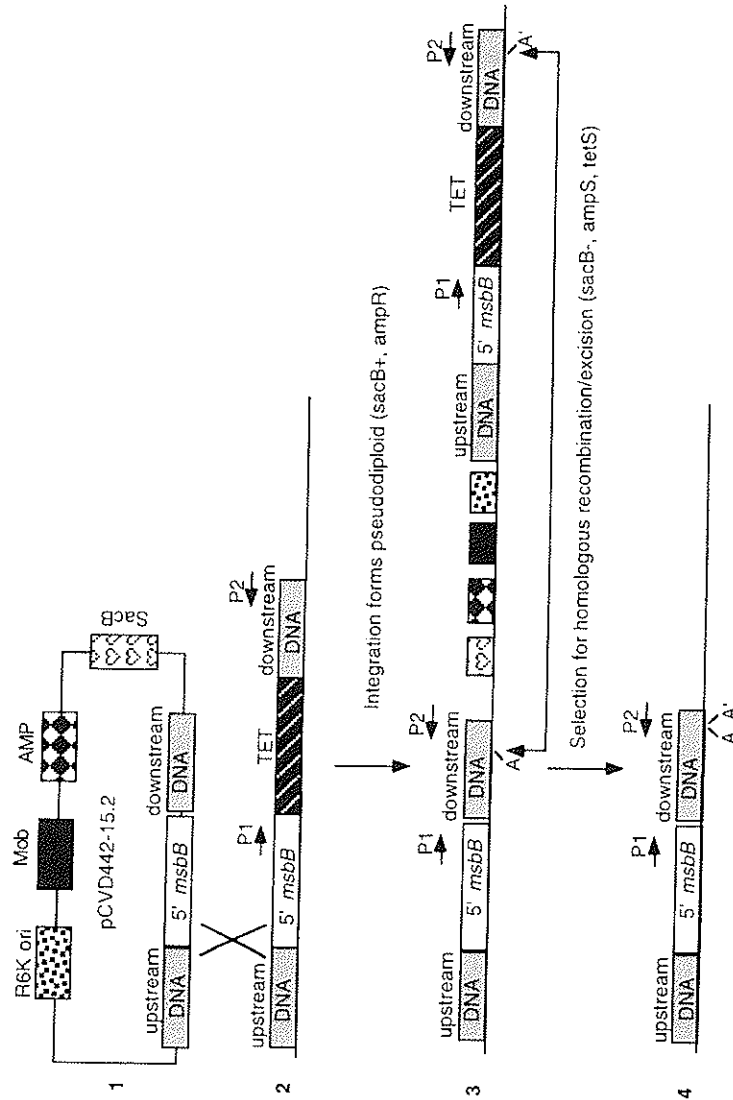


Figure 9.1. 1) pCVD442-*msbB* vector, 2) homologous recombination with the Δ *msbB* chromosomal copy in *Salmonella* YS26 (derived from YS82, Low *et al.*, 1999a) which contains a tetracycline resistance determinant (TET) in *msbB* referred to as *msbB1* below, 3) the chromosomally integrated vector resulting in both *msbB1* and the disrupted *msbB* not containing tetracycline resistance referred to as *msbB2*, and 4) following sucrose resistance selection, the genetic organization in strain VNP20009 (sec #3 for an example of possible crossover locations A and A'). *R6Kori*, the plasmid origin of replication, *Mob*, the mobilization element in order for this plasmid to be transferred from one strain to another. *Amp* the beta-lactamase gene which confers sensitivity to β -lactam antibiotics such as carbenicillin and ampicillin. *SacB*, the gene which confers sensitivity to sucrose. Note: not drawn to scale.

the wild-type allele (Russell *et al.*, 1989). In this way we constructed an *msbB* knock-out mutation (Low *et al.*, 1999b).

- (3) Use of a suicide vector to introduce knock-outs: In various situations the use of the Bochner method for obtaining deletions is not convenient, for example in *msbB*⁻ strains which are sensitive to the Bochner medium. As another approach, a partial or complete deletion knock-out can be introduced into a strain on a conjugative plasmid which cannot replicate except in the donor strain from which it is transferred. If the plasmid also carries a selectable antibiotic resistance marker as well as metabolite sensitivity marker such as *sacB* (which confers sensitivity to sucrose), it is possible to sequentially select for introduction of the plasmid, which then integrates into the chromosomal allele of the knocked out gene, or flanking DNA, followed by selection for loss of the plasmid (*sac* gene) by a different crossover on the other side of the knock-out, to result in a deletion on the chromosome. This is essentially the same type of system reported by Donnenberg and Kaper (1991). In this way, we introduced the final deletion in *msbB* in strain YS1646 (Low *et al.*, 1999b), as indicated in *Figure 9.1*.

Generalized transduction

In order to move selectable markers from one strain to another we used bacteriophage P22 for generalized transduction (Davis *et al.*, 1980; Sternberg and Maurer, 1991).

Anti-tumour efficacy of VNP20009

The anti-tumour activity of VNP20009 has been evaluated in a tumour panel comprising murine transplantable tumours and human tumour xenografts (*Table 9.1*). A single bolus intravenous injection of VNP20009 (1×10^4 to 1×10^6 cfu/mouse) retards the growth of subcutaneously implanted B16-F10 melanoma. The anti-tumour effect of the anti-neoplastic drug cyclophosphamide, given at 200 mg/kg once weekly for 3 weeks, is equivalent to or worse than VNP20009. Functional T-cells, B-cells, or NK-cells are not required for VNP20009 activity, since similar activity occurs in *nu/nu*, SCID and beige mice (Luo *et al.*, 1999).

The anti-tumour effect of VNP20009 on human tumour xenografts derived from lung carcinomas A549 and HTB117, colon carcinomas DLD1 and WiDr, Lox melanoma, and breast carcinoma MDA-MB-231 has also been evaluated (*Table 9.1*). A single intravenous injection of VNP20009, ranging from 1×10^6 to 3×10^6 cfu/mouse, significantly inhibits the growth of the tumours, paralleling observations made in murine tumour models. Growth inhibition was particularly impressive in late stage DLD1 tumours, where VNP20009 almost completely inhibited the tumour growth (Luo *et al.*, 1999).

An intravenous injection of VNP20009 (2×10^6 cfu/mouse) also significantly inhibited the growth of lung metastases of B16-F10 melanoma, compared to untreated controls (Luo *et al.*, 1999). In athymic nude mice and SCID mice, similar experiments produced comparable results, suggesting again that the inhibitory effect of VNP20009 does not depend on the presence of functional T- and B-cells. In addition, live bacteria, but not dead bacteria, inhibited the growth of lung metastases. Another attenuated *Salmonella* strain M2 (a.k.a. YS7212, Pawelek *et al.*, 1997) exhibited similar anti-tumour activity in murine tumour models (Zheng *et al.*, 1997).

Table 9.1. Effect of VNP20009 on tumour growth

Tumour inhibition*	Treatment	%
B16-F10 melanoma	Cyclophosphamide	92
	VNP20009 1×10^4 cfu	81
	VNP20009 1×10^5 cfu	91
	VNP20009 1×10^6 cfu	96
Lox human melanoma	VNP20009 2×10^6 cfu	87
DLD1 human colon carcinoma		
Late stage	VNP20009 2×10^6 cfu	97
Early stage	VNP20009 2×10^6 cfu	64
WiDr colon cancer	VNP20009 2×10^6 cfu	52
A549 lung cancer	VNP20009 2×10^6 cfu	62
MDA-MB-231 breast cancer	VNP20009 3×10^6 cfu	73
HTB177 lung carcinoma	VNP20009 1×10^6 cfu	66

*Compared to vehicle control at the end of experiments

Table 9.2. Tissue distribution of VNP20009 in tumour models

Tumour type	No. of <i>Salmonella</i> (cfu/g tissue)		Ratio
	Tumour	Liver	
B16-F10 melanoma	2.0×10^9	1.6×10^5	12000:1
M109 lung carcinoma	1.3×10^{10}	1.5×10^6	8800:1
Lox melanoma	4.4×10^8	1.0×10^6	420:1
C8186 melanoma	2.9×10^8	1.7×10^5	1700:1
DLD1 colon carcinoma	5.7×10^8	1.4×10^5	3900:1
SW620 colon carcinoma	5.0×10^8	2.0×10^6	260:1
HCT116 colon carcinoma	1.0×10^9	5.5×10^4	18000:1
HTB177 lung carcinoma	1.9×10^9	2.9×10^5	6800:1
DU145 prostate cancer	6.2×10^8	6.9×10^4	9100:1
MDA-MB-231 breast cancer	2.3×10^8	2.9×10^4	8100:1
Caki Renal cell carcinoma	9.3×10^8	3.8×10^5	310:1

VNP20009 accumulates preferentially in tumours rather than in normal tissues. For example, in subcutaneously implanted B16-F10 melanoma, VNP20009 was detected at levels up to 2.0×10^9 cfu/g tumour, whereas levels of VNP20009 in liver were 10,000-fold lower. This selectivity occurred in other subcutaneously implanted murine tumours, such as M109 lung carcinoma, and human tumour xenografts Lox, C8186, DLD1, SW620, HCT116, HTB177, DU145, MDA-MB-231, and Caki (Table 9.2).

Tumour-specific pro-drug-converting enzyme delivery

Tumour-localized expression of enzymes capable of specifically converting pro-drugs to cytotoxic agents remains a primary goal in the gene therapy of cancer. The ability to activate pro-drugs only within the tumour mass could potentially alleviate the devastating side effects of conventional chemotherapy on normal tissues. Through intra-tumoural injection of genetically modified viral vectors harbouring such pro-drug converting enzymes, infection and transduction of tumour cells does occur, but only along and near the needle track of such injections.

The ability to infect and transduce each cell within a tumour using viral vehicles remains an elusive goal.

Genetically attenuated *Salmonella typhimurium* possess the unique ability to replicate preferentially within solid neoplasms following systemic administration, thus providing the targeting vehicle to deliver and express pro-drug converting enzyme genes specifically to the solid tumour microenvironment. The expression of enzymes confined within the bacterium (either cytoplasmic or periplasmic) necessitates that the corresponding pro-drug be capable of bystander cytotoxicity. The pro-drug must be able to reach and penetrate throughout a tumour mass, diffuse across one or both of the bacterial membranes, become enzymatically converted to its active form, and then diffuse out of the bacterium and into tumour cells. Cytosine deaminase (CD), an enzyme found in bacteria and fungi but not mammalian cells, converts 5-fluorocytosine (5-FC), a non-toxic agent (IC_{50} of 200 mM), to the cytotoxic anti-metabolite 5-fluorouracil (5-FU) (Deonarain *et al.*, 1995). This activated agent is then converted to 5 fluorouridine 5'-triphosphate and 5 fluoro-2' deoxyuridine-5' monophosphate, resulting in the disruption of RNA and DNA synthesis with subsequent toxicity to both quiescent and proliferating cells. 5-FU is frequently used in the treatment of colorectal, stomach, and breast carcinomas. Although 5-FU is not as toxic (*in vitro* IC_{50} of 20 μ M) as some anti-cancer alkylating agents, sufficient *in vivo* concentrations of 5-FC have been achieved that eradication of retrovirally transduced human colorectal tumour cells expressing bacterial CD has resulted, even when only 2% of the total tumour cells contained the CD gene (Huber *et al.*, 1994). Moreover, 5-FU is a radiosensitizer (McGinn *et al.*, 1996), suggesting that a radiotherapy combination with CD/5-FC treatment may improve response (Khil *et al.*, 1996; Hanna *et al.*, 1997).

The *Escherichia coli* cytosine deaminase gene was cloned into the bacterial expression vector pTrc99A vector (Pharmacia) in order to place the gene under the control of the *trc* promoter in a plasmid denoted pTrc-CD, and electroporated into the attenuated, tumour-targeting *Salmonella typhimurium* M2 strain (a.k.a. YS7212, Pawelek *et al.*, 1997) resulting in the strain M2-pTrc-CD (King *et al.*, 1998; Zheng *et al.*, unpublished). The ability of M2-pTrc-CD to deliver CD activity to the tumour was examined by quantitating CD activity in both tumours and livers at various times after intravenous injection of bacteria into mice (Figure 9.2). Figure 9.2 shows that CD expression remains in the tumour over the 17-day period following bacterial inoculation. Tumours and livers were removed from animals inoculated with *Salmonella* expressing CD genes at various time points, and CD activity was determined *ex vivo*. Significant CD activity was detected in the tumours 1 day following inoculation, and activity progressively increased over 12 days. At no time was CD activity detected in the liver.

Due to the expression of CD in tumours, we examined whether 5-FC administration, following inoculation of CD-expressing bacteria, would inhibit tumour growth. To explore this possibility, we utilized the M2 strain of *S. typhimurium*, which possessed the ability to accumulate in, but had little anti-tumour activity against, the M27 lung carcinoma. Inoculation of M2-Trc-CD into tumour-bearing mice caused some tumour inhibition (Figure 9.3). However, the combination of M2-Trc-CD + 5-FC resulted in an approximately 40% inhibition of tumour volume on day 21 of the experiment (Figure 9.3). These results indicate specific delivery of CD and associated activity from 5-FC with a therapeutic effect *in vivo* from systemic delivery.

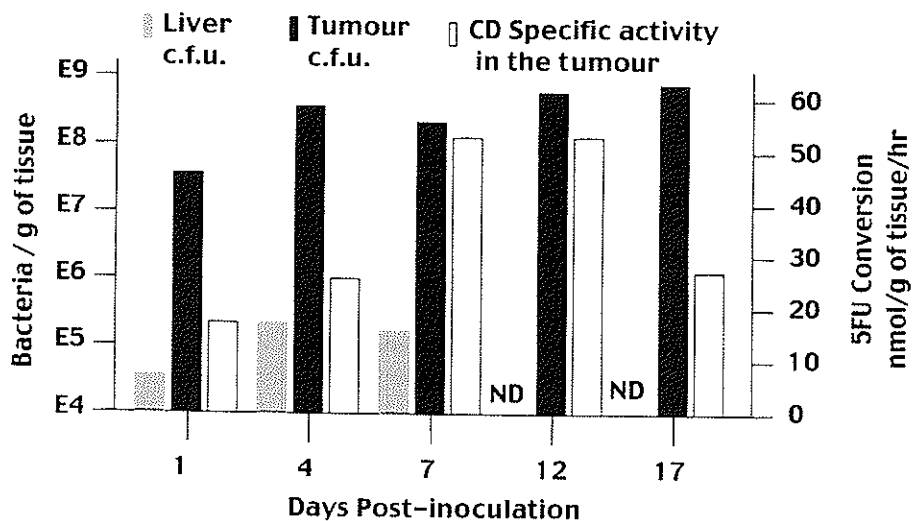


Figure 9.2. The ability of M2-pTrc-CD to deliver CD activity to the tumour was examined by quantitating CD activity in both tumours and livers at various times after i.v. injection of bacteria into animals. Tumours and livers were removed from animals inoculated with *Salmonella* expressing CD genes at various time points, and CD activity was determined *ex vivo*. Significant CD activity was detected in the tumours 1 day following inoculation, and activity progressively increased over 12 days. CD expression remained in the tumour over the 17 day period following inoculation. At no time was CD activity detected in the liver (ND, not determined).

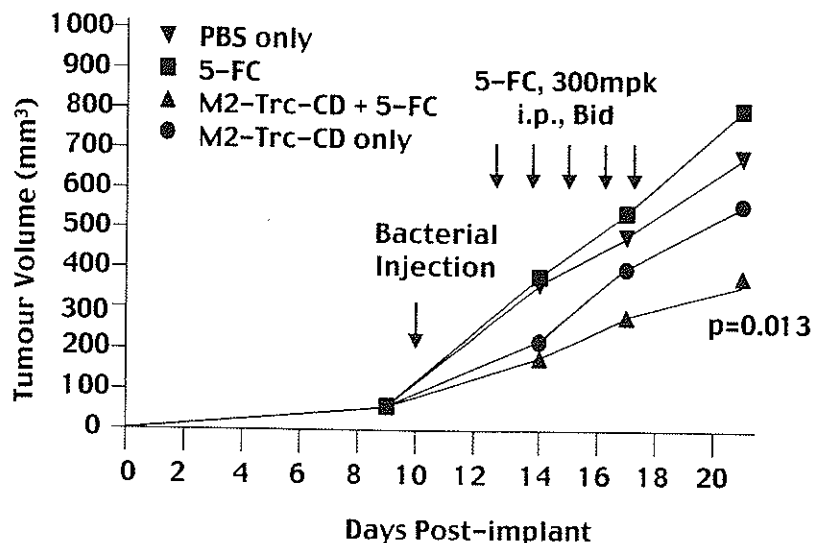


Figure 9.3. C57BL/6 mice bearing tumours of approximately 0.1 g were inoculated with 2×10^6 *S. typhimurium*, M2 strain, expressing cytosine deaminase. Commencing on Day 13, 5-FC was administered twice daily for 5 days at 300 milligrams per kilogram (mpk) per dose (* $P = 0.013$ for M2-Trc-CD + 5-FC and M2-Trc-CD alone).

Anti-tumour effects of *Salmonella* in combination with radiation

In a recent study, it was investigated whether *Salmonella* might be useful when combined with X-ray therapy for melanomas and other solid tumours. Whereas earlier reports suggested that melanomas are relatively radio-resistant compared to other tumours, a number of more recent studies have shown that X-ray treatment can indeed elicit complete responses and even local control in a significant percentage of patients (Peters *et al.*, 1992). Thus the effectiveness of combined treatments of *Salmonella* + X-rays against melanomas and other solid tumours was tested.

The effects of single X-ray doses ranging from 5 to 15 Gy, with and without i.v. injected *Salmonella*, on B16-F10 growth suppression have been investigated (Platt *et al.*, 2000). Anti-tumour activity was assessed by determining the number of days post tumour implantation needed to form 1 g tumours. *Salmonella* alone prolonged the time to 1 g from the control value of 18 ± 1 d (mean \pm S.E.) to a value of 26 ± 3 d. X-rays alone also prolonged the time to 1 g (Figure 9.3). The dose–response curve relating radiation dose to tumour growth delay was linear over the dose range studied in these experiments. The combination of *Salmonella* + X-rays showed supra-additive anti-tumour effects, with the slope of the dose–response curve being greater than expected for additivity (Figure 9.4). Supra-additivity was indicated in all 3 of the 3 X-ray dose–response experiments in mice using the B16-F10 melanoma, as shown by comparing the actual slopes of the dose–response curves obtained to those slopes expected for simple additivity (Table 9.3) (Steel and Peckham, 1979).

Tumour growth curves from the experiment in Figure 9.4 are shown in Figure 9.5, where it is seen that the combination of *Salmonella* and a single dose of 15 Gy X-rays markedly slowed B16-F10 melanoma growth and prolonged mouse survival compared to the other treatment categories. Similar results with a single dose of 15 Gy X-rays in combination with *Salmonella* were obtained with the Cloudman S91 melanoma line implanted s.c. in DBA/2J mice. A summary of additional studies (Platt *et al.*, 2000) is as follows. Higher cumulative X-ray doses of 25 Gy and 50 Gy were achieved by delivery of smaller weekly increments. When the total X-ray doses from all of our experiments were compared (range of 5–50 Gy), the results consistently

Table 9.3. X-ray dose response \pm *Salmonella* strain YS1646 vs tumour growth

Treatment	Expt #	Slope + y intercept*	r ²
X-rays only (0–15Gy)	177	y=0.605x + 18.0	0.99
	172	y=0.490x + 17.9	0.60
	185	y=0.524x + 16.4	0.99
	pooled data	y=0.514x + 17.6	0.83
<i>Salmonella</i> + X-rays (0–15Gy)	177	y=1.089x + 26.6	0.99
	172	y=1.350x + 21.2	0.99
	185	y=0.912x + 21.5	0.43
	pooled data	y=1.075x + 23.3	0.61
Expected for additivity	177	y=0.605x + 26.7	n.a.
	172	y=0.490x + 21.2	n.a.
	185	y=0.524x + 21.5	n.a.
	pooled data	y=0.514x + 23.3	n.a.

* y intercept for X-rays only = mean number of days for tumours to reach 1 g in sham X-rayed control mice (c.f. Figure 9.4); y intercept for *Salmonella* plus X-rays = mean days for tumours to reach 1 g in control mice treated with *Salmonella* only plus sham radiation. r²= correlation coefficient.

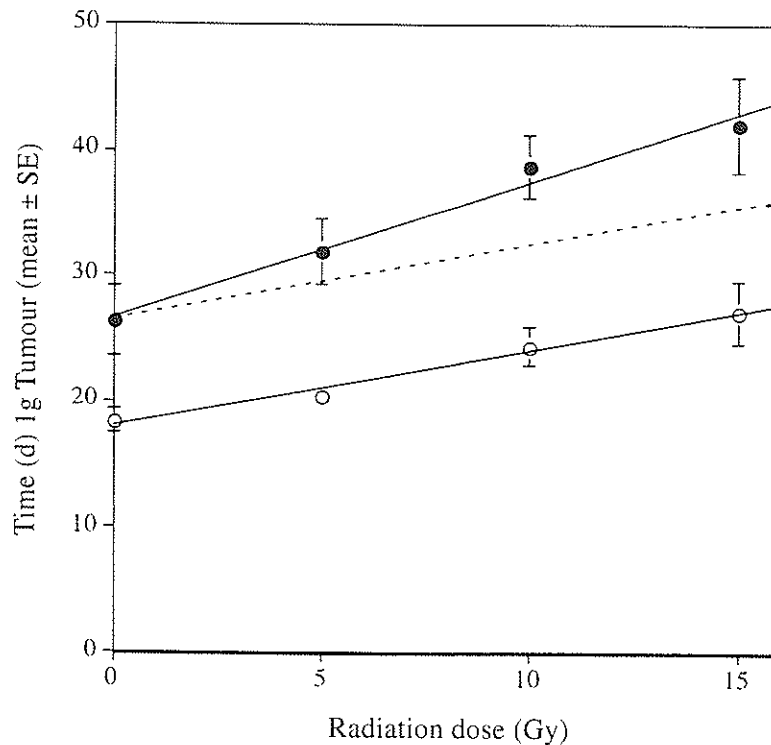


Figure 9.4. Time (d) to 1 g tumour after s.c. implantation of 5×10^5 B16-F10 mouse melanoma cells into C57B6 mice ($n=8-10$ /group), followed 7 d later, when tumours were not yet palpable, by i.v. injection of *Salmonella* YS1646. Four days after *Salmonella* injection, mice were irradiated with X-rays at doses of 5–15 Gy. Controls with or without *Salmonella* were sham irradiated for the time equivalent to 15 Gy. Points represent the mean \pm S.E. The dashed line denotes the anti-tumour effects expected from additive interactions between the X-ray and *Salmonella* treatments. All treatment categories differed significantly from the sham irradiated controls, with P values ranging from <0.0001 to 0.03. *Salmonella* + X-rays differed from X-rays alone at each X-ray dose, with P values ranging from <0.0001 to 0.002. The experiments were performed 3 times with similar results (Table 9.3). Open circles: no *Salmonella*; closed circles: with *Salmonella*.

demonstrated that tumour growth suppression with and without *Salmonella* increased with increasing X-ray dosage, and that at all X-ray doses measured, the combined effects of *Salmonella* + X-rays were greater than expected for additivity. The strongest tumour suppression and longest survival of mice were achieved at the highest cumulative dose (50 Gy) of X-rays + *Salmonella*. For this combination, B16-F10 melanomas reached 1 g an average of 100 days post tumour implantation, 6 times longer than for the sham irradiated controls, 5 times longer than with *Salmonella* alone, and about 50% longer than with X-rays alone.

The results demonstrated in two melanoma tumour models that combined administration of *Salmonella* and X-rays produced significantly greater anti-tumour effects than either of the treatments alone. With both tumour models, when treatment was initiated after tumours were palpable, numerous cases of tumour regression were documented in individual experimental animals, most frequently with the combined treatments of *Salmonella* + X-rays. While in these cases tumour growth was markedly

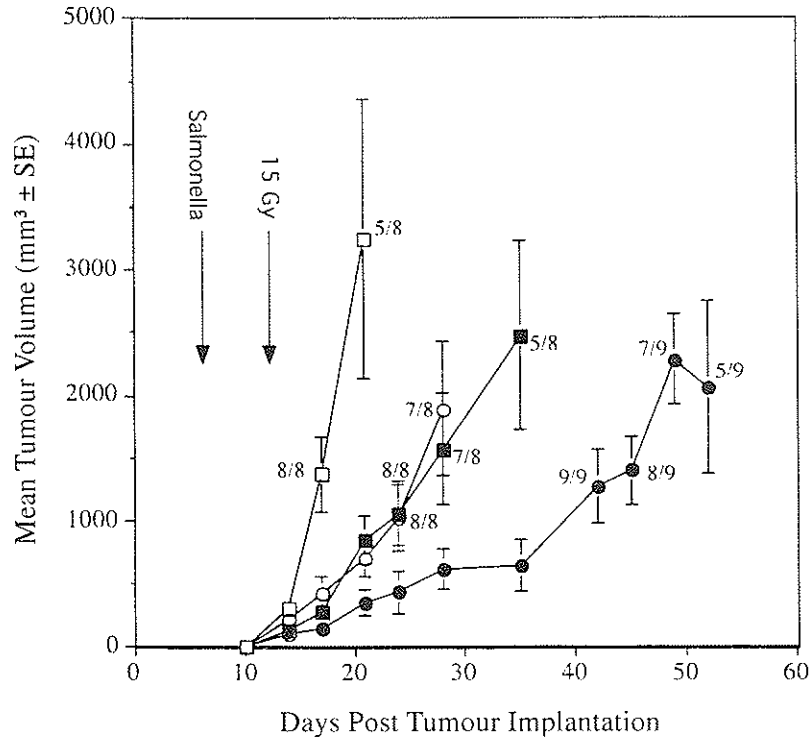


Figure 9.5. Effects of single X-ray doses (15 Gy), with and without *Salmonella* strain YS1646, on the growth of B16-F10 mouse melanomas after s.c. implantation of 5×10^5 B16-F10 cells in C57B6 mice. Open squares: sham irradiated controls; open circles: *Salmonella* only; closed squares: X-rays only; closed circles: *Salmonella* plus X-rays. Experiments were performed 3 times with similar results. The fractions on the graphs are the number of living mice/number of mice initially in the treatment group.

retarded in comparison to controls, the regressions were transient and tumours eventually recurred (data not shown). The mechanisms for this tumour escape are not understood, however one possibility, an enrichment of *Salmonella*-resistant tumour cells, did not seem to be the case, although it could not be ruled out. B16-F10 tumours that had begun growing in mice in the presence of *Salmonella* were dissociated into single cell suspensions, grown 1–2 weeks in culture with antibiotics to kill residual *Salmonella*, implanted into naive mice and again challenged with *Salmonella*, with the procedures repeated 5 times. In all cases, the '*Salmonella*-cycled' tumours, and clones derived from them, were still suppressed by *Salmonella* when re-implanted in mice, similar to control B16-F10 melanomas that had received no previous *Salmonella* exposure. Thus, there was no evidence of acquired melanoma cell resistance to *Salmonella*, and the phenomenon of tumour escape from *Salmonella* suppression could not be explained by this mechanism.

Although the mechanisms are not yet understood, the anti-tumour effects of combined treatment with X-rays and *Salmonella* are likely to be multifaceted, and our research for elucidation of the mechanism(s) is planned with the following considerations. *Salmonella* could secrete molecules which increase cellular radiosensitivity, e.g. by inhibiting repair of radiation damage. X-ray treatment, though not

lethal to all tumour cells at the doses used here, might render some of the tumour cells more vulnerable to *Salmonella* infection and/or to *Salmonella* toxins. X-rays might alter the tumour environment rendering it more accessible to *Salmonella* infection. *Salmonella* might recruit cells of the immune system to tumours (e.g. lymphocytes, macrophages, neutrophils) and X-rays might render tumour cells more vulnerable to immune attack. Another possibility, that X-rays in some fashion induce an increase in the number of *Salmonella* in the tumours, was not borne out. That is to say, *Salmonella*lg tumour were quantified over 5 days post X-ray treatment (15 Gy) and no effect of X-rays on *Salmonella* number in tumours was found (J. Pawelek, unpublished).

We used the term 'supra-additivity' as discussed by Steel and Peckham (1979) for combined radiotherapy and chemotherapy in which the effect of a combination appears to be greater than would be expected for the two agents on the basis of additivity. Since *Salmonella* amplify within tumours, the effects on bacterial density within tumours, as well as on tumour suppression, are largely independent of the initial inoculum, and therefore there is no dose-response curve for the effects of *Salmonella* alone, using tumour suppression as an end-point. The term *synergism* was not used because the term implies that the two agents were working together, and as stated above, the mechanisms underlying the supra-additive anti-tumour effects of *Salmonella* and X-rays are unknown. Whatever the mechanisms, these results suggest that the combined use of *Salmonella* and radiation would be of therapeutic value in cases where X-irradiation is indicated for the therapy of solid tumours.

Conclusions

The difficulties and peculiarities associated with tumours can, in theory, also serve as the source of tumour-specific therapies. The recently developed *Salmonella*-based anti-cancer therapy seems to benefit from and/or overcome several of the refractory features of tumours. The *Salmonella*-based system is capable of tumour-specific delivery by intravenous as well as intra-tumoural administration. The bacteria are replication competent, and thus a low dose replicates to a high dose within the tumour. *Salmonella* are highly amenable to standard genetic manipulation. Lipid A (endotoxin deficient) mutants have favourable properties for a systemic anti-tumour agent and bypass one of the primary obstacles for systemic use of gram-negative bacteria. These bacteria are highly specific, with targeting ratios often 100 to 1000 times greater than normal tissues. The system works on a broad range of tumour types including melanoma, lung, colon, breast, renal, hepatic and prostate tumours. The genetically engineered *Salmonella* have innate anti-tumour activity. These bacteria are further able to specifically deliver pro-drug-converting enzymes such as cytosine deaminase directly to the tumour and maintain high levels of the enzyme for at least two weeks. Delivery of the enzyme to the tumour results in enhanced anti-tumour activity *in vivo* in combination with the appropriate pro-drug (5-FC). *Salmonella* also combines well with X-ray therapy in pre-clinical studies and results in a significant prolongation of survival. These bacteria are antibiotic sensitive, allowing for cessation of treatment or post treatment eradication.

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References

- ALTMAN, E., ROTH, J.R., HESSEL, A. AND SANDERSON, K.E. (1996). In: *Escherichia coli and Salmonella, cellular and molecular biology: Transposons currently in use in Salmonella species*. Eds. F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter and H.E. Umbarger. Washington, DC: American Society for Microbiology.
- BACON, G.A., BURROWS, T.W. AND YATES, M. (1950a). The effects of biochemical mutation on the virulence of *Bacterium typhosum*: The induction and isolation of mutants. *British Journal of Experimental Pathology* **31**, 703–713.
- BACON, G.A., BURROWS, T.W. AND YATES, M. (1950b). The effects of biochemical mutation on the virulence of *Bacterium typhosum*: The virulence of mutants. *British Journal of Experimental Pathology* **31**, 703–713.
- BACON, G.A., BURROWS, T.W. AND YATES, M. (1951). The effects of biochemical mutation on the virulence of *Bacterium typhosum*: The loss of virulence of certain mutants. *British Journal of Experimental Pathology* **32**, 85–96.
- BERMUDES, D., LOW, B. AND PAWELEK, J.M. (2000a). Tumor-targeted *Salmonella*. Highly selective delivery vectors. *Advances in Experimental Medicine and Biology* **465**, 57–63.
- BERMUDES, D., LOW, B. AND PAWELEK, J. (2000b). Tumor-targeted *Salmonella*: Strain development and expression of the HSV TK effector gene. In: *Gene therapy: Methods and protocols*, Vol. 35. Eds. W. Walther and U. Stein, pp 419–436. Totowa, NJ: Humana Press.
- BOCHNER, B.R., HUANG, H.-C., SCHIEVEN, G.L. AND AMES, B. (1980). Positive selection for loss of tetracycline resistance. *Journal of Bacteriology* **143**, 926–933.
- CLAIRMONT, C., LEE, K.C., PIKE, J., ITTENSOHN, M., LOW, K.B., PAWELEK, J., BERMUDES, D., BRECHER, S.M., MARGITICH, D., TURNIER, J., LI, Z., LUO, X., KING, I. AND ZHENG, L.-M. (2000). Biodistribution and genetic stability of the novel anti-tumor agent VNP20009, a genetically modified strain of *Salmonella typhimurium*. *Journal of Infectious Diseases* **181**, 1996–2002.
- DAVIS, R.W., BOTSTEIN, D. AND ROTH, J.R. (1980). *Advanced bacterial genetics*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- DEONARAIN, M.P., SPOONER, R.A. AND EPENETOS, A.A. (1995). Genetic delivery of enzymes for cancer therapy. *Gene Therapy* **2**, 235–244.
- DONNENBERG, M.S. AND KAPER, J.B. (1991). Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infection and Immunity* **59**, 4310–4317.
- EISENSTADT, E., CARLTON, B.C. AND BROWN, B.J. (1994). In: *Methods for general and molecular bacteriology: Gene mutation*. Eds. P. Gerhardt, R.G.E. Murray, W.A. Wood and N.R. Krieg. Washington, DC: American Society for Microbiology.
- FIELDS, P.I., SWANSON, R.V., HAIDARIS, C.G. AND HEFFRON, F. (1986). Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 5189–5193.
- FIELDS, P.I., GROISMAN, E.A. AND HEFFRON, F. (1989). A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. *Science* **243**, 1059–1062.
- HALL, S.S. (1995). Monoclonal antibodies at age 20: promise at last? *Science* **270**, 915–916.
- HANNA, N.N., MAUCERI, H.J., WAYNE, J.D., HALLAHAN, D.E., KUFE, D.W. AND WEICHSELBAUM, R.R. (1997). Virally directed cytosine deaminase/5-fluorocytosine gene therapy enhances radiation response in human cancer xenografts. *Cancer Research* **57**, 4205–4209.
- HOISETH, S.K.J. AND STOCKER, B.A.D. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **291**, 238–239.

- HUBER, B.E., AUSTIN, E.A., RICHARDS, C.A., DAVIS, S.T. AND GOOD, S.S. (1994). Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant anti-tumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 8302–8306.
- JAIN, R.K. (1994). Barriers to drug delivery in solid tumors. *Scientific American* **271** (July), 58–65.
- JOINER K.A. (1988). Complement evasion by bacteria and parasites. *Annual Review of Microbiology* **42**, 201–230.
- KHIL, M.S., KIM, J.H., MULLEN, C.A., KIM, S.H. AND FREYTAG, S.O. (1996). Radiosensitization by 5-fluorocytosine of human colorectal carcinoma cells in culture transduced with cytosine deaminase. *Clinical Cancer Research* **2**, 53–57.
- KING, I., FENG, M., LOU, X., LIN, S., BERMUDEZ, D. AND ZHENG, L.-M. (1998). Tumor-targeted *Salmonella* expressing cytosine deaminase converted 5-fluorocytosine to 5-fluorouracil and inhibited tumor growth *in vivo*. Abstract #3484, *Proceedings of the American Association for Cancer Research* **39**, 512.
- KING, I., LUO, X., FENG, M., ITTENSOHN, M., LI, Z., BELCOURT, M., LIN, S., LE, T., PIKE, J., TROY, K., SZNOL, M., CLAIRMONT, C., BERMUDEZ, D. AND ZHENG, L.-M. (2000). Tumor therapy using *Salmonella*. *Emerging Drugs* **5**, 211–219.
- KLECKNER, N., BENDER, J. AND GOTTESMAN, S. (1991). In: *Bacterial genetic systems: Uses of transposons with emphasis on Tn10*. Ed. J.H. Miller. *Methods in Enzymology* **204**, 139–180.
- LEE, K.C., ZHENG, L.-M., XUO, X., CLAIRMONT, C., FISCHER, J., MARGITICH, D., TURNIER, J., ALMASSIAN, B., BERMUDEZ, D. AND KING, I. (2000). Comparative evaluation of the acute toxic effects in monkeys, pigs, and mice of a genetically engineered *Salmonella* strain (VNP20009) being developed as an anti-tumor agent. *International Journal of Toxicology* **19**, 19–25.
- LOW, B. (1973). Rapid mapping of conditional and auxotrophic mutations in *Escherichia coli* K-12. *Journal of Bacteriology* **113**, 798–812.
- LOW, K.B., ITTENSOHN, M., LE, T., PLATT, J., SODI, S., AMOSS, M., ASH, O., CARMICHAEL, E., CHAKRABORTY, A., FISHER, J., LIN, S.L., LUO, X., MILLER, S.I., ZHENG, L.-M., KING, I., PAWELEK, J.M. AND BERMUDEZ, D. (1999a). Lipid A mutant *Salmonella* with suppressed virulence and TNF α induction retain tumor-targeting *in vivo*. *Nature Biotechnology* **17**, 37–41.
- LOW, K.B., ITTENSOHN, M., LIN, S., CLAIRMONT, C., LUO, X., ZHENG, L.-M., KING, I., PAWELEK, J.M. AND BERMUDEZ, D. (1999b). VNP20009, a genetically modified *Salmonella typhimurium* for treatment of solid tumors. Abstract #581, *Proceedings of the American Association for Cancer Research* **40**, 87.
- LUO, X., ITTENSOHN, M., LOW, B., PAWELEK, J., LI, Z., MA, X., BERMUDEZ, D., LIN, S., ZHENG, L.-M. AND KING, I. (1999). Genetically modified *Salmonella typhimurium* inhibited growth of primary tumors and metastases. Abstract #3146, *Proceedings of the American Association for Cancer Research* **40**, 476.
- MCGINN, C.J., SHEWACH, D.S. AND LAWRENCE, T.S. (1996). Radiosensitizing nucleosides. *Journal of the National Cancer Institute* **88**, 1193–1203.
- MILLER, J.H. (1992). *A short course in bacterial genetics*. Cold Spring Harbor: Cold Spring Harbor Laboratory.
- O'CALLAGHAN, D., MASKELL, D., LIEW, F.Y., EASMON, C.S.F. AND DOUGAN, G. (1988). Characterization of aromatic- and purine-dependent *Salmonella typhimurium*: Attenuation, persistence, and ability induce protective immunity in BALB/c mice. *Infection and Immunity* **56**, 419–423.
- PAWELEK, J., LOW, K.B. AND BERMUDEZ, D. (1997). Tumor-targeted *Salmonella* as a novel anticancer agent. *Cancer Research* **57**, 4537–4544.
- PETERS, L.J., BYERS, R.M. AND ANG, K.K. (1992). Radiotherapy for Melanoma. In: *Cutaneous melanoma*, Second Edition. Eds. C.M. Balch, A.N. Houghton, G.W. Milton, A.J. Sober and S. Soong, pp 509–521. Philadelphia: J.B. Lippincott & Co.
- PLATT, J., SODI, S., KELLEY, M., ROCKWELL, S., BERMUDEZ, D., LOW, K.B. AND PAWELEK, J.

- (2000). Anti-tumor effects of genetically engineered *Salmonella* in combination with radiation. *European Journal of Cancer* **36**, 2397–2402.
- RUSSELL, C.B., THALER, D.S. AND DAHLQUIST, F.W. (1989). Chromosomal transformation of *Escherichia coli recD*- strains with linearized plasmids. *Journal of Bacteriology* **171**, 2609–2613.
- SAMBROOK, J., FRITSCH, E.F. AND MANIATIS, T. (1989). *Molecular cloning: A laboratory manual*, 2nd Edition. Cold Spring Harbor: Cold Spring Harbor Press.
- STEEL, G.G. AND PECKHAM, M.J. (1979). Exploitable mechanisms in combined radiotherapy chemotherapy: The concept of additivity. *International Journal of Radiation Oncology Biology and Physics* **5**, 85–91.
- STERNBERG, N.L. AND MAURER, R. (1991). Bacteriophage-mediated generalized transduction in *Escherichia coli* and *Salmonella typhimurium*. *Methods in Enzymology* **204**, 18–43.
- SZNOL, M., LIN, S.L., BERMUDEZ, D., ZHENG, L.-M. AND KING, I. (2000). Use of preferentially replicating bacteria for treatment of cancer. *Journal of Clinical Investigation* **105**, 1027–1030.
- ZHENG, L.-M., LUO, X., FISCHER, J., LE, T., BERMUDEZ, D., LOW, B., PAWELEK, J. AND KING, I. (1997). Attenuated *Salmonella typhimurium* inhibited tumor metastasis *in vivo*. Abstract #60, *Proceedings of the American Association for Cancer Research* **38**, 9.