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AVALIAÇÃO DO ÉSTER DE ACRIDINA COMO MARCADOR CONJUGADO À LECTINA EM HISTOQUÍMICA

LILIA DE MOURA CAMPOS

RECIFE, 2004

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Dissertação apresentada ao Mestrado em Bioquímica do Centro de Ciências Biológicas da Universidade Federal de Pernambuco como requisito parcial para obtenção do título de Mestre em Bioquímica.

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SUMÁRIO

RESUMO

Transformações neoplásicas apresentam modificações na composição e distribuição de oligossacarídeos da superfície celular de glicoproteínas e glicolipídeos, a partir desta característica, lectinas vêm sendo utilizadas como sondas no auxílio de diagnósticos histopatológicos de tecidos de mama, útero e cérebro, entre outros. Quimiluminescência é uma a técnica que possui baixos limites de detecção e amplas faixas dinâmicas. O éster de acridina vem sendo utilizado em sistemas quimiluminescentes para imunodiagnóstico conjugado a anticorpos. Neste trabalho**,** o éster de acridina foi conjugado à Concanavalina A (Con A) e empregado como marcador histoquímico quimiluminescente. Foram utilizados tecidos mamários humanos normais e diagnosticados como carcinoma ductal infiltrante (CDI). Na metodologia utilizada a emissão de fótons, durante a hidrólise do éster de acridina conjugado à Con A, que foi quantificada, expressada em unidade relativas de luz (URL) e correlacionada com a marcação do tecido, normal ou transformado. Os resultados encontrados demonstraram uma proporcionalidade de URL com a intensidade de marcação dos tecidos estudados. Os valores de URL para o tecido mamário normal ($2{,}565 \times 10^3 \pm 0{,}247 \times 10^3$) foram inferiores aos obtidos para o CDI ($1.283{,}920 \times 10^3 \pm 220{,}621 \times 10^3$). A eficiência da conjugação da lectina ao éster de acridina, a marcação diferenciada do tecido normal e CDI, e a quantificação dos resultados obtidos contribuem para diminuir a subjetividade no diagnóstico histopatológico de rotina e possibilitam o emprego do éster de acridina como marcador de lectinas para uso em histoquímica.

ABSTRACT

Neoplasic cell transformations present differences in the composition and distribution of oligosaccharides in cell surface glicoproteins and glicolipids. Lectins have been used as auxiliary tools in histopathologic diagnosis to mammary, uterus and brain tissues, for example. Chemiluminescence is a technique with low detection threshold and large dynamic spectrum. Acridinium ester (AE) conjugated to antibodies has been employed in chemiluminescent systems to immunodiagnosis. The present work aimed to conjugate AE to Concanavalin A (Con A) and use it as chemiluminescent histochemistry tool. Biopsies of normal and transformed, infiltrating duct carcinoma (IDC), mammary tissues were used. Photon emission, observed during the breakage of the chemical bound between Con A and AE, was quantified, expressed in relative light units (RLU) and correlated to the labeling of the normal and transformed tissues. Results demonstrated that there is RLU proportionality with the labeling. RLU to normal tissue ($2{,}565x10^3 \pm 0{,}247x10^3$) were lower than to IDC ($1.283{,}920x10^3 \pm 220{,}621x10^3$). Con A-AE conjugation efficiency, differential staining of normal and IDC tissues, and quantification of results contribute to decrease the subjectivity in routine histopathologic diagnoses and indicate that acrydinum ester can join other lectin marker to be used in histochemistry.

1 INTRODUÇÃO

1.1 LECTINAS GENERALIDADES

Stillmark, em 1888, foi o primeiro a descrever o fenômeno de hemaglutinação por extratos de plantas, sendo mais tarde relacionado à presença de lectinas. Estudando a toxicidade de *Ricinus communis* (mamona), ele descobriu uma proteína, a qual chamou de ricina, que era capaz de aglutinar as células vermelhas de animais e humanos (Kennedy *et al.*, 1995).

O termo “lectina” (do latim *legere* – selecionar, escolher) foi empregado para descrever aglutininas específicas de grupo sangüíneo, encontradas em sementes e outras partes de algumas plantas (Body & Shapleigh, 1954).

Goldstein e colaboradores (1980) definiram lectinas como proteínas ou glicoproteínas, de origem não imunológica, capazes de reconhecer e precipitar carboidratos, livres ou complexados, e glicoconjugados através de sítios de ligação nos quais as interações hidrofóbicas são as principais forças de interação (Sharon & Lis, 1990; Kennedy *et al.*, 1995).

As lectinas são encontradas em seres unicelulares (Kawagishi *et al*., 2001) e pluricelulares, animais (Danphy *et al*., 2002) e vegetais (Coelho & Silva, 2000).

Apesar do vasto número de informação a respeito de suas propriedades, suas funções fisiológicas específicas continuam imprecisas. As principais hipóteses são as de que as lectinas participam do mecanismo de defesa de plantas contra ataques de fitopatógenos, principalmente, durante os estágios iniciais de germinação das sementes, quando elas agiriam como fungistáticos ou bacteriostáticos (Wang & Ng, 2003). Funcionariam, também, como mediadoras da simbiose entre plantas e microorganismo

fixadores de nitrogênio (Dazzo & Truchet, 1983), proteção contra predadores (insetos e animais), armazenagem de proteínas (Ponchel & Irache, 1998). Atuariam, ainda, na mobilização e acúmulo de matérias para estoque nas sementes antes do período de dormência, bem como no processo de aumento/alongamento da parede celular (Liener *et al.*, 1986).

1.2 HISTOQUÍMICA COM LECTINAS.

Lectinas têm sido empregadas em estudos relacionados às mais diferentes áreas, como matrizes de afinidade (Green & Baenziger, 1989), descobrimento dos códigos de sacarídeos em biomoléculas, na separação de populações celulares biologicamente distintas, técnicas preparativas e analíticas de caracterização, no seqüenciamento e purificação de carboidratos e glicoconjugados. (Peumans & Van Damme, 1998; Calvo *et al.*, 2000), nos tecidos animais (Wakui *et al.*, 1996; Kiatipattanasakul *et al.*, 1998; Baintner *et al.*, 2000; Sames *et al.*,2001) na tipagem sanguínea (Khang *et al*, 1990), como imunossupressoras no transplante de medula óssea (Remani *et al.*, 1994), na caracterização e quantificação das glicoproteínas do soro e análise das imunoglobulinas humanas (Dalziel *et al.*, 1999), na síndrome de imunodeficiência adquirida humana (Astoul *et al.*, 2000), na biotecnologia farmacêutica, como molécula de mediação no direcionamento de drogas para alvos específicos (Lehr, 2000; Yamazaki *et al.*, 2000), como droga anti-neoplásica *in vitro* (Wang *et al.*, 2000).

A variação na expressão de carboidratos nos vários processos metabólicos, de desenvolvimento e de reconhecimento celular, possibilita o uso das lectinas como marcadores estruturais revelando a organização das superfícies celulares e mudanças

durante envelhecimento e patologias (Katnik-Prastowska, 1999; Astoul *et al.*, 2000; Nishimura *et al.*, 2000).

Um grande número de lectinas tem sido empregado como marcadores histoquímicos de tecidos. Dentre elas destacam-se as lectinas de amendoim (“Peanut agglutinin”; PNA), a de *Canavalia ensiformis* (Con A), de *Ulex europaeus* 1 (UEA-1), de *Dolichos biflorus* (DBA) e a de *Triticum vulgaris* (WGA) (Herling *et al.*, 2000).

A histoquímica com lectinas tem sido empregada no auxílio de diagnósticos histopatológicos, para mapeamento dos estágios de diferenciação/desdiferenciação, nível de malignidade e capacidade de metástases (Brooks, 2000; Danguy *et al.*, 2002) em tecidos da cavidade oral (Komath *et al.*, 2000; Kaneko *et al.*, 2000); cérebro humano (Nishimura *et al.*, 2000; Beltrão *et al*, 2003), baço (Dullman *et al.*, 2000); mama (Brooks & Carter, 2001; Beltrão *et al.*, 1998, 2001), útero (Pillai *et al.*, 1994), próstata (van den Brûle *et al.*, 2001) e pele (Sames *et al.*, 2001), entre outros.

1.3 CÂNCER

Neoplasia é o processo patológico que resulta no desenvolvimento de um neoplasma. Caracteriza-se pelo crescimento anormal, incontrolado e progressivo do tecido, mediante proliferação celular/tumor com neoformação (WHO, 2000). Popularmente e indistintamente chamado de câncer, é responsável por altas taxas de mortalidade em todo o mundo, sendo crescente o número de casos diagnosticados a cada ano.

Pesquisa realizada no Hospital do Câncer de Pernambuco indicou que o câncer de mama aparece em terceiro lugar, dos 80 tipos de câncer, alcançando a marca dos 14,75% do total dos pacientes atendidos no período 1996 a 2000 (HCP, 2000).

O câncer de mama é 100 vezes mais freqüente em mulheres que em homens. Dentro dos fatores de riscos e etiológicos são citadas a predisposição hereditária, estado marital, procriação, e lactação, displasia mamária, câncer de útero e ovário, hormônios estrógenos, trauma, fatores étnicos e ambientais, anti-conceptivos orais e menopausa. O câncer de mama nas mulheres é a segunda maior incidência de neoplasias maligna no mundo. Neste tipo de câncer a melhora na qualidade de vida tem diminuído sua incidência. Hábitos da vida moderna como tabagismo, alcoolismo, sedentarismo e obesidade têm contribuído para aumentar o risco de câncer de mama (Parkin *et al.*,2001).

A Sociedade Americana de Câncer estimou que dos 89.000 novos casos de câncer de mama ocorrido em 1976 nos EUA, 33.000 pacientes morreram da doença em 1 ano (Krupp & Chatton, 1980). Atualmente uma em oito mulheres americana desenvolverá câncer de mama durante suas vidas (England *et al.*, 2003)

No Brasil a taxa de mortalidade por câncer de mama ainda é elevada, provavelmente os pacientes ao procurarem ajuda médica encontram-se em estádios avançados (INCA, 2003).

Nem todas as neoplasias malignas são passíveis de prevenção ou detecção precoce. O câncer de mama quando detectado precocemente propicia uma maior sobrevida. Na Europa, a sobrevida é de 91% após 1 ano e de 65% após 5 anos; nos Estados Unidos, a sobrevida é de 96,8%, no primeiro ano (Parkin *et al.*, 2001).

Nos processos neoplásicos as células sofrem alterações, ocorrendo, dentre elas, mudanças significativas no padrão de glicosilação da membrana celular. Estas

modificações são observadas quanto à composição e distribuição de oligossacarídeos da superfície celular de glicoproteínas e glicolipídeos são reconhecidos preferencialmente pelas lectinas (Brooks & Leathem, 1995; Nishimura *et al.*, 2000).

1.4 ENSAIOS QUIMILUMINESCÊNTES

Quimiluminescência (QL) é definida como a emissão de radiação eletromagnética, geralmente na faixa do visível ou infravermelho próximo, produzida por uma reação química. Este fenômeno possui aplicações diversas em química analítica, principalmente, na área da análise por injeção em fluxo, cromatografia de coluna líquida e sistemas de separação por eletroforese capilar, bem como em imunoensaios (Garcia-Campaña *et al.*, 2003).

O uso de reações de QL apresenta algumas vantagens, dentre as quais possuir baixos limites de detecção e amplas faixas dinâmicas (Baeyens *et al.*, 1998). A aplicação da QL, como instrumento analítico, depende da conjugação da substância de interesse a um dos participantes da reação quimiluminescente ou ao produto no estado excitado. A concentração da amostra desconhecida será proporcional à produção de luz total emitida ou a um parâmetro físico associado à luminescência, tal como cor ou polarização da luz emitida (Campbell *et al.*, 1985).

Análises laboratoriais baseadas nos princípios quimiluminescentes têm sido desenvolvidas para dosagens que requerem uma alta sensibilidade, como a determinação de citocinas, fator de crescimento epidérmico, e de crescimento endotelial vascular. Dentre a grande variedade de testes já disponíveis comercialmente estão aqueles utilizados na avaliação de função tireoidiana, fertilidade, marcadores tumorais,

monitoramento de drogas terapêuticas, hepatite, proteínas específicas e esteróides (Kricka, 2003).

Substâncias quimiluminescentes podem ser detectadas na faixa de fentomoles ou atomoles (10^{-15} a 10^{-18} mol), com sensibilidade superior aos ensaios espectofotométricos (10^{-6} a 10^{-9} mol) e fluorimétricos (10^{-9} a 10^{-12} mol) (Campbell *et al.*, 1985). Vários compostos orgânicos exibem QL em condições apropriadas, dentre os quais destacam-se o luminol, isoluminol, éster de acridina ou seus derivados como alguns dos marcadores utilizados em imunoensaios quimiluminescentes (Roda *et al.*, 2000).

O éster de acridina foi introduzido em imunoensaios a partir da necessidade de substituir os marcadores radioisotópicos que estavam se tornando poucos populares devido à sua curta meia vida, ao perigo potencial à saúde e aos problemas quanto aos dejetos gerados (Weeks *et al.*, 1986). Desde então o éster de acridina tem sido foco de pesquisas para ampliar sua aplicação nas mais diversas áreas de Biotecnologia. (Adamezyk *et al.*, 2001).

2 RELEVÂNCIA DO TRABALHO

A especificidade de ligação das lectinas a carboidratos tem encontrado aplicação nas áreas médica e biológica. Lectinas têm sido usadas para estudar as propriedades de ligações a diferentes tipos de tecidos humanos com lesões benignas e malignas (Beltrão *et al.,* 1998, 2001, 2003; Sames *et al.,* 2001). Éster de acridina, por sua vez, tem sido utilizado com sucesso em sistema quimiluminescentes para imunodiagnóstico (Kricka, 2003). Com o intuito de buscar novos compostos que possam ser utilizados como marcadores em conjugados de lectinas, o éster de acridina será utilizado em histoquímica com lectinas de tecidos mamários humanos. Tendo em vista o número de casos de câncer de mama no Estado de Pernambuco, diagnosticados no Hospital do Câncer de Pernambuco, e seu impacto na sociedade, este tecido será utilizado como modelo para avaliação do potencial do éster de acridina conjugado à lectina de *Canavalia ensiformis* (Con A) como ferramenta auxiliar para diagnóstico em Histopatologia, encontrando, assim, uma nova aplicação para este composto, tradicionalmente empregado em ensaios quimiluminescentes em imunodiagnóstico.

3 OBJETIVOS

3.1 GERAL

Avaliar o potencial do éster de acridina conjugado a lectina de *Canavalia ensiformis* (Con A) como marcador celular em histoquímica com lectinas de tecidos mamários humanos.

3.2 ESPECÍFICOS

- Conjugar o éster de acridina à Con A;

- Detectar a emissão de fótons durante a hidrólise do éster de acridina conjugado a Con A;

- Estabelecer a correlação entre os fótons emitidos, a área de marcação e o diagnóstico.

4 ARTIGO CIENTÍFICO

EVALUATION OF ACRIDINIUM ESTER CONJUGATED TO CONCANAVALIN A AS CHEMILUMINESCENT HISTOCHEMISTRY MARKER

(A ser submetido ao Biotechnology Letters)

EVALUATION OF ACRIDINIUM ESTER CONJUGATED TO CONCANAVALIN A AS CHEMILUMINESCENT HISTOCHEMISTRY MARKER

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ABSTRACT

Cell differentiations/dedifferentiations include differences in the composition and distribution of oligosaccharides in cell surface glicoconjugates. Lectins have been used as auxiliary tools in histopathologic diagnosis of many pathologies of mammary, uterus and brain tissues, for example. Chemiluminescence is a technique with low detection threshold and large dynamic spectrum. Acridinium ester (AE) conjugated to antibodies have been employed in chemiluminescent systems to immunodiagnosis. This work aims to use Con A-AE as chemiluminescent histochemistry tool. Biopsies of normal and transformed, infiltrating duct carcinoma (IDC), mammary tissues were used. Photon emission, observed during the breakage of the chemical bound between Con A and AE, was quantified, expressed in relative light units (RLU) and correlated to the labeling of the normal and transformed tissues. Results demonstrated RLU proportionality with the labeling. RLU to normal tissue ($2{,}565x10^3 \pm 0{,}247x10^3$) were lower than to IDC ($1.283{,}920x10^3 \pm 220{,}621x10^3$). Con A-AE conjugation efficiency, differential staining of normal and IDC tissues, and quantification of results contribute to decrease the subjectivity in routine histopathologic diagnoses and indicate that Acrydinum Ester can join other lectin marker to be used in histochemistry.

INTRODUCTION

Molecular switches for fluorescence have been attracting considerable attention, especially because of high sensitivity of luminescence signals (Suzuki *et al.*, 2003).

Chemiluminescence (CL), defined as a process in which excited molecules or atoms generated from chemical reactions release the excess of energy in light form has been known to be a powerful analytical technique that exhibits high sensitivity and selectivity. The benefits of chemiluminescent methods include ultra sensitive detection limits (attomole-zeptomole), rapid assays and a broad range of analytical applications (Kricka, 2003).

Historically, luminol and isoluminol were the first chemiluminescence compounds to be used as labels (Campbell *et al.*, 1985) but the more sensitive acridinium ester labels quickly super exceeded them. As effective labels for biomacromolecules, acridinium esters have received great interest as a basis for the development of high-performance chemiluminometric assays using labeled molecules (Yang *et al.*, 2002).

Lectins, (glyco)proteins that reversible bind free or conjugated carbohydrates, have found an increasing number of applications in medicine and biological processes (Kennedy *et al.*, 1995). Traditionally they are conjugated to peroxidase, fluorescein isothiocyanate (FITC) or biotinylated to be used in histochemistry, flow cytometry analysis (Brooks & Leathem, 1995; Nishimura *et al.*, 2000).

As histochemical marker, lectins have been applied to characterize mammary (Beltrão *et al.*, 1998, 2001), uterine (Remani *et al.*, 1994) and cerebral neoplasic tumors (Nishimura *et al.*, 2000, Beltrão *et al.*, 2003), and many others. Lectin histochemistry

has been used as an extra marker besides the traditional immunohistochemistry to characterize normal and transformed tissues.

In this work acridinium ester is proposed as the labeling molecule for Concanavalin A, Con A, to be used as auxiliary histochemistry tool to help the clinical-pathological evaluation of infiltrating duct carcinoma, a human mammary tumour of high incidence in the State of Pernambuco – Northeast Brazil.

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MATERIALS AND METHODS

Human mammary specimens: twelve formalin-fixed and paraffin-embedded malign mammary tissues, diagnosed as infiltrating duct carcinoma, and four normal mammary tissues were obtained from the Hospital do Câncer of the State of Pernambuco, Brazil.

Con A conjugation with acridinium ester: Acridinium ester (AE) was conjugated to Con A according to Weeks *et al.* (1986). Briefly, Con A (500 µL containing 2 mg of protein) was incubated with 15 µL of acridinium ester solution (0,2 mg diluted in 400 µL de N,N-dimethylformamide), for 1 h at 25°C. The conjugate (AE-Con A) was applied to a column of Sephadex G-25, previously equilibrated with phosphate buffer pH 7,2. Aliquots (200µL) were collected and protein spectrophotometrically determined at 280 nm. Protein peak was protein content and chemiluminescence assayed.

Lectin Histochemistry. Four-micrometer paraffin sections were cut and microscope slides were prepared. Sections were deparaffinized in xylene and hydrated through graded alcohol (100-70%). Tissue slices were incubated with Con A-AE (100 µg/mL) for two hours at 4°C. All solutions were prepared in 10 mM phosphate buffer, containing 0.15M NaCl (PBS), pH 7.2. After each step, the slices were immersed in PBS for two 5-min washes. Lectin binding inhibition assays were accomplished incubating each lectin solution with D-glucose (0.02 to 0.5 M) for 15 min prior to its incubation with the tissue. Following steps were as described previously for the binding protocol.

Measurement of chemiluminescence: Luminometry was performed using a Magic Lite Analyzer (Ciba Corning Diagnostics, Walpole). The emission intensity was

determined as relative light units (RLU) using 5 μL of sample. Duplicate measurements routinely exhibit precision lower than 5%.

RESULTS AND DISCUSSION

A large number of research immunoassays and emerging clinical tests have been developed in a chemiluminescent format (Kricka, 2003).

Conjugation of acridinium ester to antibodies and enzymes has been widely used in analytical and clinical research (Goto *et al.*, 2002; García-Campaña *et al.*, 2003; Kricka, 2003). Here, lectin labeling with acridinium ester was developed using Con A and the conjugate, Con A-AE, was used as chemiluminescent histochemistry tool.

Luminescence was evaluated as a function of tissue area. It was observed that photon emission was proportional to the tissue area incubated with Con A- AE. From $1cm^2$ to $1/8cm^2$ a decrease in relative light units (RLU) was achieved when infiltrating duct carcinoma was labeled with the conjugate (Figure 1).

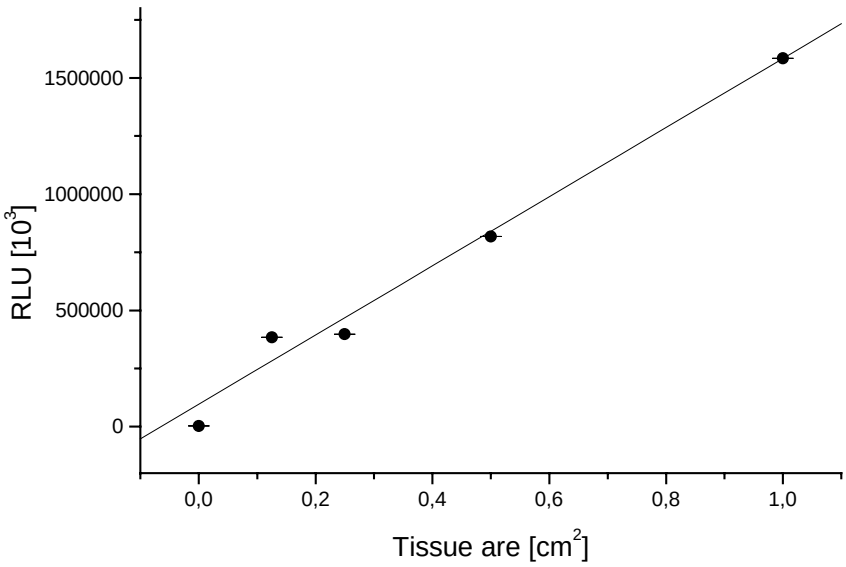

Figure 1: Con A-AE labeling proportionality to infiltrating duct carcinoma tissue area. Values of r and p were 0,98 and 0,002, respectively.

The proportionality of RLU per area of tissue helps to standardize the expected emission of light for each stage of dedifferentiation to which the investigated tissue presents.

The RLU counting for the infiltrating duct carcinoma was much higher than that estimated for the normal mammary tissue (Table 1). These results are according to Beltrão *et al*. (1998) who demonstrated that Con A specifically labeled IDC. Change in the glycosilation pattern of glycoconjugates in the cell membrane, mainly as over expression, is a feature observed in many tumours.

Table 1: Chemiluminescence of Con A-AE labeled and inhibited tissues.

Tissue	Labeled (RLU)	Lectin inhibited (RLU)
Infiltrating duct carcinoma	$1{,}283.920 \times 10^3 \pm 220.621 \times 10^3$	$0.647 \times 10^3 \pm 0.046 \times 10^3$
Normal	$2{,}565 \times 10^3 \pm 0.247 \times 10^3$	$0.192 \times 10^3 \pm 0.032 \times 10^3$

Lectin inhibition binding assay was best performed with D-glucose (300 mM). The assay assured that resulting counting of RLU was obtained via lectin carbohydrate binding site that recognized glucose/mannose residues in cell membrane and not by unspecific binding between acridinium ester and such residues of glycoconjugates also present in cell membrane (Table 1).

These results is of great importance since the use of others labels such as peroxidase and FITC showed to be effective to indicate difference in staining intensity in transformed and normal tissues but not to quantify how much is correspondent to such transformations per area. The sensitivity, dynamic range and diversity of chemiluminescent assays, notably in immunoassays, protein and nucleic acid blotting, have found another application in lectin histochemistry as showed here in this work.

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5 CONCLUSÕES

O presente trabalho permite concluir que:

- o éster de acridina foi conjugado com sucesso a Con A;

- o conjugado mostrou-se um marcador estável para tumores de tecido mamário humano, normal e diagnosticado como carcinoma ductal infiltrante;

- a emissão de unidades relativas de luz pela quebra química do conjugado Con A éster de acridina é proporcional à área de tecido avaliado;

- a emissão de URL foi decorrente do reconhecimento lectina-glicoconjugados da superfície celular dos tecidos normais e transformados como comprovado pela abolição da mesma quando da inibição dos sítios de reconhecimento a carboidrato da Con A por glicose.

- o conjugado Con A-EA se mostrou um promissor marcador quimiluminescente em histoquímica com lectinas.

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